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Ovarian differentiation and development in cachara *Pseudoplatystoma fasciatum*

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One thousand five hundred cachara or tiger shovelnose catfish *Pseudoplatystoma fasciatum*, obtained from induced reproduction, were used to determine the onset of ovarian differentiation and development and to record the main characteristics of this process. Samples were collected from 0 to 240 days post-fertilization (dpf) and the results classified into stages I–XII. Ovarian formation was histologically detected for the first time when juveniles measured mean ± s.d. 51·5 ± 8·3 mm total length (L_T) at 39–45 dpf (stages I–V), with intense somatic cell proliferation originating in the ovarian cavity. Both L_T and age of fish had a positive correlation ($P < 0{\cdot}001$) with ovarian differentiation, but L_T showed a greater correlation ($r^2 = 0{\cdot}95$) than age ($r^2 = 0{\cdot}85$), especially during the initial stages of development. From stages VI to VII, the ovarian cavity was enlarged and undifferentiated oogonia were present. At stage VIII, small projections formed in the ovarian stroma towards the ventral region of the gonad (future ovarian lamellae) and the basal membrane and differentiated oogonia nests could be seen. At stages IX and X, the germ cells entered meiosis and folliculogenesis was completed by stages XI and XII, which can be considered late in comparison to other Siluriformes. This study has demonstrated that ovarian differentiation in *P. fasciatum* begins with an intense proliferation of squamous epithelial cells (somatic cells) during the early stages of development and that sex inversion protocols could, thus, be applied successfully before this period. Furthermore, the results have demonstrated that both size and age can influence gonad differentiation and development in this species.

Key words: age; folliculogenesis; germ cells; size; somatic cells.

INTRODUCTION

Pseudoplatystoma fasciatum (L. 1766), commonly known in Brazil as cachara, and known worldwide as tiger shovelnose catfish, is a large fish of the order Siluriformes. Native to the hydrographical basins of South America, it occupies a unique ecosystem, the Pantanal (Paraguay rivers tributaries), which is shared by Brazil, Bolivia, Paraguay and Argentina (Roubach *et al.*, 2003). It is also of great importance to aquaculture, mainly because of the meat quality and growth performance (Roubach *et al.*, 2003; Campos, 2005). Another important characteristic of this species is the sexual dimorphism of the juveniles. The females are larger and grow faster than males (Romagosa

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et al., 2003), suggesting that monosex female rearing could potentially increase production in captivity.

In teleosts, the mechanisms that control sex determination and differentiation, which lead to differences in morphological, physiological and behavioural traits are highly diverse and usually governed by genetic and environmental factors (Volff *et al.*, 2007; Stelkens & Wedekind, 2010). Much evidence indicates that in many fish species with genetic sex determination, environmental factors such as temperature may overwhelm the effect of genetic factors at the edge of the temperature tolerance threshold (Baroiller *et al.*, 2009; Piferrer *et al.*, 2012). Environmentally induced sex inversion is widely used in aquaculture to increase the production of the gender that has higher growth rates or to prevent energy destined for production from being diverted to the fattening process (Devlin & Nagahama, 2002).

Histological studies on gonad differentiation are thus of great importance for developing useful methods and tools aimed at prioritizing the gender of the species of interest to aquaculture (Hunter & Donaldson, 1983; Foyle, 1993; Strussmann *et al.*, 1996*b*; Nakamura *et al.*, 1998).

The aim of this study was to histologically describe the process of ovarian differentiation and development in *P. fasciatum* and identify the timing of sexual differentiation and its characteristics, such as the proliferation of somatic and germ cells, the formation of specific arrangements of somatic cells that originate in the ovarian cavity, the meiotic process of the germ cells and folliculogenesis.

MATERIALS AND METHODS

ANIMALS

Larvae for this study were obtained from the Centro de Aquicultura da Universidade Estadual Paulista – UNESP (CAUNESP) and from Mar & Terra Fisheries. At 15 days post-fertilization (dpf), the larvae were transferred from incubators and kept under continuous water circulation at densities of between 4 and 6 fish l^{-1}. The physico-chemical variables of the water were monitored and the fish were fed a commercial extruded diet containing 40% crude protein six times a day (to apparent satiation) throughout the experiment. Fish were sampled every 3 h up to 24 h post-fertilization (hpf), followed by collection every 6 h up to 48 hpf and daily until 6 dpf. Subsequently, samples were taken at 13, 20, 27 39, 45, 57, 70, 100, 113, 120, 150, 180 and 240 dpf. The fish were euthanized by prolonged anaesthesia with 0·1% benzocaine. The total length (L_T) of larvae was measured to the nearest 0·01 mm and juveniles > 10 cm were measured to the nearest 1 mm. Body mass (M; g) was determined using an analytical balance.

SAMPLE PREPARATION FOR LIGHT AND ELECTRON MICROSCOPY

Whole larvae, the medial region of body of juveniles and the gonad tissue (when anatomically discernible) were used for light and electron microscopy analysis.

Samples destined for light microscopy were fixed in Karnovsky solution (2% glutaraldehyde and 4% paraformaldehyde solution in Sorensen buffer, 0·1 M, pH 7·2) (Karnovsky, 1965) for at least 24 h. After fixation, samples were photographed using a MZ8 stereomicroscope equipped with a LEICA DFC 280 camera (www.leica-microsysytems.com) and subjected to analysis.

For light microscopy, samples were embedded in histosec, cut into 5 µm sections and stained with haematoxylin and eosin, while the remaining samples were embedded in historesin (Kit Leica HistoResin; www.leicabiosystems.com), cut into 3 µm sections and stained with

TABLE I. Mean ± S.D. body mass (M) and total length (L_T), and age (days post-fertilization, dpf) and sexual development rate (%) of *Pseudoplatystoma fasciatum* juveniles during gonad differentiation and development. Number of individuals: 39 to 45/60 to 70 dpf ($n = 100$); 100 to 113/120 to 150 dpf ($n = 50$); 180 dpf ($n = 30$); 240 dpf ($n = 30$)

Biometric data			Sexual development rate (%) juveniles		
Juveniles			Gonads		
M (g)	L_T (mm)	Age (dpf)	Undifferentiated	Female	Male
0·66 ± 0·26	51·5 ± 8·3	39–45	63·0	17·0	20·0
1·96 ± 0·65	67·7 ± 7·9	60–70	37·5	37·5	25·0
4·35 ± 0·98	81·2 ± 15·3	100–113	–	40·0	60·0
12·49 ± 2·08	124·7 ± 11·6	120–150	–	35·6	64·4
15·98 ± 2·84	135·7 ± 8·9	180	–	66·7	33·3
119·83 ± 13·27	241·0 ± 8·8	240	–	60·0	40·0
Overall average of sexual development rate (%)				51·0	49·0

haematoxylin–phloxine and using the periodic acid-Schiff + iron haematoxylin + metanil yellow method (Quintero-Hunter *et al.*, 1991). Analysis was performed using a LEICA DM2500 photomicroscope equipped with a LEICA DFC 285 camera and the Leica Application Suite (LAS) software.

For transmission electron microscopy, the gonads were fixed in 2·5% glutaraldehyde, immersed in 1% osmium tetroxide solution for 2 h and dehydrated. Pre-infiltration was performed with araldite resin and acetone (1:1 ratio) and infiltration took place in pure resin. Sections (0·5 μm) were cut using a glass blade and stained with 1% toluidine blue in saturated boric acid. The best blocks were selected for ultra-fine sectioning (70 nm) with a diamond blade and sections contrasted in uranyl acetate and lead citrate before being electron-micrographed with a transmission electron microscope (JEOL 100CX II e JEOL 1010; www.jeol.com).

STATISTICAL ANALYSIS

Data are shown as mean ± S.D. The Lilliefors test (5%) was used to verify a normal distribution. The logarithmic regression equation and the Spearman non-parametric test ($P < 0·05$) were used to analyse the correlation between L_T and age and the stage of development. Statistical analysis was performed using the software SAS (Version 9.0, SAS Institute, Inc.; www.sas.com)

RESULTS

BIOMETRIC DATA, AGE AND SEXUAL DEVELOPMENT

The M (g), L_T (mm) and ages (dpf) of female *P. fasciatum* juveniles throughout gonad development as well as the female:male sex ratio are summarized in Table I.

