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Progesterone-like Substance in the Ovaries, Hepatopancreases, and Hemolymph of Female *Uca arcuata*

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Jin-Taur Shih and Yuh-Ming Wang (1993) Progesterone-like substance in the ovaries, hepatopancreases, and hemolymph of female *Uca arcuata*. *Bull. Inst. Zool., Academia Sinica* **32**(2): 120-126. Our purpose was to find whether or not fiddler crab (*Uca arcuata*) tissues contained a progesterone-like substance. Ethanol extracts were prepared from the ovaries, hepatopancreases, and hemolymph of female *U. arcuata*. Using a chromatograph with a RP-C18 colum, a progesterone-like substance was identified in all samples of extracted steroid residues according to a retention time similar to a progesterone standard. All samples showed a positive reaction to progesterone via radioimmunoaasay.

Key words: *Uca arcuata*, Ovary, Hepatopancreas, Hemolymph, Progesterone-like substance.

A sex steroid-like substance in crustaceans was first found in the ovaries and eggs of the American lobster (*Homarus americanus*) (Donahue 1940, 1948). Sasser and Singhas (1988) used column chromatography and radioimmunoassay (RIA) to analyze the hemolymph of *Callinectes sapidus* and penaeid shrimp; an estradiol-like substance was found in both species. Recently a progesterone-like substance as identified via high pressure liquid chromatography (HPLC) was found in the body extract of female *Uca arcuata* (Shih *et al.* 1990). Shih (1992) also reported finding both progesterone-like and estradiol-like substances in the hemolymph of *Uca arcuata*, and that these sex steroid-like substances reached peak levels two to three months before breeding.

In an *in vitro* study, Sandor (1981) reported that ovarian tissue in *Portunus trituberculatus* converted progesterone to 11-deoxycorticosterone. Enzymes involved in the conversion of progesterone to hydroxyprogesterone, testosterone and deoxycorticosterone have been isolated in the ovaries of *Portunus trituberculatus* (Teshima and Kawazawa 1971). In addition, ecdysteroids were detectable by RIA in the ovaries of *Carcinus maenas* (Lachaise and Hoffmann 1977). Ecdysteroids have also been detected in the oocytes, follicle cells and hemolymph of *Orchestia gammarellus* (Zerbib 1976, Rateua and Zerbib 1978, Blanchet *et al.* 1979).

There is still a lack of either qualitative or quantitative evidence showing the existence of a crustacean (crab) containing sex steroids in its ovaries, hepatopancreases, adipose tissues and/or hemolymph. Furthermore, the location of sex steroid biosynthesis in crustaceans is still unknown. However, before we can find this location, it is im-

portant to know if sex steroids exist in various crustacean tissues. This study reports on the detection of a progesterone-like substance by HPLC and RIA in the steroid residues of *Uca arcuata* ovaries, hepatopancreases, and hemolymph.

MATERIALS AND METHODS

Preparation of tissues

Female *Uca arcuata* with carapace widths between 2.0-2.5 cm were collected in the K area of the Tanshui mangrove swamp in Taiwan (Shih *et al.* 1991). Specimens were rinsed, wrapped with tissue paper to absorb excess water, and frozen prior to use. The crabs used in this study were collected throughout their breeding season (egg-carrying in April and May). Crabs collected during their prereproductive period (January to March) had a relatively high gonad somatic index (Shih 1992) and large ovaries; for this reason, one pooled hemolymph sample was prepared from crabs collected between January and March, 1989. Ovaries and hemolymph from nonreproductive crabs (collected between October and December) were used for comparison.

Crabs collected on the same date were weighed and measured to record carapace widths. After removing the carapace, the ovaries and hepatopancreases of individual crabs were removed, placed in cold absolute ethanol, and stored in a refrigerator. The Hemolymph of crabs collected during the same period was pooled and kept in a freezer ($-22°C$).

