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In vitro metabolism of steroid hormones by ovary and hepatopancreas of the crustacean Penaeid shrimp *Marsupenaeus japonicus**

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SUMMARY: Radioimmunoassay (RIA) was applied to identify selected metabolites obtained in *in vitro* incubations of ovarian and hepatopancreatic tissues from female *Marsupenaeus japonicus* in previtellogenic and vitellogenic maturation stages. Depending on the precursors used, progesterone, 17α-hydroxyprogesterone or testosterone, it was possible to observe the presence of several enzymatic activities through metabolites detected in incubations. The present study provides evidence that the ovary of the shrimp *M. japonicus* is capable of synthesising 17β-estradiol from progesterone, evidencing the presence of 17α-hydroxylase, C_{17}-C_{20} lyase, 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase. Enzymatic activities of aromatase, 3β-HSD and 17α-hydroxylase were detected in hepatopancreas, but not that of C_{17}-C_{20} lyase. These results suggest that 17β-estradiol could be the vitellogenesis stimulating ovarian hormone (VSOH), and together with former *in vivo* and *in vitro* studies this leads us to propose that in fact there may be a physiological role for steroid hormones in the maturation cycle of crustaceans.

Key words: crustacean, decapods, hepatopancreas, hormones, maturation, ovary, reproduction, steroids.

RESUMEN: METABOLISMO *IN VITRO* DE HORMONAS ESTEROIDES EN EL OVARIO Y EN EL HEPATOPÁNCREAS DEL LANGOSTINO *MARSUPENAEUS JAPONICUS* (CRUSTACEA, PENAEIDAE). – Se utilizó el radioinmunoensayo (RIA) para identificar los metabolitos seleccionados obtenidos en incubaciones in vitro del ovario y del hepatopáncreas de hembras en estado de maduración previtelogénico y vitelogénico de *Marsupenaeus japonicus*. Dependiendo del precursor utilizado: progesterona, 17α-hidroxiprogesterona o testosterona fue posible observar la presencia de diversas actividades enzimáticas a través de metabolitos detectados en las incubaciones. El presente estudio demuestra que el ovario del langostino *M. japonicus* es capaz de sintetizar 17β-estradiol a partir de progesterona y pone en evidencia la presencia de 17α-hidroxilasa, C_{17}-C_{20}liasa, 17β-hidroxiesteroide deshidrogenasa (17β-HSD) y aromatasa. La actividad enzimática de la aromatasa, 3β-HSD y 17α-hidroxilasa se detectó en el hepatopáncreas, pero de C_{17}-C_{20}liasa. Estos resultados sugieren que el 17β-estradiol podría ser la hormona estimuladora de la vitelogénesis (VSOH), y conjuntamente con anteriores estudios in vivo e in vitro nos dirige a proponer que de hecho debería existir una función fisiológica para las hormonas esteroides en el ciclo de maduración de los crustáceos.

Palabras clave: Crustáceos decápodos, hepatopáncreas, hormonas, maduración, ovario, reproducción, esteroides.

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INTRODUCTION

Control of gonadal maturation, particularly of ovary maturation, is a major problem for the development of crustacean culture, since several species do not mature without external manipulation. Particular attention has been given to hormonal factors involved in vitellogenesis (Laufer and Downer, 1988). Although yet to be fully elucidated, this process is known to be controlled by hormonal factors that can be either neuroendocrine or other, such as steroid hormones (Van Herp and Payen, 1991). In previous studies several vertebrate-like steroids were identified in the haemolymph, ovary and mandibular organ of decapods (Couch *et al.*, 1987; Fairs *et al.*, 1989) and were shown to be biologically active. The *in vivo* stimulation of vitellogenesis in marine penaeid prawns by progesterone (Kulkarni *et al.*, 1979; Van Herp and Payen, 1991) and in fresh water species by progesterone (Sarajoni *et al.*, 1985) or by estrone and 17β-estradiol (Sarajoni *et al.*, 1986) was reported. Also, 17α-hydroxyprogesterone was demonstrated to stimulate vitellogenesis in marine penaeids (Nagabhushanam *et al.*, 1980; Yano, 1987) and to increase oocyte diameter in *Litopenaeus vannamei* (Tsukimura and Kamemoto, 1988). Nevertheless, other researchers reported lack of response to either 17α-hydroxyprogesterone or 17β-estradiol as vitellogenin stimulating hormones in *Penaeus esculentus* (Koskela *et al.*, 1992).

