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The Role of the Hepatopancreas of Young Female *Mictyris brevidactylus*

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ABSTRACT

This report was aimed to study the physiological function of the hepatopancreas of zero-year old female *Mictyris brevidactylus* (carapace width 3.1-6.3 mm). The hepatosomatic index (HSI) of young crabs were 5.6-9.4% during July-September 1966. The HSI value started dropping from October and reached 4.8% in late November. The fat and protein contents of hepatopancreas were high during July-September (fat: 33.9-95.4 mg/g of hepatopancreas; protein: 6.9-14.6 mg/ g of hepatopancreas). During October-November, both fat and protein levels dropped to one third both that of August and September. When the steroid extract of hepatopancreas was analyzed by HPLC and immunoassays, the progesterone-like and estradiol-like substances were detected. The possible physiological function of the hepatopancreas of young female crab is discussed.

Key words: *Mictyris brevidactylus*, Hepatopancreas, Progesterone-like substance.

Introduction

The hepatopancreas of crustaceans is an important digestive organ. It has the function of food digestion and nutrient storage. For instance, the hepatopancreas of crabs contains large amount of fats and glycogens (Bahn, 1984; Robinson and Dillman, 1985). In female crab, the physiology of the hepatopancreas is correlated to the growth of the ovary (Kyomo, 1988). Many enzymes involved in metabolism are found in the hepatopancreas of crab (Dharale and Masurekar, 1986; Keeran and Lee, 1987; Batel *et al.*, 1988). Sanders (1983a, 1983b) isolated insulin-like peptide from the hepatopancreas of *Homerus americana*. This substance stimulated the glycogenesis to increase in the hepatopancreas of *Homarus americana* (Keller and Andrew, 1973; Sanders, 1983b).

Recently, sex steroid-like substances were detected in the hepatopancreas of female *Uca arcuata*, *Uca borealis* and *Mictyris brevidactylus* (Shih and Wang, 1993; Tseng, 1996; Shih, 1997). Shih (1993) reported that progesterone-like substance was detected in the hemolymoph of zero-year old female *M. brevidactylus* which had only immature ovaries. In vertebrates, progesterone is usually synthesized in gonads. Thus the source of progesterone-like substance in young *M. brevidactylus* could not be ovaries. Though Shih and Liao (1997) found that the hepatopancreas of female *M. brevidactylus* converted ^{3}H-cholesterol to sex steroid-like substances *in vitro* it is still untested whether sex steroids exist in the hepatopancreas of young crab. Therefore, this report was aimed to study the role of the hepatopancreas of zero-year old female *M. brevidactylus*. Studies included measurements of protein and fat contents and the detection of progesterone-like substance of the hepatopancreas of this crab during its early somatic growth period.

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Materials and Methods

Protien and fat determination

Zero-year old female *Mictyris brevidactylus* were collected in the Tanshui mangrove swamp of northern Taiwan during March-November 1996. After chilling in a refrigerator for 10-15 min, each crab was weighed and dissected to remove hepatopancreas. The pooled hepatopancreases were homogenized in 0.1 M phosphate buffer saline (pH 7.4) and centrifuged (1000 x g) at 4℃ for 10 min. One third of the supernatant was used for protein determination and the rest was used for fat determination. The protien assay was according to Bradford (1976). The bovine serum albumin was used for standard curve preparation. To extract the fat, supernatant was mixed with methanol and chloroform (v/v, 1:2) (Shih and Tseng, 1997). After shaking the mixture in a separatory funnel, the organic phase was recovered. This solution was filled into a vial which was dried in a oven and weighed. This vial was placed in a oven at 60℃ for 10 h to dryness. The vial with the extracted fat was weighed and the net weight of the fat was obtained.

Extraction of steroids

Separate preparation of pooled hepatopancreas was placed in absolute ethanol for steroid extraction. The extraction of steroids was carried out using procedure described by Shih (1997). Hepato-pancreases were homogenized twice in cold absolute ethanol in a mortar. The pooled homogenate was filtered. The filtrate was extracted twice in 10 volumes of methanol and chloroform (1:2, v/v). The organic phase was air-dried. The extract was dissolved in 70 ml of methanol and mixed with 30 ml of $CaCl_2$ (1.0 M). The mixture was refrigerated overnight. The precipitate was removed by filtering. The above procedure was repeated once. The steroids in the aqueous phase were extracted by dichloromethane. In dichloro-methane extract of 40 ml, 8 ml each of water, 0.1 N NaOH, and 0.1 N acetic acid were added and mixed in a separatory funnel. After removing the aqueous phase, the residue in the dichloromethane was evaporated to dryness. The resultant extract was designated as the steroid extract used for this study.