Extraction of steroids

The extraction of steroids from ovaries and hepatopancreases was carried out using the procedure described by Huang *et al.* (1983) and Shih *et al.* (1990). Ovaries or hepatopancreases were homogenized twice in cold absolute ethanol (10 times tissue volume) in a mortar. The pooled homogenate was centrifuged at 4°C, 8,000 x g for 10 min. Supernatant was then extracted twice in 10 volumes of methanol and chloroform (2:1, v/v). Organic solvent was evaporated to dryness. This methanol extract was then dissolved in 14 ml of methanol and mixed with 7 ml of $CaCl_2$ (1.0M), thus creating a methanol concentration of about 70% (Ismail *et al.* 1972) which was kept in a refrigerator overnight. After low speed (3,000 x g) centrifugation, the supernatant was again treated with $CaCl_2$ as above, and the final supernatant was again evaporated to dryness. The collected residue was dissolved in 20 ml of dichloromethane and washed twice with 4 ml each of water, 0.1N NaOH, and 0.1N acetic acid. After removing the aqueous phase, the residue in the dichloromethane was evaporated to dryness. The resultant residue was designated as the extracted steroid residue used for this study. Because the *U. arcuata* hemolymph contained water, it was necessary to further separate the aqueous phase by adding diethyl ether to the hemolymph. The ether phase containing dissolved steroids was collected; after the ether was evaporated, the residue was dissolved in absolute ethanol and subjected to the same procedure as described for the ovaries.

Chromatography

The HPLC system used for this study was composed of a Knauer HPLC pump (Type 364), a sample injector, a Spectral photometer, (Nr./No. 731 879), a Chromatocorder 11 (SIC, Japan) and a column of RP-C18. The column was first equilibrated with eluent (20% water:80% methanol) for at least one hour. If not otherwise stated, UV-absorbing steroids were detected in all chromatograms at 254 nm; attenuation was

set at 16 a.u./cm, and optimum flow rate was set at 0.5 ml/min. A sample of 20 ul was injected for each run. Extracted steroid residues were dissolved in 0.5 to 1.0 ml of 80% methanol, filtered through a millipore membrane (0.45 um) and prepared for HPLC. Progesterone (pregn-4-one-3,20-dione) (Sigma Co., USA) was used as a standard. The progesterone stock solution had a concentration of 2.0ug/ml of 80% methanol.

Preparation of sample for radioimmunoassay (RIA)

Extracted steroid residues were dissolved in 0.01 M phosphate buffer saline (PBS, pH 7.40) (Yu *et al.* 1990, Shih 1992) containing 0.1% gelatin (PBSG); this PBSG soluble residue was used for the RIA. For each batch of samples, one sample was measured by RIA at dilutions of 1/2, 1/4, and 1/8 to check for contamination. All samples were then tested for progesterone using a Biodata Progesterone Maia Kit (Code 12274, Milano, Italy). The progesterone antibody was prepared from rabbits. The working RIA range for progesterone was 0.07-33.0 ng/ml of saline. Any assay with a concentration below the minimum limit was rejected. Cross-reaction of progesterone antiserum to estradiol was less than 0.01%. In this study, the observed concentration of progesterone-like substance was 0.18-2.16 ng/ml of PBSG.

RESULTS

As shown in Fig. 1a, retention time for the authentic progesterone (20ng) was 14.60 minutes. The extracted hemolymph steroid residues from female *U. arcuata* were analyzed by HPLC; results are shown in Fig. 1b. Substances with high optical density were not well separated before 10 minutes, except for peaks 2, 3, and 4. However, peaks 5,