MORPHOLOGICAL DESCRIPTION OF OVARIAN DIFFERENTIATION AND DEVELOPMENT

L_T and age were positively correlated ($P < 0·001$) to the stages of ovarian differentiation, as illustrated in Fig. 1. L_T was better correlated with ovarian

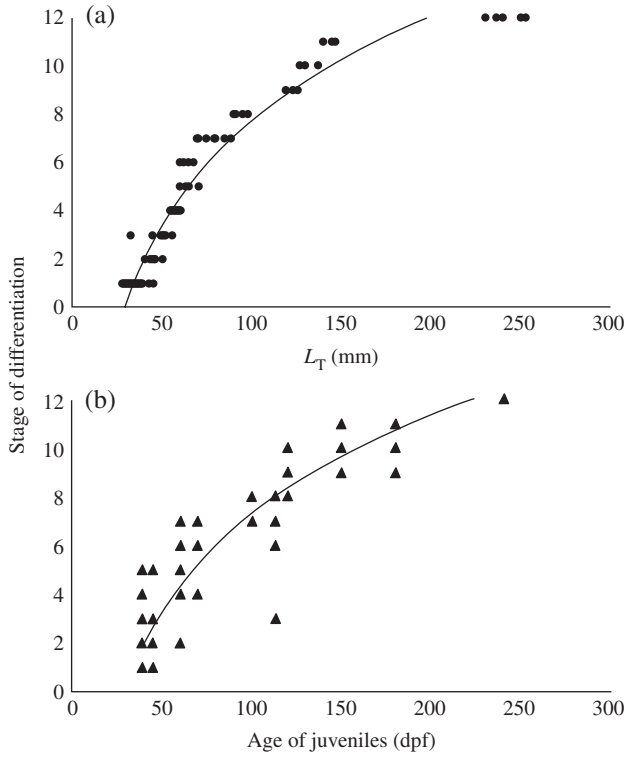

FIG. 1. Correlation between (a) total length (L_T) and (b) age in days post-fertilization (dpf) during the stage of ovarian differentiation of *Pseudoplatystoma fasciatum* juveniles. The curves were fitted by (a) $y = 6{\cdot}2398 \ln x - 6{\cdot}5909$ ($r^2 = 0{\cdot}95$) and (b) $y = 5{\cdot}8045 \ln x - 19{\cdot}387$ ($r^2 = 0{\cdot}85$) (both $P < 0{\cdot}001$).

differentiation ($r^2 = 0{\cdot}95$) than age ($r^2 = 0{\cdot}85$) and the XII stages of ovarian development described in this study were determined by the size and not by the age of the fish.

UNDIFFERENTIATED GONADS

Juveniles (stage I)

Macroscopically, the undifferentiated gonads of juveniles with $L_T = 36{\cdot}0 \pm 5{\cdot}1$ mm were visible as slim elongated structures that were positioned above the kidneys and extended through the whole coelomic cavity, from the urogenital papillae to the gas bladder (Fig. 2).

These gonads comprised two lobules individually suspended by mesenteric projections, which rested above the gas vesicle. The gonads were positioned dorsally to the intestine, ventrally to the kidney, ventro-laterally to the gas bladder and next to a blood vessel. The exterior region was rectilinear and the mesogonadium connected the gonad to the mesentery. The gonads consisted mainly of somatic cells which were randomly distributed and faced different directions [Fig. 3(a)].

The gonads also contained a few primordial germ cells (PGCs), which were individually surrounded by somatic cells. The PGCs were small and oval shaped, but larger

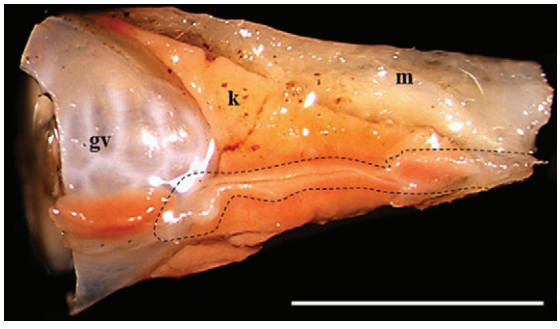

FIG. 2. Stereomicrographs of the medial region of the body of *Pseudoplatystoma fasciatum* juveniles (mean ± S.D. total length, $L_T = 51{\cdot}5 \pm 8{\cdot}3$ mm, stage I). Location of the undifferentiated gonad (.....); k, kidney; gv, gas vesicle; m, muscle. Bar = 0·5 cm.

than the somatic cells. They had a voluminous basophilic nucleus and electro-dense euchromatin. Their cytoplasm, on the other hand, was scarce and acidophilic [Figs 3(a) and 4(a)].

The somatic cells had cytoplasmic extensions, which adhered to the PGCs and surrounded the whole germ cell [Fig. 4(a)]. These somatic cells were found in large numbers throughout the gonad and were of different types and shapes (fusiform, elliptic and round) [Figs 3(a) and 4(b)]. The electron density of the nuclear euchromatin varied according to the characteristics of the cells [Fig. 4(b)].

BEGINNING OF OVARIAN DIFFERENTIATION

Juveniles (stages I–V)

The first morphological signs of gonad differentiation were observed in juveniles measuring $51{\cdot}5 \pm 8{\cdot}3$ mm L_T and aged between 39 and 45 dpf; 63% of the sample had undifferentiated gonads while 17% were females (ovaries) and 20% were males (testes) (Table I). Ovarian differentiation to this point could be divided into five developmental stages (Fig. 5). During this period, a straight ovarian cavity was formed and extended along the mesovarium [Fig. 3(b)]. The presence of somatic cells in the ventral region, close to the ovarian cavity, was also observed [Fig. 3(b)]. The ovary was enveloped by simple squamous epithelium and dense connective tissue, the PGCs transitioned to undifferentiated oogonia and the gonad tissue consisted of isolated undifferentiated oogonia surrounded by a great number of somatic cells [Fig. 3(c)].

Juveniles (stages VI and VII)

During this period, an increase in the ovarian cavity could be observed in juveniles with $L_T = 66{\cdot}6 \pm 7{\cdot}9$ mm [Fig. 3(d)]. The tunica albuginea, which was composed of the ovarian surface epithelium and the adjacent connective tissue, could be seen for the first time on the ovarian cortex [Fig. 3(d)]. Even though the somatic cells surrounded the germ cells, they could be seen perpendicularly to the surface epithelium verging towards the interior of the gonad [Fig. 4(c)]. During these stages, the undifferentiated oogonia remained individualized and surrounded by somatic cells in the gonad stroma [Fig. 3(d), detail].