Steroid hormones were identified in various tissues and in the haemolymph of several decapod crustaceans. In the ovary of *Penaeus monodon*, estrogens such as estrone and 17β-estradiol were detected by means of gas chromatography/mass spectrometry with selected ion monitoring (GC/MS; SIM) (Fairs *et al.*, 1990). The same steroids were shown to be present in *Parapenaeus fissurus* using radioimmunoassay (RIA) (Jeng *et al.*, 1978). Also using RIA, 17β-estradiol was detected in the ovary of mature females of *Homarus americanus* (Couch *et al.*, 1987). Additionally, progesterone and related compounds were found in several other crustacean species (Couch *et al.*, 1987; Fairs *et al.*, 1989; Kanazawa and Teshima, 1971; Ollevier *et al.*, 1986). Testosterone was detected in *Parapenaeopsis stylifera* (Nagabhushanam *et al.*, 1980) and in the haemolymph and testis of *H. americanus* by means of RIA (Burns *et al.*, 1984b). Several steroids were identified in the crayfish *Pontastacus leptodactylus* (Ollevier *et al.*, 1986) and in the haemolymph, eggs and ovary of *Nephrops norvegicus* (Fairs *et al.*, 1989) using GC/MS with SIM.

Steroid hormone synthesis in the ovary has already been demonstrated (Kanazawa and Teshima, 1971; Teshima and Kanazawa, 1971) and although little is known of its action, it has been proposed that vitellogenesis in crustaceans is under control of steroid hormones (Quackenbush, 1992). Based upon initial studies made in the ovary of the amphipod *Orchestia gammarellus* (Junera *et al.*, 1977), it has also been hypothesised that the ovary contains a vitellogenin stimulating ovarian hormone (VSOH), playing a similar role to that of 17β-estradiol in egg laying vertebrates. It was also hypothesised that this VSOH could be a steroid hormone (Van Herp and Payen, 1991). A putative role of VSOH has been attributed to several steroid hormones such as progesterone (Kulkarni *et al.*, 1979; Yano, 1985), 17α-hydroxyprogesterone (Nagabhushanam *et al.*, 1980; Tsukimura and Kamemoto, 1988; Yano, 1987) and 17β-estradiol (Couch *et al.*, 1987; Fairs *et al.*, 1990).

In former studies, the presence of several important enzyme systems known to be involved in biosynthesis and catabolism of steroids in vertebrates was investigated in crustaceans. Hydroxysteroid hydrogenase (HSD) activity has been observed in all crustacean species' tested (Bjorkhem and Danielson, 1971; Blanchet *et al.*, 1972; Burns *et al.*, 1984a; Callard *et al.*, 1978; Gilgan and Idler, 1967) and particularly 17β-HSD (conversion of androstenedione to testosterone) was shown to be present in substantial concentrations (Swevers *et al.*, 1991). Other enzymes such as 3β-HSD/isomerase (conversion of pregnenolone to progesterone) and C_{17}-C_{20} lyase (conversion of 17α-hydroxyprogesterone to androstenedione) were also detected in the same animal at feeble levels (Swevers *et al.*, 1991). However, no traces of 17α-hydroxylase (conversion of progesterone to 17α-hydroxyprogesterone) and aromatase could be observed (Swevers *et al.*, 1991).

In the present study, the authors aimed to contribute to the clarification of the metabolism of steroid hormones in marine crustaceans. This paper deals with the bioconversion of steroid hormones in the prawn *M. japonicus*, and attempts to determine the presence of four enzyme systems necessary to catalyse the bioconversion of progesterone into 17β-estradiol in the ovary. In addition, a possible role of the hepatopancreas in this bioconversion process was also assessed.

MATERIAL AND METHODS

Animals

Female shrimp *Marsupenaeus japonicus*, in different stages of maturation, were obtained from the shrimp farm EURODAQUA located at Castro Marim, Algarve (Portugal). The animals were raised and bred on the shrimp farm and kept in earth pounds containing salt water under natural photoperiod. Shrimp groups were selected by the apparent maturation stage based on external observation of ovary morphology (Tan-Fermin, 1989). Body length (average length 15.3±0.8 cm) and weight (average weight 27±6 g) were recorded, and ovaries and the hepatopancreas were collected and subsequently processed for the *in vitro* tissue incubation, histology, reversed-phase high-performance liquid chromatography (RP-HPLC) and radioimmunoassay (RIA) analysis.