High-performance liquid chromatography

Organic solvents used were LC grade from Alps Chemical Company, Taiwan. Authentic steroids were from Sigma Co., (St. Louis, MO, USA.) The HPLC system used for this study was composed of a Knauer HPLC pump (Type 364, Germany), a sample injector, a spectro-photometer, (N_r/No_o. 731 879), a Chromatocorder 11 (SIC, Japan) and a column of LiChroCART 250-4, RP-C18 (4.0 mm x 244 mm, Merck, Germany). The elution procedure was carried out according to Huang *et al.* (1983). The elution was first performed by an isocratic elution with solvent A (water: methanol: acetonitrile: isopropanol, 55: 32: 6.5: 7.5, v/v) for 15 min, followed by a linear gradient elution built up to 80% of solvent B (water: methanol: n-butanol, 40: 40: 20, v/v) within 35 min.

Detection of progesterone-like substance

Steroid extract was dissolved in 1.0 ml of methanol, filtered through a millipore membrane (0.45 μm), and prepared for HPLC. Sixty micro-liters of this sample were placed in a vial and air dried. This sample was assayed for steroid. Authentic steroid standards were: progesterone (pregen-4-one-3,20-dione), estradiol (17-beta-estradiol), and aldosterone (4-pregnen-18-alpha, 11 beta, 21-diol-3,20-dione). A sample of 20 μl was injected for each chromatograph. For identity examination, the eluates which had OD_{254} peaks including the estradiol-like or progesterone-like substance were collected. The eluates with no distinct OD peaks were also collected at 1 to 2-min intervals.

For each hepatopancreas steroid extract, 3 runs were carried out in order to collect enough substance for steroid identification assay. All eluates were air-dried in preparation for steroid assays. The dried residues were dissolved in 1.0 ml of 0.1 M phosphate buffer saline (pH 7.4) containing 0.1% gelatin (PBSG) (Shih, 1997). This mixture was incubated at 50℃ in a waterbath for 1 h and used for steroid assay. The steroid assays were carried out according to protocol provided by Chiron/Diagnostics Corporation, E. Walpole, MA, U.S.A. The Kits, ASC:180 Estradiol + E and ASC:180 Progesterone + E were used for estradiol and progesterone respectively. In this study, the observed concentrations of progesterone-like

substance were 0.10-40 ng/ml of PBSG. The observed concentrations of estradiol-like substance were 20.0 to 550.0 pg/ml of PBSG. Assay with a concen-tration below the minimum limit was rejected. In this study, 71.4-78.2% of the progesterone-like substance (detected in hepatopancreas steroid extract) applied to the column was recovered from chromatography. The recovery efficiency of estradiol-like substance from chromatography was 68.3-73.3%.

Results

Physiology of hepatopancreas

According to Shih (1995), the sex of zero-year old *M. brevidactylus* is distinguished in late June. Thus in this study, young crab collection was started from early July. The carapace width of crab were 3.1-4.8 mm, and the wet body weight were 0.06-0.13g in July. These crabs grew fast and their carapace width reached 6.3 mm during August-September. However, the zero-year old crabs can be easily distinguished from adults until November. In December, the appearance of young crab was similar to adults and was not collected for study.

The hepatosomatic index (HSI) values of crabs were high (9.2-9.4%) from July to August (Table 1). But this value decreased from September and reached to 4.8% in November. The fat contents were 33.9-45.2 mg/g of hepatopancreas during July-August. A peak value of 95.4 mg/g of hepatopancreas in September decreased to 25.8 mg/g of hepatopancreas in November. The protein contents were 11.7-14.6 mg/g of hepatopancreas during July-August. This value started dropping from September and reached to 3.1 mg/g of hepatopancreas in November.