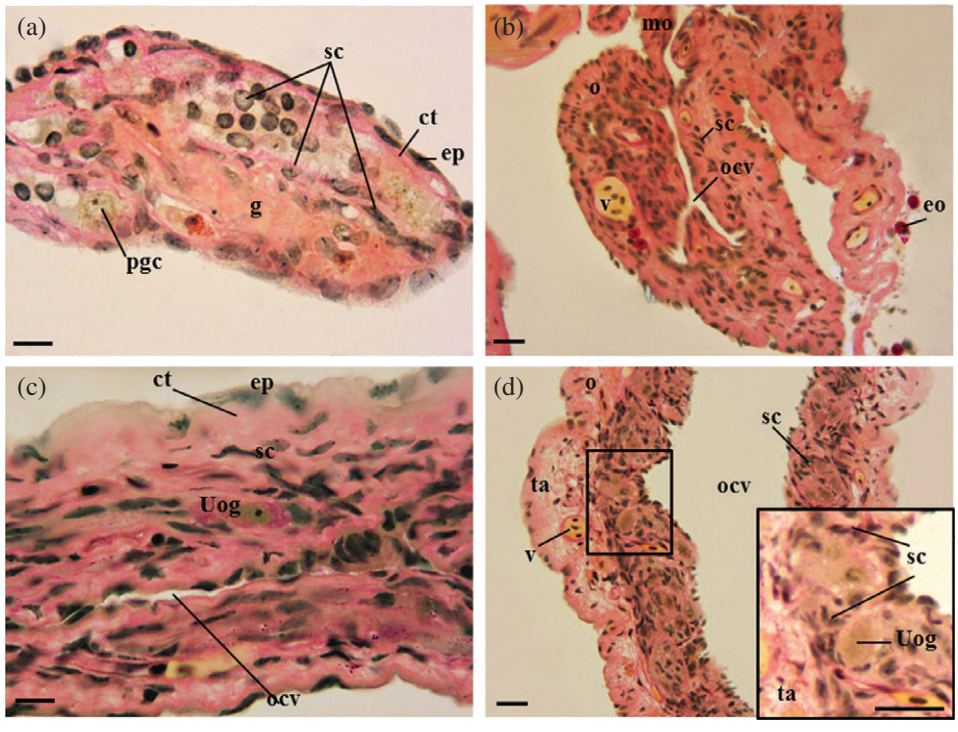

FIG. 3. Photomicrographs of the gonads of *Pseudoplatystoma fasciatum*. (a) Undifferentiated gonads (stage I): longitudinal section of an undifferentiated gonad (g), presence of primordial germ cells (pgc) isolated by somatic cells (sc) and the presence of a serous epithelium composed of simple squamous epithelium (ep) and dense connective tissue (ct) on the external region of the gonad, bar = 10 μm. (b, c) Beginning of ovarian differentiation, juveniles with mean ± S.D. total length, $L_T = 51{\cdot}5 \pm 8{\cdot}3$ mm (stages I–V): (b) ovary (o) showing the beginnings of the ovarian cavity (ocv), somatic cells (sc) directed towards the ovarian cavity (ocv), mesovarium (mo), blood vessels (v) and eosinophils (eo), bar = 20 μm and (c) undifferentiated oogonia (Uog) surrounded by several somatic cells (sc), ovarian cavity (ocv), simple squamous epithelium (ep) and dense connective tissue (ct), bar = 10 μm. (d) Juveniles with $L_T = 66{\cdot}6 \pm 7{\cdot}9$ mm (stages VI and VII): longitudinal section of the ovary (o), tunica albuginea (ta), blood vessels (v), somatic cells (sc) and oogonia distributed in large quantities in the ventral region of the gonad close to the ovarian cavity (ocv), bar = 20 μm. Detail showing undifferentiated oogonia (Uog) and the somatic cells (sc) directed towards the ovarian cavity, bar = 20 μm. Staining: (PAS/IH/MY).

The oogonia nuclei were initially elliptical, but as gonad development progressed they acquired a more rounded shape [Fig. 6(a), (b)]. The nucleus was basophilic [Fig. 3(d)] with moderately condensed euchromatin [Fig. 6(a), (b)]. Clusters of heterochromatin were present near the nuclear membrane and the nucleolus was decentralized. The cytoplasm was dense and dark, probably due to the presence of a great number of mitochondria containing several cristae [Fig. 6(a), (b)].

Juveniles (stage VIII)

At the beginning of this stage, in juveniles with $L_T = 81{\cdot}2 \pm 15{\cdot}3$ mm, small projections (future lamellae) could be seen forming from the tunica albuginea towards the ovarian cavity [Fig. 7(a)]. Mitotic proliferation of undifferentiated oogonia could also

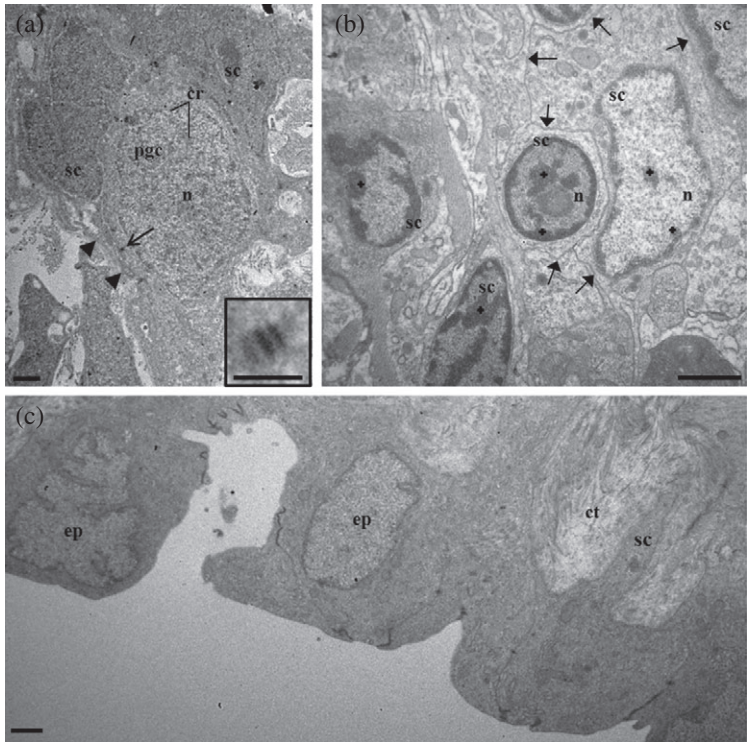

FIG. 4. Transmission electronmicrographs of the gonads of *Pseudoplatystoma fasciatum* juveniles: (a), (b) mean ± S.D. total length, $L_T = 51{\cdot}5 \pm 8{\cdot}3$ mm (stage I) and (c) $L_T = 66{\cdot}6 \pm 7{\cdot}9$ mm (stages VI and VII). (a) Primordial germ cells (pgc) associated with somatic cells (sc) showing electro-dense nucleus (n) of the primordial germ cells filled with euchromatin (cr), cytoplasmic extensions (◂) of somatic cells (sc) and desmosomes (⟵). (b) Different types and shapes of somatic cells (sc) showing the border of the cytoplasmic membrane (→) of the somatic cells and the presence of heterochromatin (✚). (c) Somatic cells (sc) associated and located perpendicularly to the serous epithelium comprised by the epithelium (ep) and dense connective tissue (ct). Bars = 2 μm [(a)–(c)]

be observed. Some undifferentiated oogonia had slightly basophilic and light nuclei, which did not contain heterochromatin in their centre or periphery [Fig. 7(b)]. The euchromatin had a low nuclear electron density and the nucleolus was centralized [Fig. 6(c)]. In the cytoplasm of these cells there were fewer mitochondria, mainly the elongate type, which were often associated with lysosomes. The smooth endoplasmic reticulum connected the internal region of the cytoplasm to the nucleus by cellular junctions. Invaginations could be seen on the plasma membrane [Fig. 6(c)].

Clusters of germ cells, which developed into cords, could be observed during oogonia proliferation and somatic cells were present in the periphery of these cords [Figs 7(c) and 6(a)]. Inside the cords, however, the oogonia were joined to one another by cellular junctions and cytoplasmic bridges, with no somatic cell being present between them [Fig. 6(a)].

At the end of this stage, the ovary increased in size, small ovarian lamellae and many nests of differentiated oogonia could be seen [Fig. 7(d)]. Somatic cells were present between the nests and around the small lamellae [Fig. 7(d)]. The external epithelial

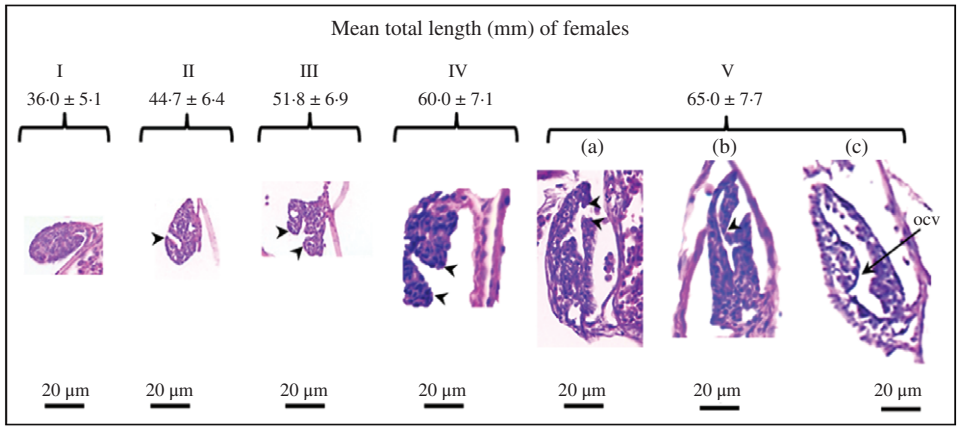

FIG. 5. Photomicrographs of the gonad of female *Pseudoplatystoma fasciatum* (mean ± S.D. total length, L_T in mm at stages I–V) highlighting the external morphological aspects and the transition of an undifferentiated gonad into ovary (transverse section). The formation and progressive enlargement of the ovarian cavity can also be observed during these five stages of gonad development: I, undifferentiated gonad; II, beginning of somatic elongation progression; III, somatic elongations; IV, increase in the size of the gonad and the somatic elongations; V(a), approximation of the somatic elongations; V(b), closure of the somatic elongations; V(c) ovarian cavity (ocv). ◂, the somatic elongations. Staining: haematoxylin phloxin (HP).

cells (somatic cells) could be seen migrating towards the nests of oogonia cells giving rise to the pre-follicular cells [Fig. 7(e)].