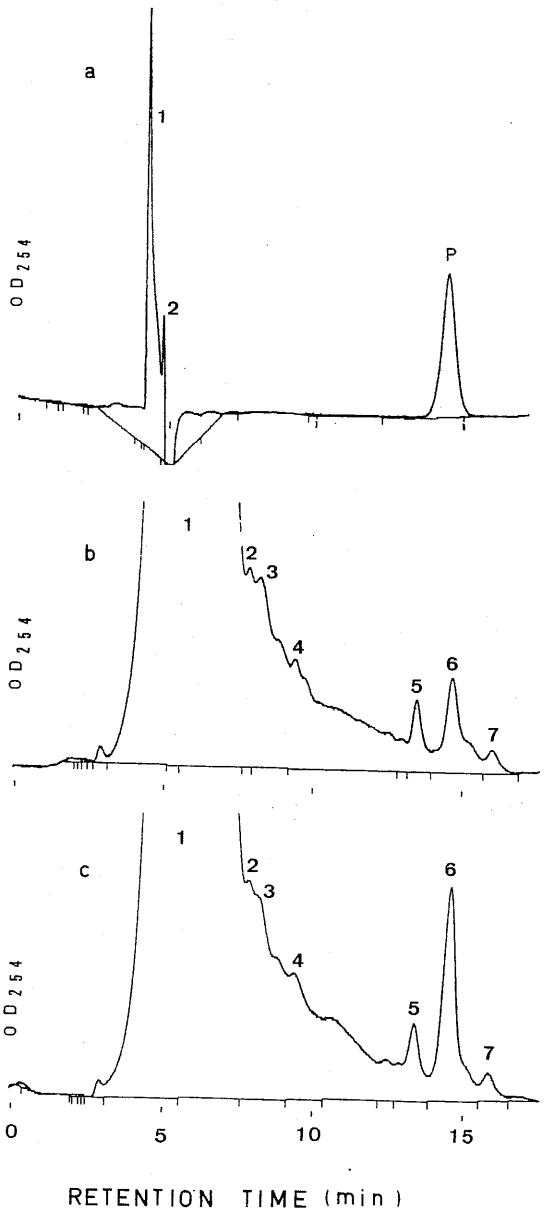

Fig. 1. HPLC chromatograms of progesterone standard and extracted steroid residues from the hemolymph of female *Uca arcuata*. (a) The progesterone standard (P, 20ng) was eluted with water:methanol (20:80) for 17 minutes on a RP-C18 column; optimal flow rate was 0.5ml/min. UV-absorbing steroids were detected at 254 nm and the attenuation was set at 16 a.u./cm. (b) Extracted steroid residue run through HPLC without the progesterone standard. (c) Sample run through HPLC with the progesterone standard (20 ng).

6, and 7 were well separated; peak 6 had a retention time of 14.68 minutes, which was similar to the progesterone standard. In order to assure the identity of the hemolymph progesterone, a chromatography was taken of the sample with the progesterone standard (20 ng). The No. 6 peak in Fig. 1c represents the progesterone standard and had a retention time of 14.59 minutes. The substance with a retention time of 14.68 minutes in Fig. 1b was therefore identified as a progesterone-like substance.

An HPLC of extracted steroid residue from *U. arcuata* ovaries (March 1989) is shown in Fig. 2a. The No. 7 peak had a retention time of 14.71 minutes, which was similar to progesterone standard (Fig. 2b) and thus identified as a progesterone-like substance. Similarly, the extracted hepatopancreases steroid residue (March 1989) were either run through an HPLC alone or co-run with the progesterone standard (Fig. 3). This progesterone-like substance had a similar retention time as the progesterone standard (14.68 minutes, peak No. 8 in Fig. 3a). As shown in Table 1, a hepatopancreas sample (January 1989) and a hemolymph sample (October to December 1989) containing a substance having a retention time similar to the HPLC estradiol standard, which was thus identified as an estradiol-like substance.

The extracted steroid residues were further analyzed by radioimmunoassay for steroids (Table 1). All samples showed positive reaction to progesterone. The ovary and hepatopancreas residues (January 1989) had very low RIA-detected amounts of progesterone-like substance, and were therefore not taken into account quantitatively. However, the other samples listed in Table 1 showed quantifiable measurements (the recovery efficiencies for progesterone-like substance were not calculated). The levels of progesterone-like substance in ovary steroid residues were 0.56-3.42 ng per gram of wet body weight. Hepatopancreas and hemolymph residues had lower levels of progesterone-like substance— 0.39-0.52 ng and 0.06-0.15 ng per gram of wet body weight, respectively.