Chemicals

Authentic steroids were obtained from Sigma (St. Louis, MO). All solvents were Lichrosolv grade, purchased from Merck KGaA, (Darmstadt, Germany). Kits for RIA were purchased from DPC, Diagnostic Products Corporation (Los Angeles, CA).

Histological methods

After dissection, a small piece of ovary was fixed in Bouin's fixative for 24 h and then processed by standard histological methods. Paraffin blocks were sectioned 5 µm thick, and stained with Mayer's hemalumen-eosin. Females were assigned to different experimental groups according to the ovary's maturation stage (Tan-Fermin 1989, 1990). There were two experimental groups, one comprising vitellogenic stages, characterised by the presence of vitellogenic ova (typically rose coloured), and one comprising previtellogenic ovaries in which only oogonia and oocytes with typical previtellogenic violet coloration could be observed. Stages in which cortical rods could be observed were included in the vitellogenic group. Post spawning stages were not considered for this study. The images representative of each of the stages mentioned above are shown in Figure 1.

In vitro incubation of the ovary and hepatopancreas

The ovary and hepatopancreas of *M. japonicus* were incubated *in vitro* in the presence of steroid hormone precursors. The incubation procedure was adapted from Monteiro *et al*. (2000) and Swevers *et al*. (1991). The ovaries, weighing 0.15±0.06 g in stage II, 1.86±0.33 g in stage III and 2.83±0.16 g in stage IV, as well as hepatopancreas (0.9±0.2 g for all stages), were dissected in two similar portions and lightly minced in a 0.1 M phosphate buffer (0.25 M in sucrose) with a pH of 7.4. Half of the organ was used as a control and the other half was incubated with the steroid hormone precursor (progesterone, 17α-hydroxyprogesterone or testosterone dissolved in propylene glycol) at the final concentration of 5 µM. Incubations were carried out at room temperature and stopped after 8 hours by addition of ethanol. After extraction from the incubation medium, steroid hormone fractions were first isolated by RP-HPLC and then quantified by RIA.

Extraction and purification of steroid hormones

Samples were kept in absolute ethanol at -20°C until used. Steroid hormones were obtained from the incubation medium by a liquid-liquid extraction in dichloromethane/water (3:1 v/v, 3x8 mL). The efficiency of extraction for this method was calculated to be approximately 90% when known concentrations of standard steroids were used. Dichloromethane fractions were concentrated under a stream of nitrogen. Cold acetone was added to the crude extract and left overnight at -20°C to precipitate lipids. Supernatant was collected, evaporated under a stream of nitrogen, dissolved in 100 µL of ethanol and filtered through a 0.2 µm membrane prior to injection on the RP-HPLC system.

Preliminary steroid isolation by RP-HPLC

A Varian Star Workstation system was used, consisting of a Varian Star 9012 Solvent Delivery System, a manual injection valve from Rheodyne, Model 7161, equipped with an injection loop of 100 µL and a Varian Polychrome / PolyView 9065 Diode Array detector with a 4.7 µL cell. The effluent was monitored at 254 nm using the Varian LC Star Workstation Software, version C2: eluents were acetonitrile and deionised water, using a gradient of 33% to 100% of acetonitrile for 45 min with a flow of 1 mL/min in a Nucleosil 100-5 C18 column (125 x 4 mm i.d., 5 µm particle size, from Macherey-Nagel). The column was kept at 45°C in a Column Thermostat from Spark Holland (Mistral version 1.3). Authentic steroids from Sigma