The oogonia and the pre-follicular cells were joined by desmosomes [Fig. 8(a)] and some oogonia displayed mitotic activity during the formation of the nests. The basal membrane could be seen enveloping and separating the germ-line cysts [Fig. 8(a)].

The differentiated oogonia were round and larger than the undifferentiated ones. Their nucleus was round, voluminous, light and slightly basophilic [Fig. 7(e)] with internal areas of euchromatin and an evident single central nucleolus [Fig. 8(a), detail]. The cytoplasm had eosinophilic granulation, was slightly acidic and light [Fig. 7(e)]. This characteristic was due to a reduction in the number of mitochondria, which in turn were round and did not possess very evident cristae. These mitochondria were often associated with lysosomes [Fig. 8(b)]. Furthermore, the presence of polyribosomes [Fig. 8(c)] and some organelles could also be evidenced in the cytoplasm, such as the Golgi complex [Fig. 8(d)], rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) [Fig. 8(e)].

As juvenile development progressed, the ovaries became full of oogonia due to an increase in the number of germ-line cysts [Fig. 7(d)].

OVARIAN STROMA FORMATION

Juveniles (stages IX and X)

Stage IX and X juveniles with $L_T = 124{\cdot}7 \pm 11{\cdot}6$ mm, when compared to those of previous stages, showed progressive enlargement of the ovary and ovarian lamellae. Germ-line cysts containing oocytes in various meiotic prophase I stages were present forming the ovarian stroma [Fig. 7(f)].

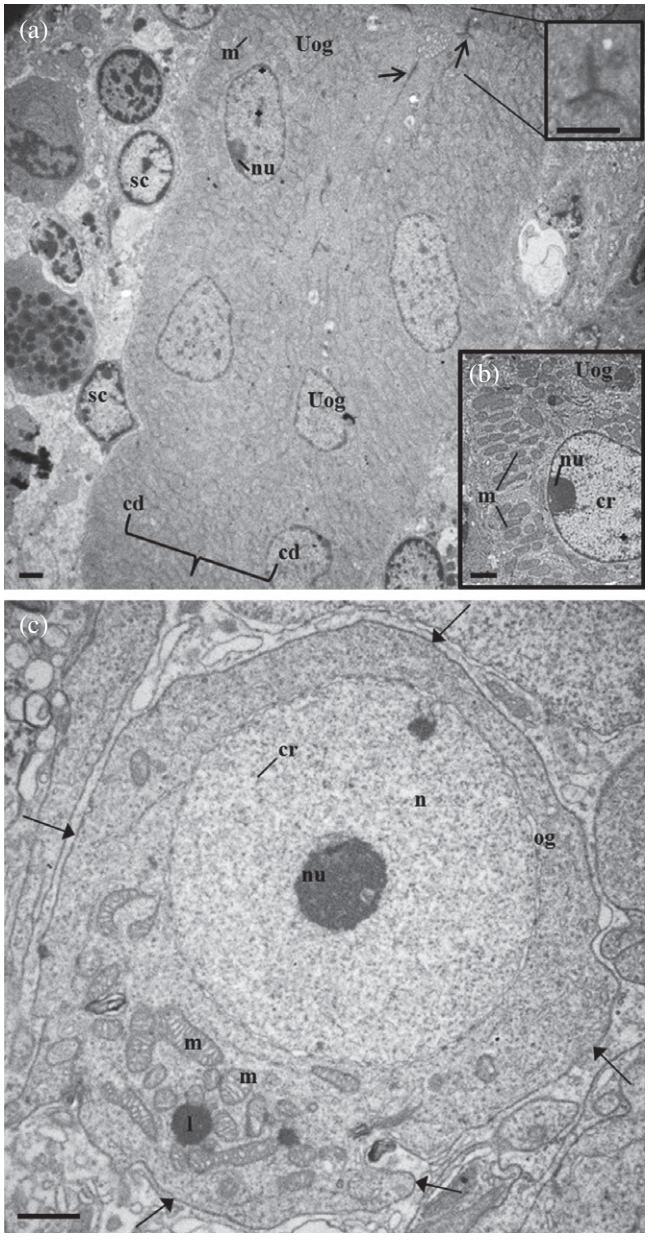

FIG. 6. Transmission electronmicrographs of the ovary of *Pseudoplatystoma fasciatum* juveniles at the beginning of ovarian differentiation. (a, b) Mean ± S.D. total length, $L_T = 66{\cdot}6 \pm 7{\cdot}9$ mm (stages VI and VII) and (c) $L_T = 81{\cdot}2 \pm 15{\cdot}3$ mm (stage VIII). (a) Group of undifferentiated oogonia (Uog) arranged into cords (cd) and the presence of somatic cells (sc) externally to these cords. The oogonia were interconnected by adhesion junctions (⟵). (a, b) Undifferentiated oogonia (Uog) with nucleus (n) containing moderately condensed euchromatin (cr), some heterochromatin (+), decentralized nucleolus (nu) and cytoplasm containing large quantities of mitochondria (m). (c) Oogonia (og) transitioned from undifferentiated to differentiated, showing nucleus (n) with less electro-dense euchromatin (cr) and centralized nucleolus (nu). Cytoplasm containing mitochondria (m) associated to lisosomes (l). Plasma membrane (⟵) present on the external region of the cytoplasm. Bars = 2 μm.