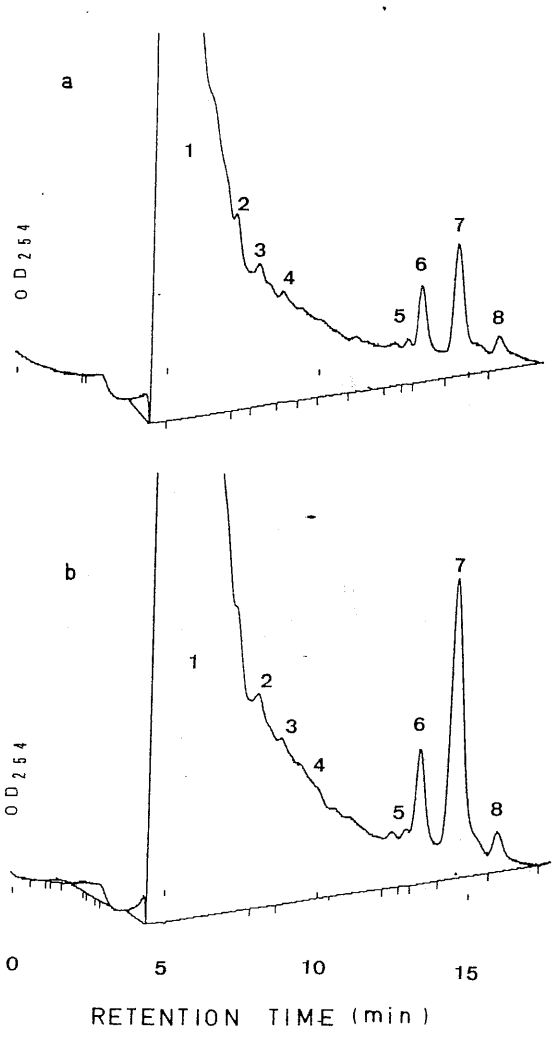

Fig. 2. HPLC chromatograms of extracted steroid residues from the ovaries of *U. arcuata*; chromatography procedures were the same as those described in Fig. 1. (a) Extracted steroid residues run through HPLC without the progesterone standard. (b) Sample run through HPLC with the progesterone standard (20 ng).

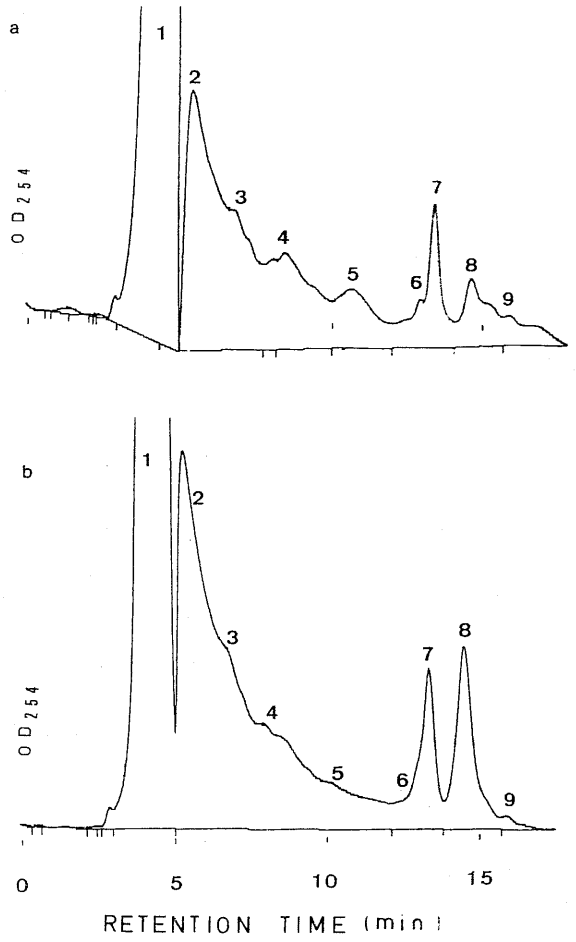

Fig. 3. HPLC chromatograms of extracted steroid residues from the hepatopancreases of female *U. arcuata*; chromatography procedures were the same as those described in Fig. 1. (a) Extracted steroid residues run through HPLC without the progesterone standard. (b) Sample run through HPLC with the progesterone standard (20ng).

DISCUSSION

Results from our study indicate that a progesterone-like substance exists in *U. arcuata* ovaries, hepatopancreases, and hemolymph. These results confirm the findings of Shih *et al.* (1990), who reported that progesterone was detectable via HPLC in the whole body extract of female *U. arcuata*, while estradiol or cortisol were only occasionally detectable. However, in that study they did not specify in which tissues the sex steroids were located. Recently, Shih (1992) found that both progesterone-like and estradiol-like substances were detectable by RIA in female *U. arcuata* hemolymph; most of those hemolymph samples were not processed before being subjected to RIA.