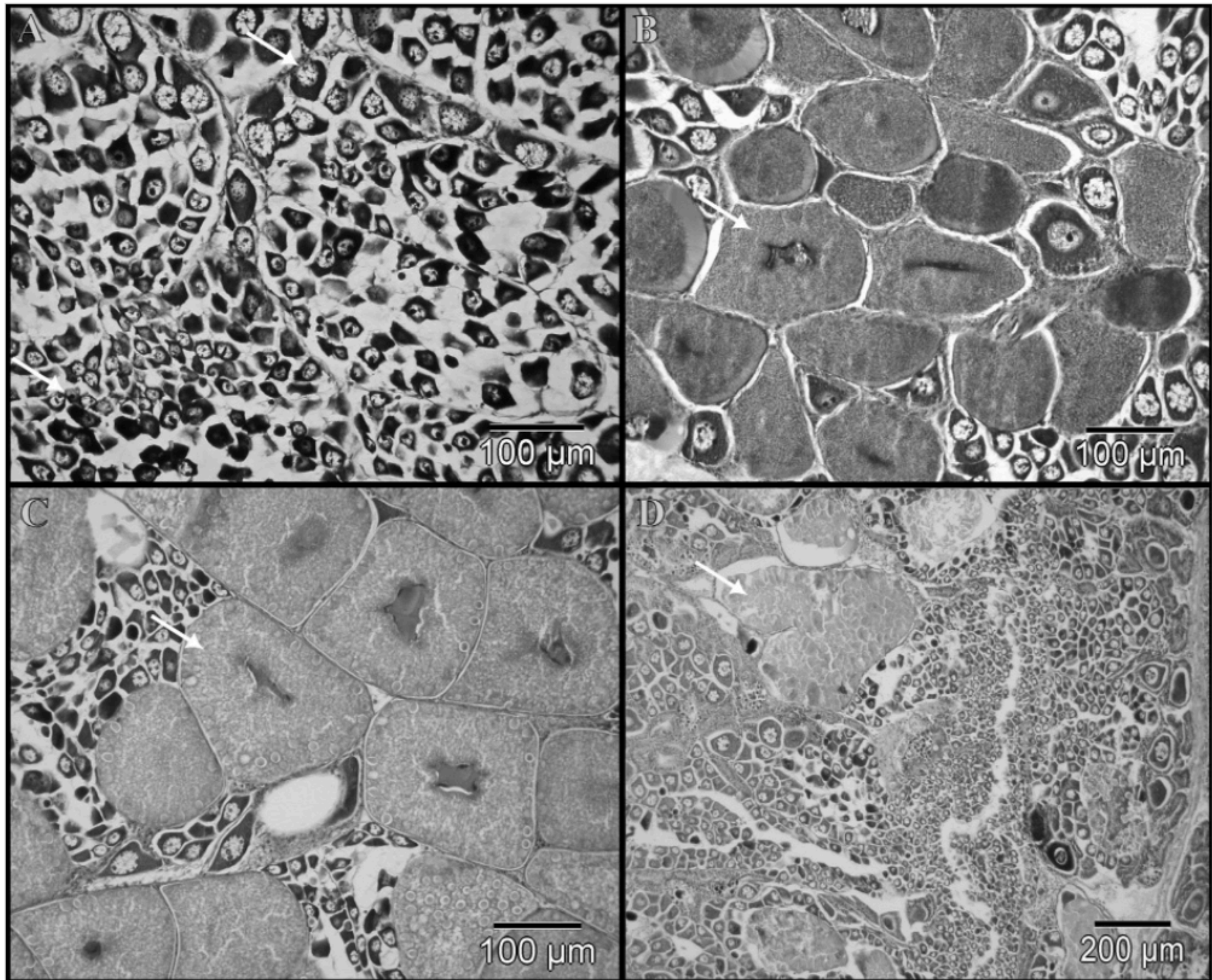

FIG. 1. – Haematoxylin and eosin stained 5 µm sagittal section through the ovary *Marsupenaeus japonicus*. Scale bars are shown in each image. A, previtellogenic ovary showing oogonia and oocytes, females at this stage of maturation were assigned to the previtellogenic experimental group; B, vitellogenic ova presenting yolk accumulation at the cytoplasm. Females at this stage were assigned to the vitellogenic experimental group; C, completely matured ovaries, peripheral cortical rods (cr, pointed by white arrows) could be observed; D, ovary representative of a post spawning stage, residues of lipids are observed (pointed by arrows). A new cycle restarts with the development of oogonia and immature oocytes.

were used to attempt an identification of the steroids extracted from the incubation medium. This identification was based not only on the retention times, but also on the UV spectra of the authentic steroids. Matches were achieved using the Varian Star Workstation Software PolyView Section. Fractions containing the steroids 17β-estradiol, testosterone and 17α-hydroxyprogesterone were collected for quantification by RIA. This previous isolation was performed to diminish the risk of cross-reactions when using radioimmunoassay. Fractions were then lyophilised and dissolved in gelatine buffer (12.5 mM NaH_2PO_4, 42.3 mM Na_2HPO_4, 164.8 mM NaCl, 0.1% Azide and 0.1% gelatine, pH 7.4).