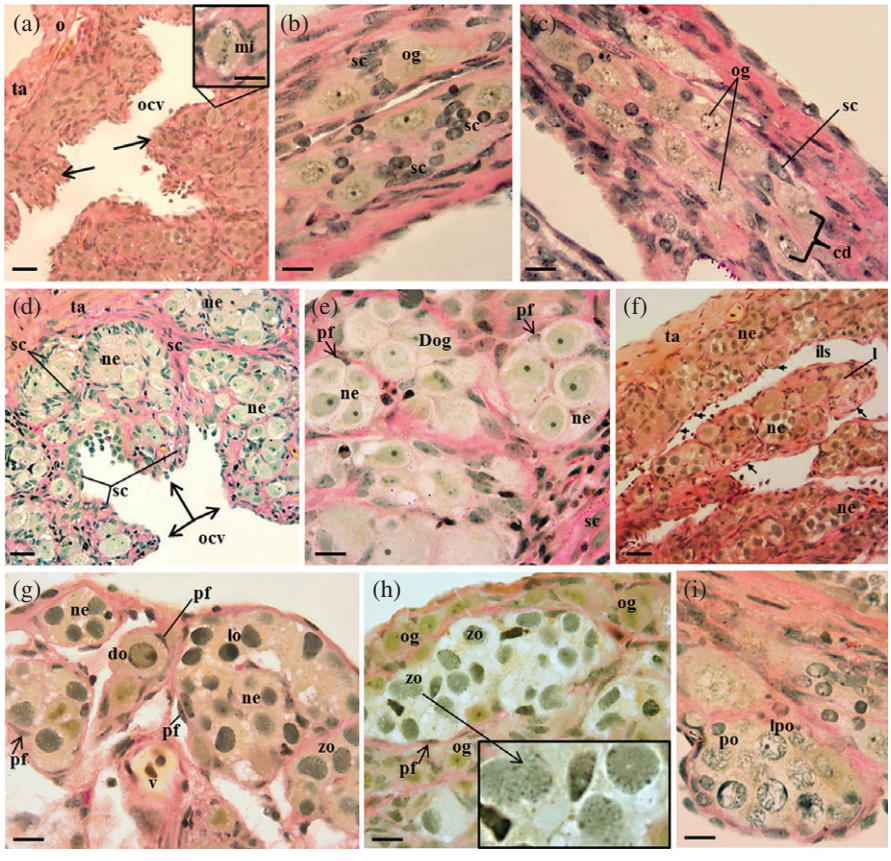

FIG. 7. Photomicrographs of the ovary of *Pseudoplatystoma fasciatum* juveniles. (a–e) Beginning of ovarian differentiation, mean ± S.D. total length, $L_T = 81{\cdot}2 \pm 15{\cdot}3$ mm (stage VIII). (f–i) Formation of ovarian stroma in juveniles with $L_T = 124{\cdot}7 \pm 11{\cdot}6$ mm (stages IX and X). (a) Ovary (o) with projections (⟵), which will develop into the ovarian lamellae; tunica albuginea (ta) and ovarian cavity (ocv). Bar = 20 μm. Detail showing the germ cells in mitosis (mi). Bar = 10 μm. (b) Isolated oogonia (og) among somatic cells (sc). Bar = 10 μm. (c) Cords (cd) containing oogonia (og) and somatic cells (sc). Bar = 10 μm. (d) Distribution of somatic cells (sc) around the small lamellae (⟵) and the oogonia nests (ne). Tunica albuginea (ta), ovarian cavity (ocv). Bar = 20 μm. (e) Nests (ne) of differentiated oogonia (Dog) with nucleus and light cytoplasm. Pre-follicular cells (pf) surrounding nests. Bar = 10 μm. (f) Ovary highlighting the enlargement of the ovarian lamellae (l), tunica albuginea (ta), somatic cells (⟵). Germ-line cysts containing nests of cells (ne) at different stages of prophase I and the formation of the ovarian stroma containing interlamellar spaces (ils). Bar = 20 μm. (g) Germ-line cysts containing nests of oocyte in meiosis I at leptotene (lo) and zigotene (zo) stages. Diplotene (do), pre-follicular cells (pf) and blood vessels (v) were also observed between the cysts. Bar = 10 μm. (h) Zygotene oocytes (zo) surrounded by pre-follicular cells (pf) and oogonia nests (og) close to these meiotic cysts. Bar = 10 μm. (i) Germ-line cysts containing nests of pachytene (po) and late pachytene (lpo) oocytes. Bar = 10 μm. Staining: (PAS/IH/MY).

The prophase oocytes had a round nucleus with various patterns of euchromatin condensation [Fig. 7(g)]. The cytoplasm, however, remained scarce, slightly acidophilic and with few evident mitochondria [Figs 7(g) and 9(a)]. The germ-line cysts were surrounded by pre-follicular cells and consisted mainly of nests containing oocytes in leptotene, zygotene and pachytene [Fig. 9(a)]. At this prophase period, there was an

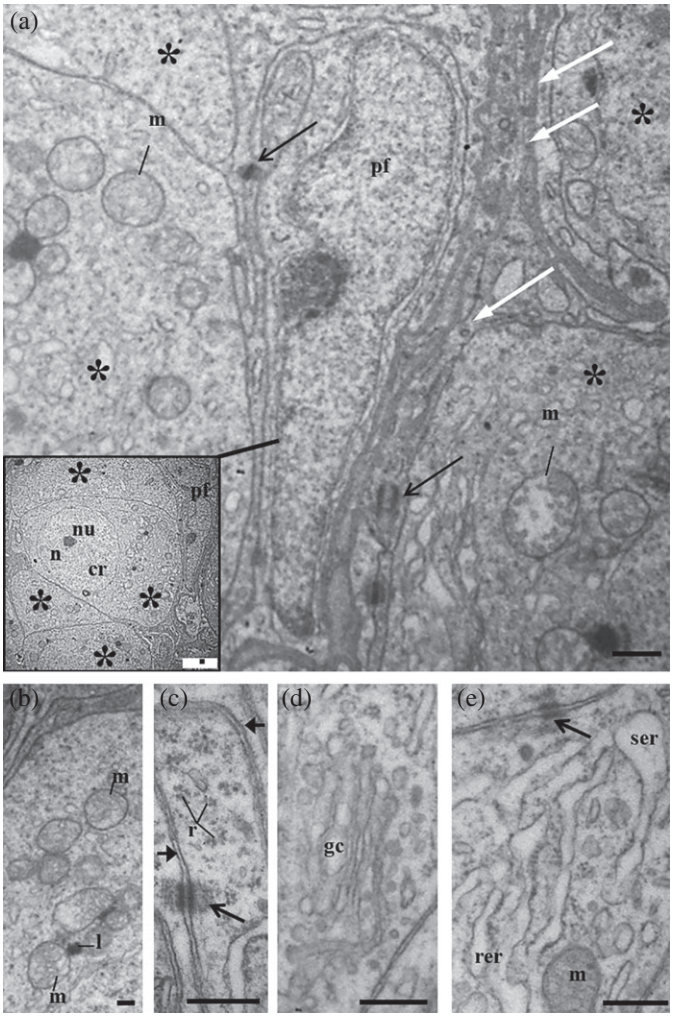

FIG. 8. Transmission electronmicrographs of the ovary of *Pseudoplatystoma fasciatum* juveniles mean ± S.D. total length, $L_T = 81{\cdot}2 \pm 15{\cdot}3$ mm (stage VIII) at the beginning of ovarian differentiation. (a) Nests containing differentiated oogonia (⁎) and surrounded by pre-follicular cells (pf) strongly attached to its base by desmosomes (⟵). First signs of the basal membrane (white arrow). Detail highlighting differentiated oogonia (⁎) with round and light nucleus (n), decondensed euchromatin (cr) and centralized nucleolus (nu). Bar = 1 μm. (b) At higher magnification of the cytoplasm: round mitochondria (m) connected to lisosomes (l). (c) Presence of poly-ribosomes (r), desmosomes (⟵), and the border and interdigitations of the plasma membrane (⟵). (d) Golgi complex (gc). (e) Rough endoplasmic reticulum (rer), smooth endoplasmic reticulum (ser) and mitochondria (m). (b–e) Bar = 0·5 μm.

intermediate phase in which differentiated oogonia transitioned to leptotene oocytes. During this phase, the nucleus had an irregular shape and its membrane was not very evident, heterochromatin was present and the nucleolus was evident, but no longer centralized [Fig. 9(b)]. The nuclei of the leptotene oocytes were strongly basophilic [Fig. 7(g)], did not possess evident nucleolus and the border between the nucleus and cytoplasm was not very distinct [Fig. 9(c)].

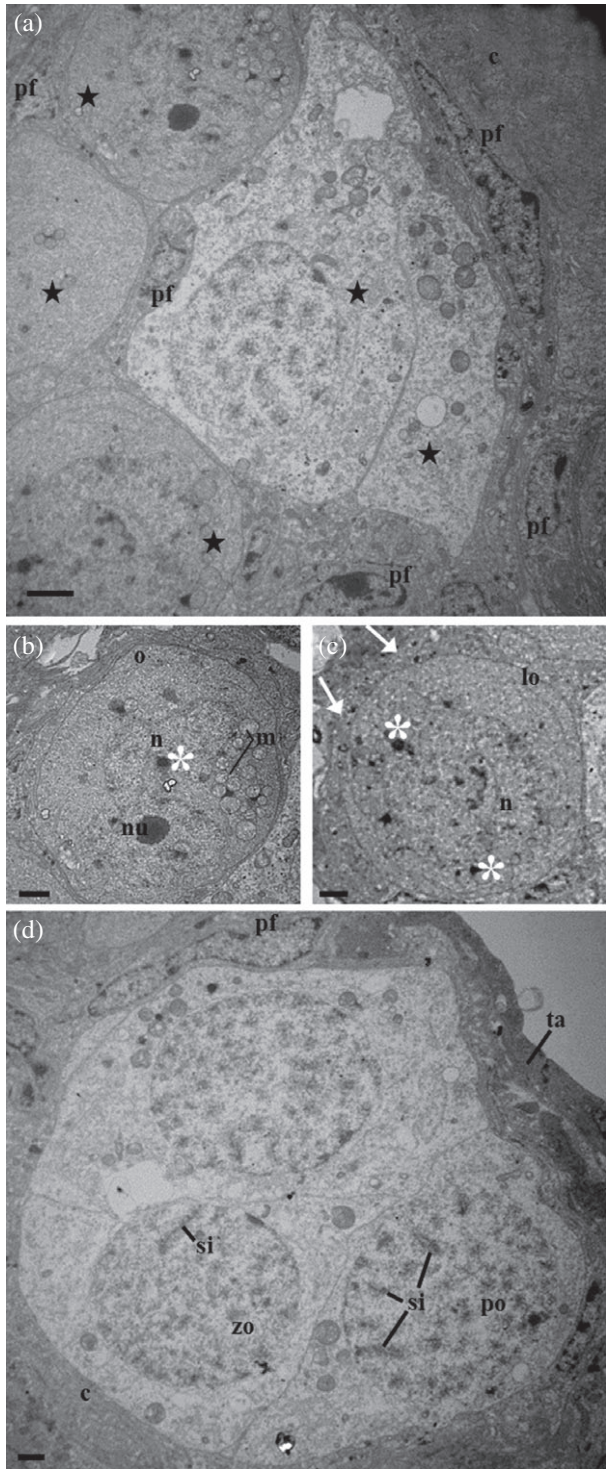

FIG. 9. Legend on next page.

