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Note

Variations in vertebrate-type steroids during testicular maturation in the marine crab *Charybdis natator* (Herbst)

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Abstract

Testosterone in the haemolymph, hepatopancreas and testis were quantified using radioimmunoassay, during different stages of testicular maturation in the marine crab, *Charybdis natator*. Testosterone was not detectable in the haemolymph, when the testis was in stage I spermatogonia, and it gradually increased during stage II spermatocytes and III spermatids and reached a peak level during stage IV spermatozoa. In the testis, testosterone showed a peak levels during stages III spermatids and IV spermatozoa, respectively.

Over the last ten years, it has become evident that the vertebrate-type steroids are also present in a variety of invertebrate species belonging to different phyla (Sandor, 1980; DeLoof and DeClerk, 1986). In the haemolymph of the crustacean *Astacus leptodactylus,* the presence of pregnenolone, testosterone and 6β-hydroxytestosterone were detected in females by gas chromatography-mass spectrometry (Ollevier *et al.*, 1986). Testosterone was shown to be present in serum and testes of *Homarus americanus* (Burns *et al.*, 1984). The activation of vitellogenesis synthesis is under the control of several hormones (Carnevali *et al.*, 1995; Peyon *et al.*, 1997). Sex steroids are natural inducers of gonadal sex differentiation in fishes. The presence of steroid-synthesizing enzymes during early stages of the developing testes has also been demonstrated through the administration of radioactive steroid precursors (Takahashi and Iwasaki, 1973). A few investigators have demonstrated the onset of steroidogenesis prior to the initiation of gonadal differentiation in other fish (Nakamura and Nagahama, 1993). It is still uncertain whether sex steroids are the natural inducers of sexual differentiation in the medaka, since the quantity of sex steroids in embryos has not actually been measured, except for the recent study on the measurement of estradiol-17β (E_2) in eggs before and after fertilization (Iwamatsu *et al.*, 2005). Vertebrate-type steroids such as 17β-estradiol (DeLoof and DeClerk, 1986), testosterone (Burns *et al.*, 1984; Fairs *et al.*, 1989) and progesterone (Kanawaza and Teshima, 1971; Yano, 1985) have been detected in malacostracan crustaceans. Fragmented evidence even suggests a functional role for some of these compounds in crustaceans (Sarojini, 1963; Nagabhushanam and Kulkarni, 1981). The present study attempts to measure testosterone in haemolymph, hepatopancreas and testes in *Charybdis natator* at various stages of testicular maturation.

Materials and methods

Male marine crab *C. natator* with different stages of gonadal maturation, weighing 100 ± 20 g, were procured from the landing centre at Vellapatti, Tuticorin. They were brought to the laboratory and were acclimatatised to laboratory conditions. Following acclimatization, the animals were anaesthetized using MS 222 (100μg/l) for a period of 10-15 minutes. First, the haemolymph was withdrawn from the intra-arthroidal space for radioimmunoassay (RIA) of testosterone using an appropriate anticoagulant (7.5% sodium citrate solution) by 24 G needle fitted to disposable syringes. Later the animals were killed, and the testes and hepatopancreas were dissected out. One part of the testes and hepatopancreas was stored at -70°C to estimate the testosterone by RIA and another part was fixed using Bouin's fluid for histological investigation.

Bouin's fixed testes was dehydrated in an ethanol series and cleared in xylene before infiltrating with paraffin. Six microns paraffin sections were cut using a Leica microtome and stained with Haematoxyline and eosin (Bullock *et al.*, 1976). Photographs were taken using a Zeiss microscope (Akioskop2).

Five hundred microlitres of haemolymph was pipetted out in a stoppered test tube containing 10 ml of HPLC grade diethyl ether. The contents were vortexed well for 5 minutes. To this 2 ml of methanol was added and the entire components were further vortexed for 3 minutes.

The tubes were allowed to stand over night at 4°C. Following day, the organic phase was separated out. The extraction procedure was repeated using the remaining undissolved contents. Both the organic phases were pooled and allowed to dry at room temperature. The residue was reconstituted in 1 ml of PBS before further proceeding to RIA.

One gram of tissue was homogenized in 3 ml of cold TDW. It was mixed with 5 ml of diethyl ether in a stoppered test tube and vortexed for 5 minutes. Then it was mixed with 10ml of methanol and vortexed for 3 minutes. The mixture was allowed to stand for over night at 4°C. Later, the organic phase was separated in another tube. The above extraction procedure was once again repeated with 10 ml methanol and both the organic phases were pooled and allowed to dry at room temperature. The residue was further dissolved in 2 ml of methanol. From this, 1 ml of methanol containing steroid was separated and dried at 50°C. After complete drying, the residue was reconstituted in 500 ml of PBS before further proceeding to RIA (Shih, 1997).