Radioimmunoassay

The solid-phase RIA was performed according to the instructions included in the Diagnostic Products Corporation kits to quantify 17β-estradiol, testosterone and 17α-hydroxyprogesterone. Radioactivity in tubes was measured using a 1275 Minnigamma Gamma counter (LKB Wallac). Conversion rates ranged from 0.02 to 0.05%.

Statistical Analysis

Differences between the level of synthesised steroids in control and in the precursor incubations were tested at a significance level of 5% using the

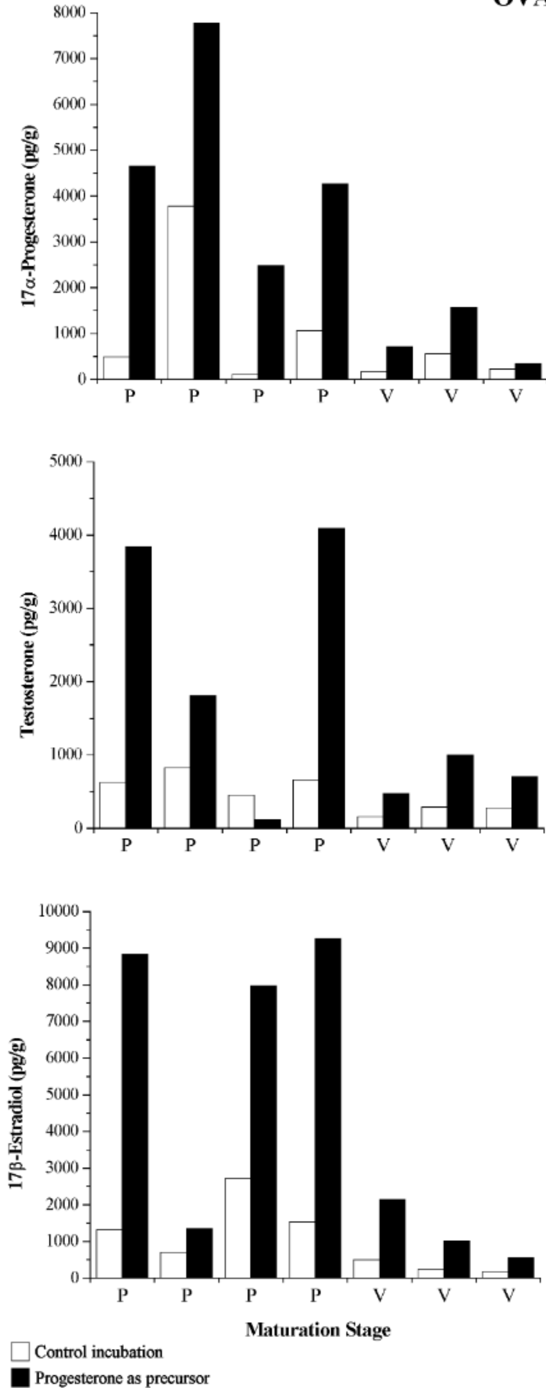

FIG. 2. – Concentrations of 17α-hydroxyprogesterone, testosterone and 17β-estradiol in the *in vitro* incubations of *Marsupenaeus japonicus* ovarian tissue in the presence or absence (control incubation) of progesterone as a steroid precursor. Values are expressed in pg/g of wet weigh. Each pair of columns corresponds to one animal. P, previtellogenic females; V, vitellogenic females. Controls are significantly lower ($p<0.05$) for 17α-hydroxyprogesterone, ($p<0.01$) for testosterone and 17β-estradiol.

non-parametric Wilcoxon matched pairs test. All tests were performed using the Statistica 5.5 software (Satsoft, Inc. 1999 edition).

RESULTS

The maturation stage of each female was determined by histology. Animals were assigned to the previtellogenic group when the ovary did not have any vitellogenic ova, as represented in Figure 1A. Animals with ovaries containing vitellogenic-maturing ova (Fig. 1B), or mature ova in which cortical rods were already observed at the periphery of the cell (Fig. 1C) were assigned to the vitellogenic experimental group. Animals that had already ovulated were not considered for this study (Fig. 1D).