The nuclei of zygotene oocytes were less basophilic than those of leptotene oocytes, were slightly granular, with chromosomal condensation in small clumps, and already possessed synaptonemal complexes. The cytoplasm was light and evident [Figs 7(h) and 9(d)].

The pachytene oocytes also had a granular nucleus; however, the granules were concentrated, forming cross-over chromosomes [Figs 7(i) and 9(d)]. As meiosis progressed, pachytene oocytes could be seen with granules concentrated closer to the periphery of the nucleus [Fig. 7(i)]. During prophase I, the oocyte nests were surrounded by the basal membrane [Fig. 9(c)].

COMPLETE OVARIAN FORMATION

Juveniles (stage XI)

The stroma, the ovarian cavity and the ovarian lamellae of the juveniles with $L_T = 135{\cdot}7 \pm 8{\cdot}9$ mm showed greater volume than those of smaller fish [Fig. 10(a)]. The diplotene oocytes separate from the nest during early phases of folliculogenesis and were surrounded by pre-follicular cells with basophilic nuclei and acidophilic cytoplasm [Fig. 10(b)].

As ovarian development progressed, isolated perinuclear oocytes could be seen. These were larger and individually surrounded by follicular cells, forming the ovarian follicle [Figs 10(c) and 11(a)]. The cytoplasm was considerably larger and gradually became basophilic, with elongated and round mitochondria. The nuclei contained variable quantities of nucleoli [Figs 10(c) and 11(a)].

The basal membrane, which supported the germinal epithelium (squamous epithelial cells or somatic cells and germ cells), bordered the ovarian follicle and the lamellae along the gonad [Figs 10(c) and 11(a)]. The pre-thecal cells appeared superimposing the follicular cells through small cytoplasmic extensions [Fig. 10(c)]. As development progressed, it was observed that the ovarian follicles were surrounded by many pre-thecal cells, which would become thecal cells to form the theca layer [Fig. 10(d)].

The somatic cells present in the lamellae were distant from one another, resulting in the formation of the extravascular spaces. The ovarian stroma was thus formed by a loose connective tissue, in which blood vessels and fibroblasts could be found among the somatic cells initially present [Fig. 10(e)]. The fibroblasts were connected by cytoplasmic extensions, forming a network within the lamellae [Fig. 11(b), (c)]. The germinal epithelium rested on top of the basal membrane, enveloping the ovarian lamellae [Fig. 10(c)].

FIG. 9. Transmission electronmicrographs of the ovary of *Pseudoplatystoma fasciatum* juveniles mean ± S.D. total length, $L_T = 124{\cdot}7 \pm 11{\cdot}6$ mm (stages IX and X) showing the formation of the ovarian stroma. (a) Germ-line cysts containing nests of oocytes in initial prophase I (★) surrounded by pre-follicular cells (pf). (b) Oocyte (o) at the stage between differentiated oogonia and leptotene oocyte, with irregular nucleus (n), heterochromatin (white asterisk) and evident nucleolus (nu). (c) Round leptotene oocyte (lo) with nucleus (n) containing high electron-dense euchromatin and the presence of heterochromatin in its interior (white asterisk). The border between the nucleus and the cytoplasm was not evident and the basal membrane (white closed arrow) was present around the whole cyst. (d) Meiotic cyst (c) containing zigotene (zo) and pachytene (po) oocytes displaying euchromatin condensation and were surrounded by pre-follicular cells (pf). Tunica albuginea (ta) surrounding the gonadal tissue. Zigotene and pachytene oocytes showing nucleus with euchromatin condensed in clumps and evident nuclear synaptonemic complexes (si). Bars = 2 μm.

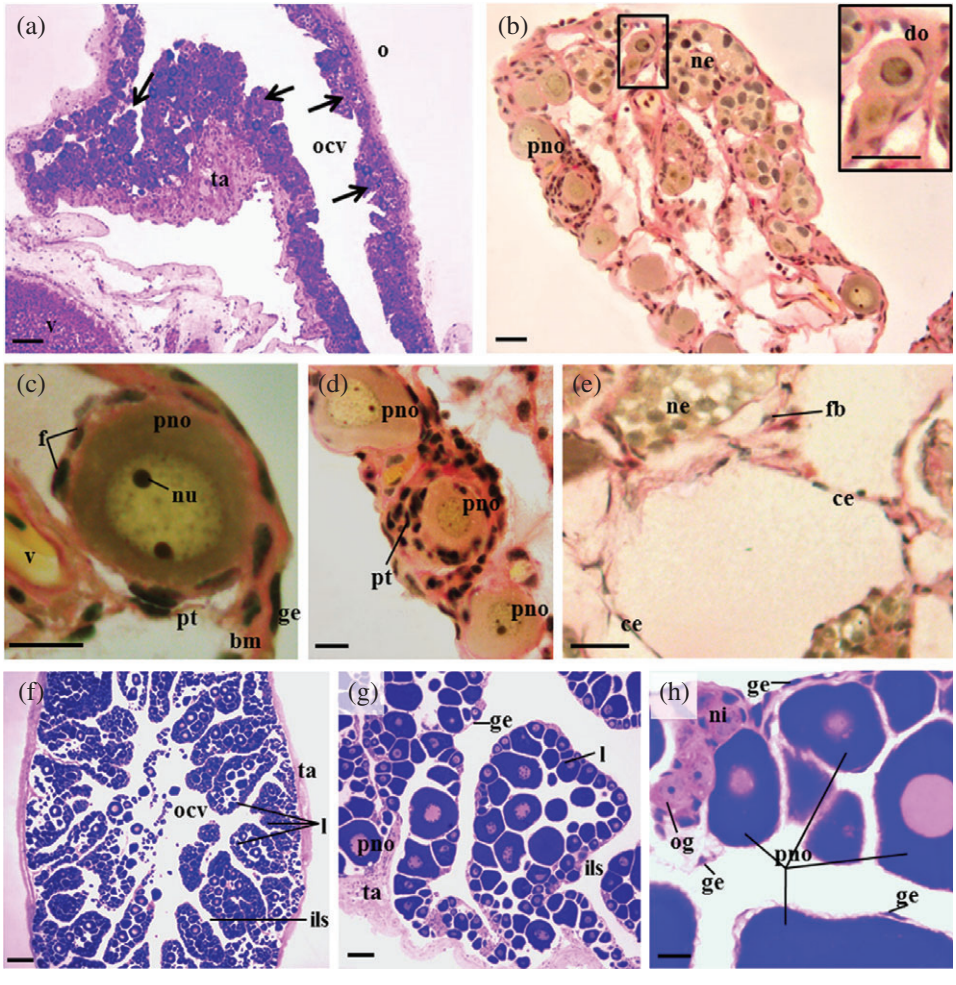

FIG. 10. Photomicrographs of the ovary of *Pseudoplatystoma fasciatum* juveniles showing complete ovarian formation. (a–e) Mean ± S.D. total length, $L_T = 135{\cdot}7 \pm 8{\cdot}9$ mm (stage XI) and (f–h) $L_T = 241{\cdot}0 \pm 8{\cdot}8$ mm (stage XII). (a) Longitudinal section of the ovary (o) with lamellae (⟵) and enlarged ovarian cavity (ocv). Tunica albuginea (ta) and blood vessel (v). Bar = 200 μm. (b) Germ-line cysts containing oocyte nests (ne) in different stages of prophase I and also highlighting diplotene oocytes (do). Also present are follicular complexes, which are formed by the perinuclear oocytes (pno). Bar = 20 μm. (c) Perinuclear oocyte (pno) showing follicular cells (f), basal membrane (bm), nucleolus (nu), pre-thecal cells (pt), germline epithelium (ge) and blood vessel (v). Bar = 10 μm. (d) Evidence of pre-thecal cells (pt) surrounding the perinuclear oocyte (pno), which will subsequently form the theca layer. Bar = 10 μm. (e) Network of somatic cells, considered fibroblasts (fb), in the ovarian stroma and the presence of cytoplasmic extensions (ce), and nests (ne) of germ cells in prophase I. Bar = 20 µm. (f) Completely formed ovary with the presence of the tunica albuginea (ta), interlamellar spaces (ils), ovarian cavity (ocv) and ovarian lamellae (l). Bar = 200 µm. (g) Well-established germ-line epithelium (ge) and ovarian stroma containing perinuclear oocytes (pno) and completely formed ovarian lamellae (l). Tunica albuginea (ta) and interlamellar space (ils). Bar = 20 μm. (h) At higher magnification: perinuclear oocytes (pno), nests of quiescent oogonia (og) and germ-line epithelium (ge). Bar = 10 μm. Staining (a) haematoxylin phloxin (HP), (b–e) PAS/IH/MY and (f–h) haematoxylin–phloxin (HP).