The levels of testosterone in the extracts of haemolymph, hepatopancreas and testes were measured by RIA following the method developed and validated by Lamba *et al.* (1983). Five hundred microlitres of reconstituted serum extract was taken in assay tube and 100μl of anti-testosterone and 10,000-10,500 counts per min (cpm) of H^3-testosterone (1,2,6,7-H^3) in 100μl of PBS was added. The reaction mixture was vortexed and left at room temperature for 1 hour and then incubated overnight at 4°C. Following the incubation, 200 μl of ice-cold dextran-coated charcoal were added and vortexed briefly before leaving for 20 minutes to absorb the free steroids. The tubes were centrifuged at 1000g for 10 minutes at 4°C and the supernatant was decanted to a scintillation vial containing 10 ml of scintillation fluid. The vials were vortexed gently and counted for 1 minute using a liquid scintillation counter (Wallac 1409 DSA) after maintaining in darkness overnight. The detectable limit of the present assay system was 10 pg/ml. Standard curves were obtained by processing tubes containing 0-1000 pg of unlabelled testosterone (Sigma, United States) in a similar manner as described for unknown samples, after selecting the antibody titre for 50% binding. For non-specific binding, 100 μl of H^3- testosterone (c. 10,000 cpm) were added to an assay tube containing 0.6 ml of PBS and the tubes were processed in the same manner as the standards. Total counts were obtained by directly adding 100μl of H^3-testosterone to 10 ml of scintillation fluid. The levels of hormones of unknown samples were calculated using the standard curve. Data were expressed as means ± standard error of means (SEM.).

Results

During the period of testicular maturation, several histological changes were noticed in stage I, II, III and IV testes (Figs. 1-4). The testes of stage I was filled with immature spermatogonia (Fig. 1), whereas stage II, mainly contained maturing spermatocytes (Fig. 2). Stage III contained matured spermatids and in the stage IV spermatozoa.

Changes in the level of testosterone in haemolymph, hepatopancreas and testes are shown in Figure 5. The testosterone level in haemolymph was also below detectable limits, when the testes mainly contained stage I