The concentrations of 17α-hydroxyprogesterone, testosterone and 17β-estradiol obtained in the incubations of the ovarian tissue in the presence or absence of a steroid hormone precursor (control

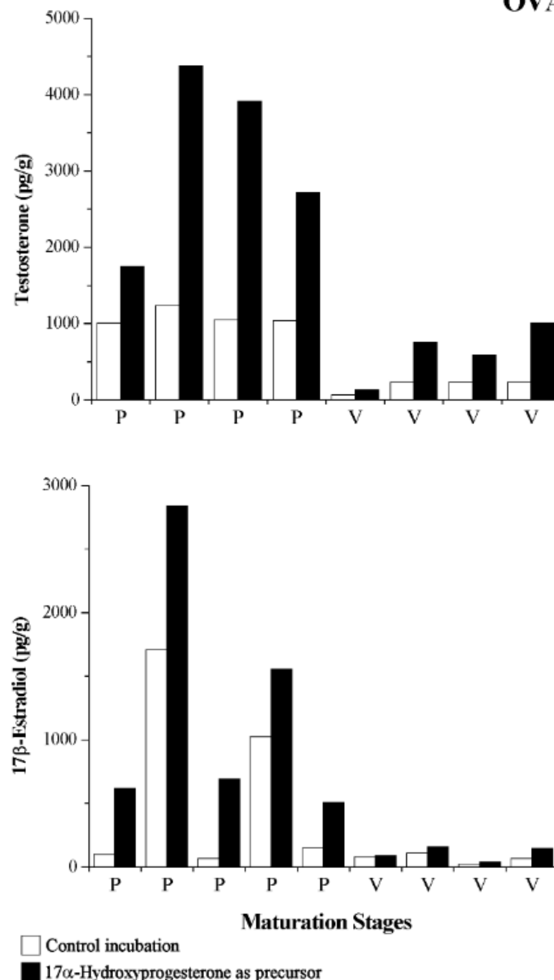

FIG. 3. – Concentrations of testosterone and 17β-estradiol in the *in vitro* incubations of *Marsupenaeus japonicus* ovarian tissue in the presence or absence (control incubation) of 17α-hydroxyprogesterone as a steroid precursor. Values are expressed in pg/g of wet weigh. Each pair of columns corresponds to one animal. P, previtellogenic females; V, vitellogenic females. Controls are significantly lower ($p<0.05$) for both for testosterone and 17β-estradiol.

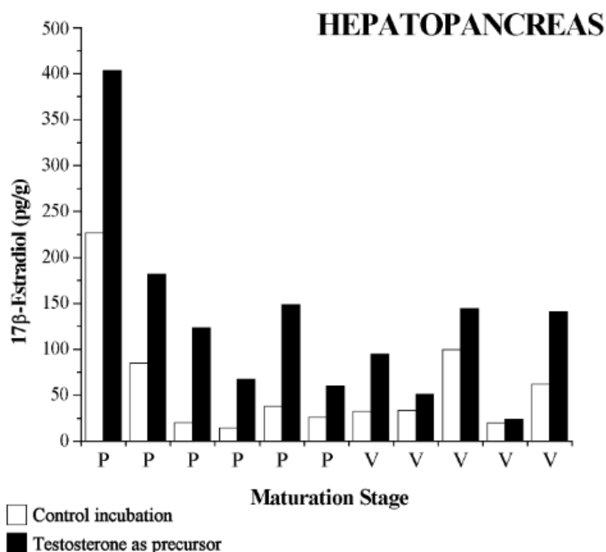

FIG. 4. – Concentration of 17β-estradiol in the *in vitro* incubations of *Marsupenaeus japonicus* hepatopancreatic tissue in the presence or absence (control incubations) of testosterone as a steroid precursor. Values are expressed in pg/g of wet weigh. Each pair of columns corresponds to one animal. P, previtellogenic females; V, vitellogenic females. Controls are significantly lower: ($p<0.01$).

incubations) are presented in Figures 2 and 3. Compared to control, the concentrations were significantly higher for 17α-hydroxyprogesterone ($p<0.05$); testosterone ($p<0.01$) and 17β-estradiol ($p<0.01$) when progesterone was used as precursor in the incubation medium (Fig.2). When 17α-progesterone was used as precursor, both testosterone and 17β-estradiol concentrations were also significantly higher than control ($p<0.05$), as demonstrated in Figure 3. However, it is evident that compared to previtellogenic stages, females in vitellogenic stages had lower levels of all measured steroid hormones.

