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The stage of ovarian development affects organ expression of vitellogenin as well as the morphometry and ultrastructure of germ cells in the freshwater prawn *Macrobrachium amazonicum* (Heller, 1862)

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Abstract

The objective was to characterize vitellogenin expression in the ovary and hepatopancreas, and to describe the morphometry and ultrastructure of oocytes, in the freshwater prawn *Macrobrachium amazonicum* at various stages of ovarian development. Five ovarian stages were defined: (I) immature, (II) maturing, (III) mature, (IV) spawned, and (V) reorganized. Ovaries and hepatopancreas were analyzed by immunohistochemistry for vitellogenin expression. Vitellogenin expression in both ovary and hepatopancreas was predominantly widespread, beginning at Stage I, peaking at Stage III, and decreasing in Stages IV and V. Characterization of the ovary included measurement of the following germ cell types: oogonia (OG), and previtellogenic (PV), early vitellogenesis (EV), advanced vitellogenesis (AV), and mature (M) oocytes. Mean ± SD diameter of OG and EV oocytes in Stages I (14.2 ± 5.5 and 119.8 ± 15.7 μm) and II (17.9 ± 4.8 and 114.3 ± 34.6 μm), respectively, were significantly different from that in Stages IV (16.6 ± 4.7 and 107.0 ± 24.6 μm) and V (14.4 ± 4.1 and 101.0 ± 25.2 μm). Both scanning and transmission electron microscopy enabled identification of EV, AV and M oocytes based on the presence of a nucleus, and the organization and distribution of yolk in the cytoplasm. In summary, vitellogenesis occurred both in the hepatopancreas and ovary, with the ovary expressing vitellogenin starting as early as Stage I. This process promoted accumulation of yolk and growth of oocytes, thus favoring sexual maturation of females. This knowledge may be applied to improve production of farmed *M. amazonicum*.

Keywords: Prawn; Yolk; Morphology; Ovarian stages; Vitellogenesis

1. Introduction

Vitellogenesis is the process that occurs during female reproduction in which the oocyte accumulates large quantities of yolk [1,2]. In crustaceans, the main yolk proteins are vitellogenin and vitellin [3,4]. Vitellogenin synthesis was reported to occur in the ovary as well as in extraovarian tissues, such as adipose tissue [5–9] and hepatopancreas [4,10–14], followed by its release into hemolymph, and absorption and processing by the oocyte to form vitellin [14,15].

Vitellogenin is essential for development of prawn oocytes, embryos, and early larvae, as well as to ensure survival of the young until they are self-supporting [9,15]. Limited immunohistochemical studies have addressed the

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dynamics and localization of vitellogenin during oocyte maturation in *Macrobrachium rosenbergii* [16,17]. However, such studies have not been performed in *Macrobrachium amazonicum* (Heller, 1862), a freshwater prawn species native to the Amazon region that is reproductively active year-round [18]. It has a great potential for culture, because of fast growth, resistance to disease, and ease of management [19,20]. Therefore, this species is extensively exploited by artisan fisheries, mainly in the North and Northeast of Brazil [21]. In fact, it is considered the South American prawn species with the greatest potential for aquaculture [22].

The aims of the present work were to characterize vitellogenin expression in the ovary and hepatopancreas, as well as to describe ovarian structure based on morphometry and ultrastructure of oocytes in the freshwater prawn *M. amazonicum* at various stages of ovarian development.

2. Materials and methods

2.1. Animals

Freshwater prawns of the species *M. amazonicum* were collected monthly from January to December 2007, in the Northeastern state of Pará, Brazil (1°13'25''S, 48°17'40''W). Live prawns were taken to the laboratory, sexed, and females were classified on the basis of the size of their ovary as observed through the external carapace, following a scoring system adapted from the literature [23]. Accordingly, five ovarian stages were defined: (I) immature, (II) maturing, (III) mature, (IV) spawned, and (V) reorganized. The animals were anesthetized (by placing them on ice) and then killed. The ovaries and hepatopancreas were dissected and processed as described below.

2.2. Immunohistochemistry

Whole ovary and hepatopancreas samples were fixed in 4% paraformaldehyde solution for 24 h and submitted for routine histological processing. Fixed samples were embedded in paraffin, cut in 5-μm thick histological sections and deparaffinized in decreasing concentrations of ethanol. Sections were then rinsed in 0.1 M phosphate buffered saline (PBS), and boiled in 0.1 M sodium citrate buffer for 30 min at 70°C for antigen retrieval. After cooling, samples were incubated in 3% hydrogen peroxide in methanol for 30 min at 27°C. Sections were then washed in PBS and blocked with 10% normal goat serum (16210072, Invitrogen, Burlington, ON, Canada) for 1 h. After washing in PBS, samples were incubated in a rabbit anti-salmon vitellogenin polyclonal primary antibody (1:50; V01402201, Biosense Laboratories AS, Bergen, Norway) overnight at 4°C. Samples were washed again in PBS, and then incubated in a peroxidase-conjugated anti-rabbit IgG secondary antibody (1:500; PA1-28564, Bioamerica, Inc., São Paulo, SP, Brazil) for 2 h at room temperature. Washing in PBS was followed by visualization of reactive sites with DAB (ImPACT DAB Peroxidase substrate SK4105, Vector Laboratories, Inc., Burlingame, CA, USA) and slide evaluation using a photomicroscope (Zeiss Axiostar Plus, Oberkoshen, Germany). Negative controls were incubated in PBS instead of primary antibody, followed by incubation in secondary antibody.

2.3. Morphometry of oocytes

Whole ovaries, in various stages of development, were fixed in Bouin's solution for 24 h and subjected to routine histological processing. Samples were embedded in paraffin, and 5 μm thick longitudinal sections of the median region of the gonad were stained with hematoxylin and eosin. Samples from three animals at each of the ovarian stages defined above were selected. Based upon previously reported criteria [24], slides were scored based on the following five germ cell types: oogonia (OG), previtellogenic oocytes (PV), early vitellogenesis oocytes (EV), advanced vitellogenesis oocytes (AV), and mature oocytes (M). In addition, only germ cells displaying a nucleus and nucleoli were measured. Slides were evaluated under an Olympus CH30 microscope (Melville, NY, USA), and measurements were done with an ocular micrometer coupled to a 10× eyepiece and a 40× objective adjusted for a correction factor.

2.4. Statistical analysis

Oocyte diameter means were analyzed by ANOVA, and Tukey's post-test was applied for mean separation when significant differences were detected. Differences were considered significant at $P \leq 0.05$. Data were analyzed with Bioestat 5.0 software (Sociedade Civil Mamirauá, Belém, PA, Brazil) [25].

2.5. Scanning electron microscopy (SEM)

Fragments of ovarian tissue were fixed for 24 h at 4°C in Karnovsky's solution (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). Thereafter, tissues were post-fixed in 1% osmium tetroxide solution buffered with sodium cacodylate (0.1 M, pH 7.2) for 2 h at room temperature. The fragments were then dehydrated in a graded ethanol series, and critical point dried using CO_2. Specimens were mounted on stubs,