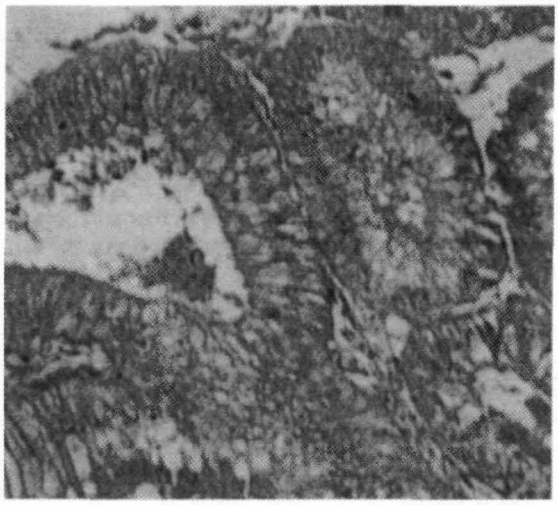

Fig.1. Stage I immature spermatogonia

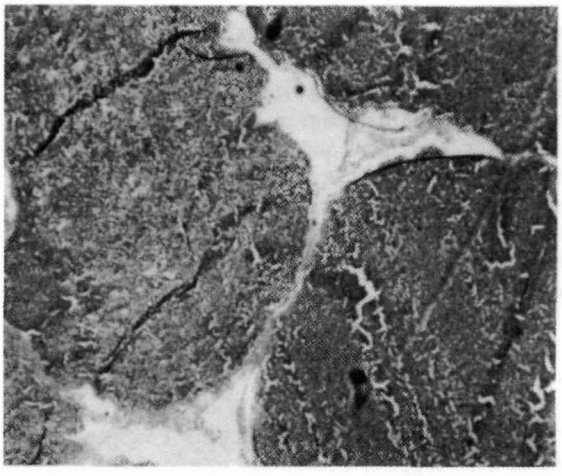

Fig. 2. Stage II maturing spermatocytes

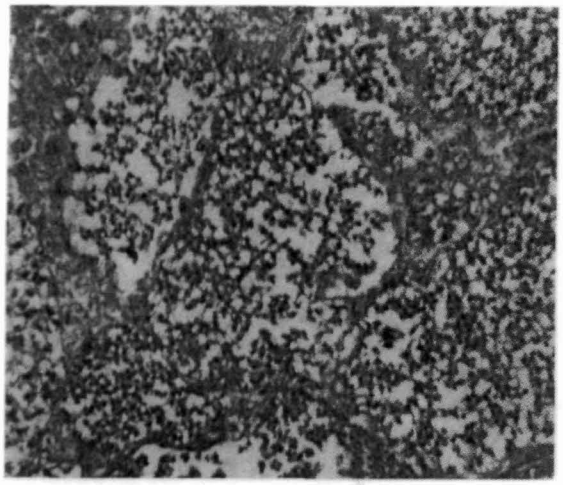

Fig. 3. Stage III matured spermatids

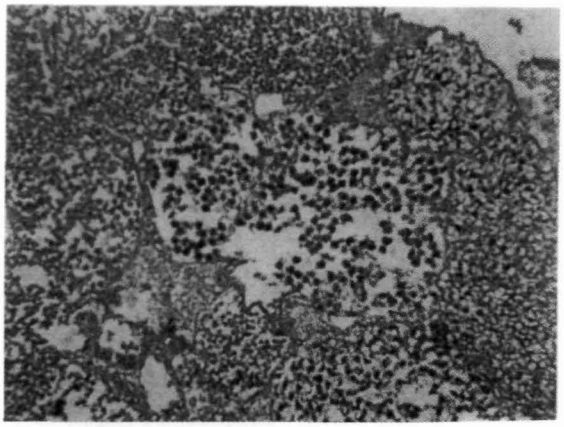

Fig. 4. Stage IV spermatozoa

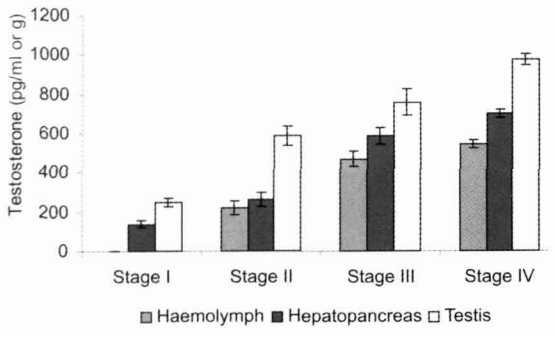

Fig. 5. Changes in the levels of testosterone in the haemolymph, hepatopancreas and testis during various stages of testicular maturation in *C. natator*

spermatogonia. Testosterone started appearing in the haemolymph during stage II testes (219.46pg/ml) and a progressive increase in the level was observed throughout the remaining stages (468.15pg/ml to 540.71pg/ml) respectively. In the hepatopancreas, however the testosterone was detectable during the immature stage I spermatogonia (136.34pg/g). The level increased steadily during the stage II spermatocytes and stage III spermatids (260.57pg/g to 585.44pg/g). During the stage IV, spermatozoa showed a peak level of testosterone (696.50pg/g). The testosterone level in the testes during stage I spermatogonia was minimum (248.69pg/g). The level of testosterone increased steadily during stage II spermatocytes (586.20pg/g) and stage III spermatids (758.12pg/g). In the stage IV spermatozoa, a peak level of testosterone (976.73pg/g) was observed as in the case of testosterone level in the haemolymph and hepatopancreas.

Discussion

In crustaceans, as in vertebrates, spermatozoa are produced through spermatogenesis (Charniaux-Cotton and Payen, 1985). Spermatogonia start meiotic division after proliferation and become spermatocytes and it differentiates into spermatozoa through spermatids. Spermatogenesis is mostly characterized by the differentiation of sperm cells and their maintenance before fertilization. In crayfish, for example, the testes contains three lobes, each built up by many tubules, whose shape changes according to the stage of spermatogenesis. During spermatogenesis, the spermatids are transformed to spermatozoa (Charmantier *et al.*, 1997).

Ronis and Mason (1996) reported that testosterone is converted to a variety of hydroxylated, oxidoreduced and sulfate-conjugated derivatives by the periwinkle. Evidence for testosterone production has only been described for some decapod crustaceans (Burns *et al.*, 1984; Fairs *et al.*, 1989), but never for mysid shrimp. Baldwin and LeBlanc (1994) found no identification of testosterone production by daphnids.

The present study clearly indicates that the testes of *C. natator* underwent a progressive change during different phases of maturation. The changes in the levels of vertebrate-type steroid (testosterone) observed in the haemolymph, hepatopancreas and testes indicated their involvement at the time of testicular maturation and their gradual increase and decrease of testosterone in haemolymph, hepatopancreas and testes. The study also demonstrates a synchronization of testicular maturation and changes in the levels of steroidal hormone. The testosterone is found to play a direct / indirect role during testicular maturation processes in *C. natator.*

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