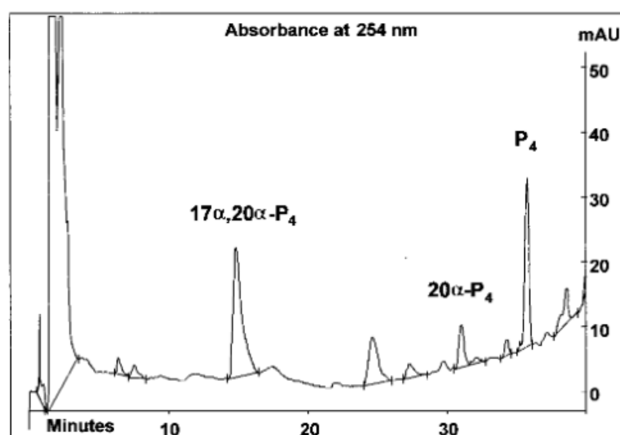

FIG. 5. – Typical RP-HPLC elution profile of the extract obtained from the medium of the ovarian tissue incubation using progesterone as a precursor (injected volume was 50 µL). Effluent was monitored at 254 nm, eluents were acetonitrile/water, flow of 1 mL/min for 45 min, in a C_{18} column (125 x 4 mm i.d., 5 µm particle size) at 45°C. Major detected steroids were progesterone (P_4); 20α-dihydroprogesterone (20α-P_4); and 17α-hydroxy-20α-dihydroprogesterone (17α,20α-P_4).

In the hepatopancreatic tissue incubations, only the level of 17β-estradiol was observed to be significantly higher ($p<0.01$) than the respective controls when testosterone was used as a precursor (Fig.4). No significant differences could be observed for the other assayed precursors, progesterone and 17α-hydroxyprogesterone. The profiles obtained for control incubations were higher than or very similar to the values obtained in the presence of the steroid precursor. However, there was no evidence of differences in steroid hormonal level related to the maturation stage in the hepatopancreas.

A typical RP-HPLC elution profile is included to show that the major metabolites of progesterone found in this study, both in controls and precursor incubations, were progesterone, 20α-dihydroprogesterone and 17α-hydroxy-20α-dihydroprogesterone (Fig. 5).

DISCUSSION

In the present study, the ability of two different tissues of the shrimp *M. japonicus*, ovary and hepatopancreas were tested for the ability to metabolise vertebrate-like steroid hormones. As pathways for synthesis of 17β-estradiol are remarkably similar through all classes of vertebrates (Kime, 1987), we looked for the intermediate steroid metabolites usually found when the conversion of 17β-estradiol from its early precursor, progesterone, takes place. This process requires action of the following enzyme systems: 17α-hydroxylase, C_{17}-C_{20} lyase, 17β-hydroxysteroid dehydrogenase (HSD) and aromatase. It is important to point out that the activities of 17α-hydroxylase and aromatase had not been detected in crustacean tissues before. Only 17β-HSD substantial concentrations had been detected in these species (Swevers *et al.*, 1991).

Using progesterone as a precursor in the *in vitro* incubations of the ovarian tissue, we obtained mainly 20α-dihydroprogesterone and 17α-hydroxy-20α-dihydroprogesterone, which confirms previous reports in another crustacean species (Swevers *et al.*, 1991; Young *et al.*, 1992) in which 20α-dihydroprogesterone was also shown to be the major metabolite produced. The concentrations of 17α-hydroxyprogesterone, testosterone and 17β-estradiol detected in these incubations of ovarian tissue

indicate the presence in this organ of "the four-enzyme system" necessary to convert steroid hormones from progesterone into 17β-estradiol. In addition, whith 17α-hydroxyprogesterone as a precursor, testosterone and 17β-estradiol were also shown to be produced, confirming the results obtained with progesterone as a precursor. As high concentrations of precursors were used, the obtained bioconversion rates were relatively low but, as shown in Figures 2, 3 and 4, the presence of precursors caused a significant increase in the concentrations of the considered steroids. Levels of steroid hormones measured in the control incubations were comparable to previously reported physiologic values (Fairs *et al.*, 1990).