Detection of progesterone-like substance

As shown in Fig. 1a, the retention times for the authentic steroids, aldosterone (A), 17-beta-estradiol (E_2), and progesterone (P_4, 100 ng each except E_2 at 500 ng) are 8.6, 28.9, and 36.7 min, respectively. The steroid extract of hepatopancreas for zero-year old female *M. brevidactylus* (July 1996) was analyzed by HPLC and the result is shown in Fig. 1b. Some peaks with optical densities (OD) were seen during the isocratic elution, a peak (P') with a retention time of 37.1 min was seen in the gradient elution. In order to identify of P' of Fig. 1b, a chromatogram was taken of the hepatopancreas steroid extract with 3 authentic steroid standards. As shown in Fig. 1c, P' of hepatopancreas extract coincided with the progesterone standard (P_4, 39.6 min, slower than that of Fig. 1a and 1b). Therefore, P' of fig. 1b was identified as a progesterone-like substance. Although there were some small OD peaks a few minutes after the gradient elution started, no distinguishable OD peak at the retention time of estradiol (28.9 min) was detected.

After applying the correction factors, contents of steroid-like substance in hepatopancreases were tabulated in Table 1. The contents of the progesterone-like substance were 5.0-11.9 ng/g of hepatopancreas during July-September 1996. The contents of estradiol-like substance were 246.3-1215.1 pg/g of hepatopancreas.

Discussion

The zero-year old female *M. brevidactylus* had high HSI values in July and early August. This value started dropping from September and reached to a year low value in November. In addition, hepatopancreas were shown having high content of fat which was 2-folds of that of July during August-September. But fat content became low (one third or forth of September) in October and November. There were similar changes of protein contents during these months. Shih (1993) found that the adult female *M. brevidactylus* had high HSI values (3.0-6.0%) in nonreproductive season (somatic growth phase, May-August). Crabs were active on their habitat feeding and grew fast during these months (Shih *et al.*, 199!; Shih, 1995). But the HSI values started dropping from October and reached 2.1-3.3% in December. On the contrary, the gonadosomatic index (GSI) were high at this time (November-December, late prereproductive season). In January, crabs started egg-carrying and were less active in their habitat. It is speculated that egg-carrying crabs used the nutrient of hepatopancreas as energy source during reproductive period. Therefore, egg-carrying crabs had the lowest HIS values (2.0%) (Shih, 1993).

Table 1. Physiological and biochemical data of the hepatopancreas of young female *Mictyris brevidactylus*.

Date of assay	1996						
	July(I)[a]	July(II)	August(I)	August(II)	September	October	November
Carapace width (mm)	3.1±0.2[b]	4.6±0.3	5.2±0.4	5.5±0.3	6.3±0.4	7.0±0.5	7.2±0.4
Wet body weight (g)	0.06±0.02	0.13±0.03	0.19±0.05	0.23±0.06	0.28±0.06	0.56±0.08	0.66±0.10
Hepatopancreas							
HSI (%)	9.2±2.8[c]	9.4±1.8	8.0±1.2	6.5±0.8	7.2±1.6	6.1± 0.5	4.8±0.7
Fat content (mg/g)	33.9	41.2	45.2	71.9±7.2[d]	95.4±6.6	43.6±10.1	25.8±7.6
Protein content (mg/g)	12.4	11.9	14.6	11.7±1.4	6.9±1.1	4.0± 0.2	3.1±0.8
Progesterone-like substance (ng/g)	—[e]	11.6		5.0	9.7		
Estradiol-like substance (pg/g)	—	307.2		246.3	1215.1		

a. Assays of July I, II, and August I, II were carried out on July 8th, 24th, August 7th, and 26th 1996, respectively
b. Carapace width and wet body weight were obtained from 25-31 crabs and are expressed as mean and standard errors.
c. Hepatosomatic index (HSI) are expressed as mean and standard errors (n=16-21)
d. Fat and protein contents are expressed as mean and standard errors (n=3)
e. Not determined.