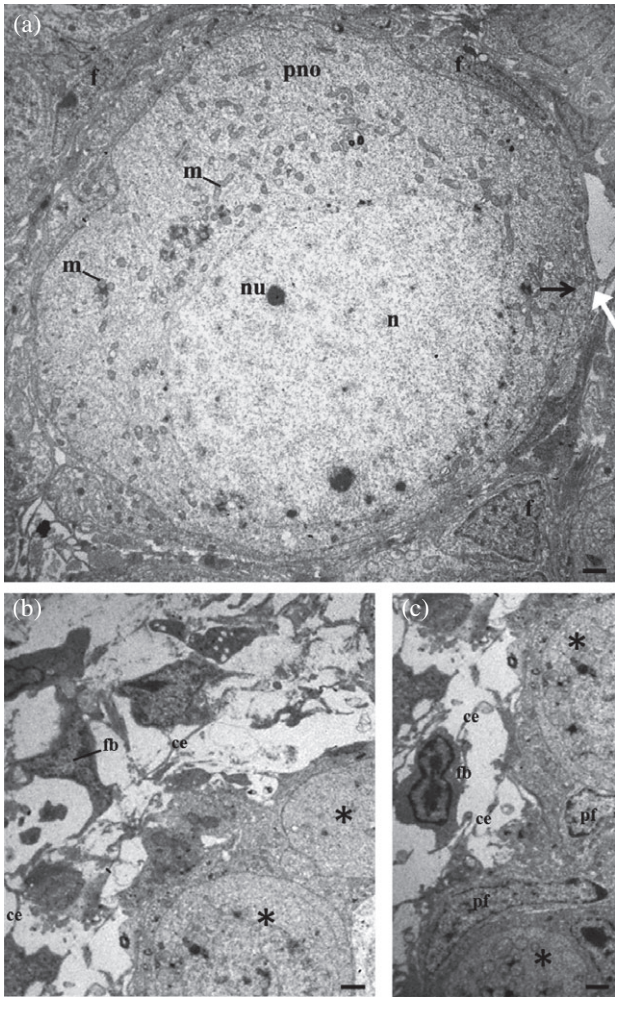

FIG. 11. Transmission electronmicrographs of the ovary of *Pseudoplatystoma fasciatum* juveniles mean ± S.D. total length, $L_T = 135{\cdot}7 \pm 8{\cdot}9$ mm (stage XI) showing complete ovarian formation. (a) Perinuclear oocytes (pno). Perinuclear nucleolus (nu) and follicular cells (f) around the oocyte. Elongated and round mitochondria (m), evident border of the cytoplasm (⟵) and basal membrane (white closed arrow). (b, c) Fibroblasts (fb) separated by cytoplasmic extensions (ce) forming a network within the lamellae. Germ-line cysts containing meiotic oocytes (*) and pre-follicular cells (pf) surrounding the cysts. Bar = 2 μm.

Juveniles (stage XII)

By this stage, the ovary of the juveniles with $L_T = 241{\cdot}0 \pm 8{\cdot}8$ mm was larger and completely formed. The tunica albuginea could be seen in the dorsal region and, in the ventral region, the ovarian lamellae occupied a large part of the ovarian cavity [Fig. 10(f)]. The ovarian stroma was comprised mainly of quiescent oogonia nests, ovarian follicles containing perinuclear oocytes, somatic and follicular cells and the germinal epithelium bordering the ovarian lamellae [Fig. 10(g), (h)]. A gap, denominated interlamellae space, was present between the lamellae in the ovarian cavity

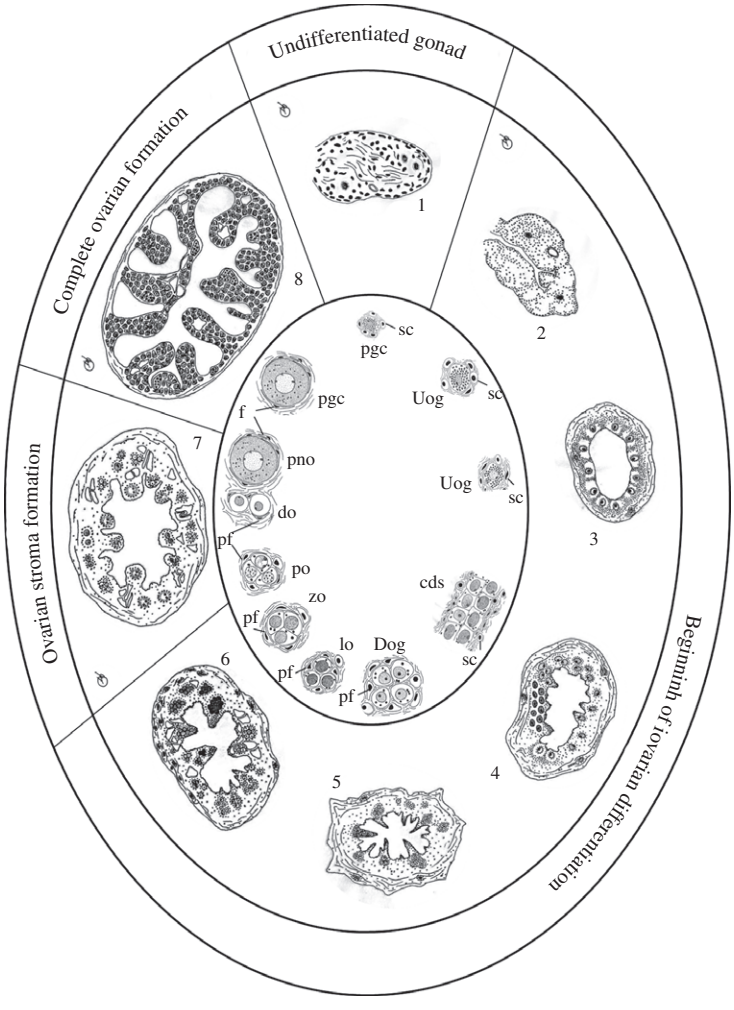

FIG. 12. Schematic illustration of ovarian differentiation and development in *Pseudoplatystoma fasciatum* highlighting the stages of gonad differentiation and the main corresponding germ cell types present, according to the following numbers mean ± S.D. total length and abbreviations.: 1 (51·5 ± 8·3/stage I): pgc and sc. 2 and 3 (51·5 ± 8·3/stages I–V) and (66·6 ± 7·9/stages VI and VII): Uog and sc. 4 and 5 (81·2 ± 15·3/stage VIII): isolated (Uog), cds (Uog and Dog), sc and nests (Dog). 6 (124·7 ± 11·6/stages IX and X): Germ-line cysts (Dog, lo, zo and po). 7 (135·7 ± 8·9/stage XI): do and pno. 8 (241·0 ± 8·8/stage XII): pno and nests of quiescent Uog. Pgc, primordial germ cells; Uog, undifferentiated oogonia; cds, cords; Dog, differentiated oogonia; lo, leptotene; zo, zigotene; po, pachytene; do, diplotene; pno, perinuclear oocytes; sc, somatic cells; pf, pre-follicular cells; f, follicular cells.

[Fig. 10(g)]. Finally, the germinal epithelium rested above the basal membrane separating and isolating the germ cells from the ovarian stroma [Fig. 10(g)]. All the main events that took place during the process of ovarian differentiation and development are summarized in Table II and Fig. 12.