In the previous study (Young *et al.*, 1992), an alternative pathway for the metabolism of progesterone in the ovary of *P. monodon* was proposed in which progesterone *in vitro* metabolites were mainly 5α-pregnanes and 20α-dihydroprogesterone. However, the endogenous presence of these 5α-pregnanes was never demonstrated, and this pathway may be an effect of the non-physiologic doses of precursor used by Young *et al.* (1992). Detection of Δ5 steroids was not considered in the present study, so we could not make a comparison to that alternative pathway. Nevertheless, the production of 20α-dihydroprogesterone as the major metabolite was consistent with our results.

In our studies, the incubations of the ovarian tissue with 17α-hydroxyprogesterone showed, independently of some natural occurring individual variability, that values of both testosterone and 17β-estradiol are higher in early stages of development (previtellogenic group) than in maturing or fully matured animals (vitellogenic group). The level of 17β-estradiol has already been shown to be maximal in animals with developing ovaries and not in mature animals (Couch *et al.*, 1987). In the present study, it was observed that a strong decrease in aromatase activity accompanies the late stage of maturation, implying a strong decrease in 17β-estradiol levels and consequent accumulation of testosterone. Also, other authors (Fairs *et al.*, 1989; Sarajoni *et al.*, 1985) have reported peaks of androgens in post- and pre-vitellogenic ovaries, associating high levels of androgens with inhibition of vitellogenesis (Fairs *et al.*, 1990). This apparent decrease in aromatase activity during late vitellogenic stages may justify the fact that this enzyme was not detected in previous studies (Swevers *et al.*, 1991), in which only animals in an advanced maturation stage were used.

In crustaceans, the hepatopancreas is an organ comparable to the liver in vertebrates, so it is expected to parallel most of its functions and presumed to play an important role in the catabolism of steroid hormones. Moreover, the crustacean hepatopancreas is well known to play a role in vitellogenesis, by producing vitellogenin during exogenous vitellogenesis (Van Herp and Payen, 1991). In the present study, concerning *in vitro* incubations of the hepatopancreatic tissue, no activity of "the four desired enzyme system" was observed when progesterone or 17α-hydroxyprogesterone were used as precursors. Only when testosterone was used as a precursor high levels of 17β-estradiol were obtained, evidencing the presence of active aromatase in this tissue. It is important, however, to note that it was possible to detect by means of HPLC the synthesis of large quantities of 20α-dihydroprogesterone and 17α-hydroxy-20α-dihydroprogesterone when progesterone was used as a steroid precursor, evidencing 3β-HSD/isomerase and hydroxylase activities. The fact that progesterone was converted into 20α-dihydroprogesterone and 17α-hydroxy-20α-dihydroprogesterone, but not into 17α-hydroxyprogesterone, may result from the physiological role of the hepatopancreas in the catabolism of steroid hormones, by which it activates different bioconversion pathways. Apparently, the enzyme activity of C_{17}-C_{20} lyase is not present in the hepatopancreas of *M. japonicus*. The presence or absence of 17β-HSD in this organ could only be determined if androstenedione were used as a precursor in the hepatopancreatic tissue incubations.

Considering the apparent decrease in ovarian aromatase activity during late exogenous vitellogenesis and the sustained levels of activity for this enzyme throughout the reproductive cycle in the hepatopancreas, one may suggest that the testosterone produced in the ovary during this stage, could be transported to the hepatopancreas and metabolised into 17β-estradiol. If, in fact, 17β-estradiol was the presumed VSOH, then this mechanism would provide the necessary 17β-estradiol to maintain exogenous vitellogenesis, paralleling the role of 17β-estradiol in egg-laying vertebrates.

In conclusion, the present study provides evidence that the ovary of the shrimp *M. japonicus*, is capable of synthesising 17β-estradiol from progesterone, evidencing the presence of 17α-hydroxylase, C_{17}-C_{20} lyase, 17β-hydroxysteroid dehydrogenase (HSD) and aromatase. In the hepatopancreas, aromatase, 3β-HSD and hydroxylase enzyme activities

were detected, but not that of C_{17}-C_{20} lyase. In future studies, the metabolism of conjugated forms of steroids in these organs will be assayed in order to obtain a more detailed illustration of the enzyme systems involved in this process. The present results, together with former *in vivo* and *in vitro* works showing the endogenous presence of steroids and their ability to induce vitellogenesis, encourage us to propose that in fact there is a physiologic role for these hormones in the crustacean species, most probably related to the maturation process.

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