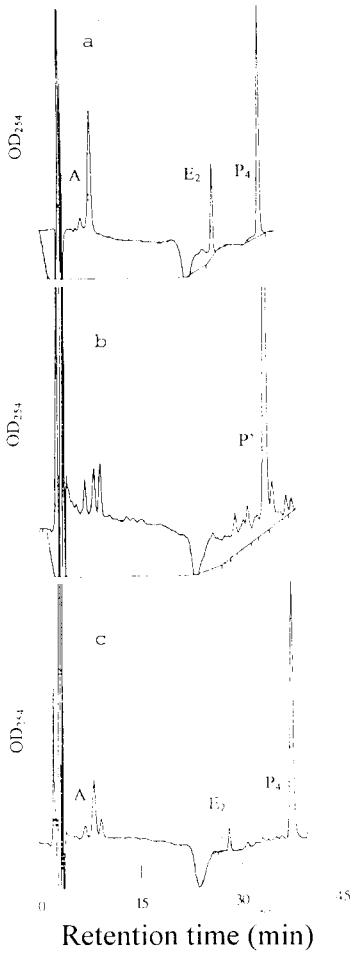

Figure 1. HPLC chromatograms of the authentic steroids and hepatopancreas steroid extract of zero-year old female *Mictyris brevidactylus* collected in July, 1996. Authentic steroids are: aldosterone (A), 17-beta-estradiol (E_2), and progesterone (P_4, at 100 ng each except for E_2 at 500 ng). The elution was first performed by an isocratic elution with solvent A (water: methanol: acetonitrile: isopropanol, 55: 32: 6.5: 7.5, v/v) for 15 min, followed by a linear gradient elution built up to 80% of solvent B (water: methanol: n-butanol, 40: 40:20,v/v) within 35 min. (a) authentic steroid standards, (b) steroid extract of hepatopancreas, P' represents a progesterone -like substance. (c) Sample of (b) run on HPLC with authentic steroid standards.

The physiology of mangrove crab hepatopancreas is not associated with the growth of ovary in the same fashion. For instance, the HSI values increased with the growth of the ovary in *Uca arcuata* (Shih, 1992) and *Sesarma intermedia* (Kyomo, 1988) and reached to peak phase during egg-carrying. In *U. borealis* which has short reproductive cycle (18-20 days), both egg-carrying and nonegg-carrying crabs have similar HSI values and protein contents in hepatopancreas (Shih and

Tseng, 1997). Shih and Chang (1991) reported that male *M. brevidactylus* also had high HSI values during May-September and low HSI in reproductive season.

A progesterone-like substance was detected in the hepatopancreas of young female *M. brevidactylus*. This result is consistent with previous results that a progesterone-like substance can be detected in the hepatopancreas and ovary of female *U. arcuata*, and *M. brevidactylus* (Shih and Wang, 1993; Shih, 1993; 1997). Recently, progesterone-like and estradiol-like substances were detected in hepatopancreas and ovary of *U. borealis* (Tseng, 1996). In this study, a progesterone-like substance was detected in the hepatopancreas while the crab had immature ovary only. This result may help to explain that progesterone-like substance was detected in the hemolymph of the zero-year old crabs (Shih, 1993). In addition, this result confirmed that the *in vitro* radioactive labeled cholesterol was converted to progesterone-like substance and its metabolites by hepatopancreas of female *M. brevidactylus* (Shih and Liao, 1997).

Though progesterone-like and estradiol-like substances were detected in hepatopancreas of young female *M. brevidactylus*, more questions are raised. Do these hormones have a physiological role in reproduction? Quackenbush (1992) treated the penaeid shrimp ovary fragments with progesterone and found that the synthesis of specific yolk protein increased by 50% compared to the control, while testosterone, estrogen, and ecdysterone exerted no effect at all. Quackenbush (1992) suggested that progesterone may have a normal biological role in shrimp ovarian maturation. In a recent review, Quackenbush (1994) stated that steroid hormones produced in the ovary of American lobster may regulate yolk production. Similar experiments have not been carried out on hepatopancreas of *M. brevidactylus*. The effect of progesterone or estradiol in reproduction of this crab needs further study.

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雌性幼齡短趾和尚蟹肝胰臟的生理功能

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摘　要

本報告是研究雌性幼齡短趾和尚蟹（背甲寬3.1-6.3mm）肝胰臟的生理功能，1996年7-9月間，幼蟹的肝胰臟體重比值（HSI）是5.6-9.4%，此值自10月下降，至11月下旬時爲4.8%。肝胰臟中脂肪和蛋白質的含量於7-9月間較高（脂肪：33.9-95.4 mg/g of hepatopancreas；蛋白質：6.9-14.6 mg/g of hepatopancreas），但至11月時，二者含量均下降至8、9月最高值的三分之一。幼齡蟹肝胰臟的類固醇萃取物經高效液體色層分析法及免疫反應測定法分析後，得知含有類助孕酮和類雌二醇，文中對此幼蟹肝胰臟的功能有所討論。

關鍵詞：短趾和尚蟹、肝胰臟、類助孕酮