In this study, the ovaries, hepatopancreases and hemolymph of female *U. arcuata* went through a purification process. For example, *U. arcuata* ovaries and hepatopancreases contain a large amount of lipids and proteins; therefore in the extraction procedure, $CaCl_2$ was applied to precipitate excess lipids from the samples. Using acid and alkaline, proteins in the samples were also washed out. Therefore, interferences from lipids in HPLC's or protein cross-reactions in the RIA's was minimized.

In order to measure the efficiency of the extraction procedure, ^{3}H-progesterone was added to the samples (with ethanol). Results showed that steroid extraction efficiency was between 63.1% and 76.1% as previously reported by Shih *et al.* (1990). Two types of extracted steroid residues (from the ovaries and hepatopancreases) were mixed with ^{3}H-progesterone at the beginning of the extraction procedure. Following HPLC analysis, the radioactivity collected from the peak region of the progesterone standard showed a recover rate more than 50%. The radioactivity (10-12%) ran out one to two minutes before the progesterone standard, which represented the degraded form of ^{3}H-progesterone.

To date, it is still in doubt whether or not crustaceans synthesize sex steroids, or what the functions of these sex steroids are for these animals. However, previous research has shown that sex steroids, ecdysteroids, or even enzymes involved in the conversion of precursors to sex steroids ex-

Table 1. Biochemical analysis of sex steroid-like substances in the ovaries, hepatopancreases, and hemolymph of *Uca arcuata*.

Date of collection	Number of crabs	Tissue used	HPLC		HPLC run with ^{3}H-progesterone	RIA-detected Progesterone-like substance (ng/g)[a]
			Progesterone-like substance	Estradiol-like substance		
1989						
January	6	Ovary	+[b]	–	+ (68.2%)[c]	Low[d]
		Hepatopancreas	+	+	+ (59.6%)	Low
March	4	Ovary	+	–	N	3.42
		Hepatopancreas	+	–	N	0.52
December	12	Ovary	+	–	N	0.56
		Hepatopancreas	+	–	N	0.39
January to March	18	Hemolymph	+	–	N	0.15
October to December	16	Hemolymph	+	+	N	0.06

a. ng of steroid per gram of wet tissue weight.
b. Symbols: "+" = positive; "–" = negative; N = Not determined.
c. Numerals in parentheses represent the % ^{3}H-progesterone recovered from HPLC.
d. These two samples showed RIA positive reactions to progesterone, but at very low concentrations.

ist in various crustacean tissues (Donahue 1940 1948, Teshima and Kawazawa 1971, Zerbib 1976, Lachaise and Hoffmann 1977, Rateua and Zerbib 1978, Blanchet *et al.* 1979, Sandor 1981, Sasser and Singhas 1988, Shih *et al.* 1990, Shih 1992). On the other hand, some researchers believe that crustacean sex steroids may come from their food sources. For instance, carnivorous crabs (family Portunidae) have many opportunities to absorb sex steroids from their food sources. However, *U. arcuata* is classified as a deposit feeder (Jones 1984); its food consists of microscopic algae, organic mud, and detritus. Shih (1990) studied the composition of the surface soil substratum in *U. arcuata* habitat; his preliminary results showed that the surface mud contains microscopic algae (diatomes), detritus, and chlorophylls. Therefore, it is unlikely that *U. arcuata* absorb sex steroids from its food sources. However, until there is evidence showing that crustacean tissues synthesize sex steroids, this possibility cannot be excluded.

In crustaceans, crabs and shrimps have scheduled breeding seasons every year. It is reasonable to suspect that crustacean breeding seasons are controlled by changes in the endogenous levels of some hormones; therefore, it is important to determine if crustaceans contain or produce sex steroids. Quantitative studies on sex steroids in the ovaries, hepatopancreases and hemolymph of *U. arcuata* are necessary. Further studies on seasonal changes in the sex steroid levels of female *U. arcuata* will contribute to a more complete understanding of the mechanisms responsible for the control of the reproductive cycle of this crab.

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弧邊招潮蟹(*Uca arcuata*)體液、卵巢和肝胰臟中的類助孕酮

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成熟雌性弧邊招潮蟹體液、卵巢及肝胰臟內的膽固醇類，經無水酒精萃取後，分別由高效能色層分析儀(HPLC)及放射免疫測定法(RIA)的鑑定，得知三種組織中均含有類助孕酮。