TABLE II. Events in the ovarian differentiation and development process in *Pseudoplatystoma fasciatum* during juvenile growth (mean ± total length, L_T)

Gonad	JuvenilesL_T (mm)	Main events
Undifferentiated gonad	36·0 ± 5·1 (stage I)	Rectilinear shape Comprised of many somatic cells but few primordial germ cells (PGCs)
Beginning of ovarian differentiation	51·5 ± 8·3 (stages I–V)	Progressive movement of somatic elongations Closure of somatic elongations, formation of the ovarian cavity PGCs transition into undifferentiated oogonia becoming isolated and surrounded by somatic cells
	66·6 ± 7·9 (stages VI and VII)	Amplification of the ovarian cavity Isolated undifferentiated oogonia and many somatic cells directed towards the ovarian cavity
	81·2 ± 15·3 (stage VIII)	Increase in the volume of the ovarian stroma (subjective data) Proliferation of undifferentiated oogonia Differentiated oogonia cluster together to form cords Small projections from the ovarian stroma form towards the ventral region of the gonad (future ovarian lamellae) Somatic cells border these projections Small nests of differentiated oogonia Evident basal membrane
Ovarian stroma formation	124·7 ± 11·6 (stages IX and X)	Formation of ovarian lamellae Somatic cells border the lamellae Germ cells go into meiosis (oocyte in prophase I): leptotene, zygotene, pachytene and late pachytene
Complete ovarian formation	135·7 ± 8·9 (stage XI)	Diplotene oocytes surrounded by pre-follicular cells Some ovarian follicles with perinuclear oocytes Formation of extravascular spaces, germline epithelium and ovarian stroma
	241·0 ± 8·8 (stage XII)	Increase in ovarian size Thickening of the tunica albuginea Ovary formed by nests of quiescent oogonia, ovarian follicles, somatic cells and follicular cells Germline epithelium borders the follicles and ovarian lamellae

DISCUSSION

Although numerous studies have addressed the importance of information about sex chromosomes and the mechanism of sex determination in fishes (Baroiller *et al.*, 2009; Piferrer *et al.*, 2012), knowledge is still scarce for *P. fasciatum*. The present results show an average sex ratio of 1:1 (51·0–49·0% to female and male, respectively), which suggests that sex determination is under genetic control.

Regarding gonad differentiation, several histological criteria are used to identify the structures that characterize gonad sexual differentiation, such as cellular proliferation, the onset of meiosis and the formation of the ovarian cavity (Devlin & Nagahama, 2002), but these criteria vary according to the species being studied.

Gonochoristic fishes often begin sexual differentiation prematurely, with the onset of meiosis. In the Japanese rice fish *Oryzias latipes* (Temminck & Schlegel 1846) for example, the gonad differentiates into ovary when meiosis starts, at just 1 day post-hatching (Satoh & Egami, 1972). In the ovary of *P. fasciatum*, meiosis started at 120 days post-fertilization. In comparison with the Argentinian silversides *Odontesthes bonariensis* (Valenciennes 1835) (Strussmann *et al.*, 1996*b*), *Odontesthes argentinensis* (Valenciennes 1835) and Odontesthes hatcheri (Eigenmann 1909) (Strussmann *et al.*, 1996*a*) and other species of the order Siluriformes, Cypriniformes and Perciformes (Nakamura & Takahashi, 1973; Meijide *et al.*, 2005; Arezo *et al.*, 2007; Çek & Yilmaz, 2007; Gao *et al.*, 2009; Villamizar *et al.*, 2012; Yamaguchi & Kitano, 2012), the meiotic process in *P. fasciatum* begins at a later stage. This is because gonad tissue consists of isolated undifferentiated oogonia surrounded by a great number of somatic cells, and, therefore, meiosis is not considered as characteristic of gonadal sexual differentiation in this species. This variation in the time of sexual differentiation is due to interspecific differences, which are often related to intrinsic factors such as behaviour and reproductive strategies (Devlin & Nagahama, 2002; Randall *et al.*, 2013).

In *P. fasciatum*, ovarian differentiation occurs with the formation of the ovarian cavity and not the onset of meiosis. At the start of ovarian differentiation (juveniles with $L_T = 51{\cdot}5 \pm 8{\cdot}3$ mm, stages I–V), the germ cells are at an early stage of development, going through a period of quiescence. Concomitantly, intense proliferation of stromal tissue at the proximal and distal extremities of the gonad leads to the formation of the ovarian cavity. In the Siluriformes channel catfish *Ictalurus punctatus* (Rafinesque 1818) (Patino *et al.*, 1996) and the Perciformes Mozambique tilapia *Oreochromis mossambicus* (Peters 1852), Nile tilapia *Oreochromis niloticus* (L. 1758) and the bluegill *Lepomis macrochirus* Rafinesque 1819 (Nakamura & Takahashi, 1973; Nakamura & Nagahama, 1985; Gao *et al.*, 2009), in which gonad differentiation occurs with the formation of the ovarian cavity, the germ cells are at a more advanced stage and meiotic oocytes are also present.

Recent studies have shown the importance of somatic cells in the identification of female and male gonads. It has been shown that the absence of germ cells does not affect ovarian or testicular development and that the phenotypic sex of fishes is independently determined by somatic cells through the expression of *Cyp19a1* (cytochrome P450, aromatase enzyme), *foxl2* (forkhead gene family, positively regulates the transcription of the gene *cyp19a1*), *amh* (Mullerian inhibitory substance) and *dmrt* (Doublesex and Mab-3-related transcription factor 1 expressed during testicular differentiation) (Marchand *et al.*, 2000; Fujimoto *et al.*, 2010; Goto *et al.*, 2012; Nozu *et al.*, 2013; Rolland *et al.*, 2013). Similarly in *P. fasciatum*, it was the histology that showed some somatic

cells were aligned and perpendicular to the cavity, already showing characteristics of an ovary, in spite of the slow differentiation and development of the few germ cells present at this stage.

In this study, a correlation between gonad differentiation, and the size and age of the fish was observed. It was also noted that the size of *P. fasciatum* was an important factor in the identification of the stage of gonad differentiation and development, suggesting that size has a higher correlation ($r^2 = 0{\cdot}95$) with ovarian differentiation than age ($r^2 = 0{\cdot}85$). Thus, animals of different ages, but of similar size had ovaries at similar stages of development. This was similar to findings by Gao *et al.* (2009) for *L. macrochirus*, Colombo *et al.* (1984) for European eel *Anguilla anguilla* (L.1758) and Blazquez *et al.* (2001) for the European sea bass Dicentrarchus labrax (L. 1758).

In this study, the germ cells showed similar characteristics to those reported by Mazzoni *et al.* (2010) in common carp *Cyprinus carpio* L. 1758, in which the ovarian cavity is formed by deep invaginations of the somatic cells (Matta *et al.*, 2002; Mazzoni *et al.*, 2010). On the other hand, in *P. fasciatum*, these cells showed a different pattern of proliferation, and the ovarian cavity has somatic elongations and projections.

In species such as *A. anguilla*, *L. macrochirus* and *C. carpio*, primordial germ cells (PGCs) and germ cells (GCs) group together to form cords when the gonad is still undifferentiated externally (Grandi & Colombo, 1997; Gao *et al.*, 2009; Mazzoni *et al.*, 2010). In this study, the cords observed were not formed by PGCs, but by undifferentiated oogonia, which changed from elliptical to round. As the gonad was already morphologically differentiated externally, it was possible to identify the oogonia as the germ cells present inside the cords. Besides being associated with the structural and ultrastructural characteristics of the gonad, the germ cells are also related to external morphological characteristics and thus are directly associated with the stage of gonad differentiation and sexual dimorphism (Dodd, 1986; Blazer, 2002; Patino & Sullivan, 2002; Grier *et al.*, 2009).

The nests of oogonia were delimited by the basal membrane, as observed by light and electron microscopy. Knowledge of the basal membrane is essential to understanding folliculogenesis and the time necessary for ovarian formation in Teleostei (Parenti & Grier, 2004). Furthermore, the basal membrane serves as a support for the germinal epithelium, which was composed of squamous epithelial cells (somatic cells) and germ cells, showing similar characteristics to those of other species from the order Siluriformes (Grier, 2002; Quagio-Grassiotto *et al.*, 2011).

The final stage of meiosis I comprises the formation of the ovarian follicle, which is separated from the germinal epithelium and stroma by a basal membrane (Grier *et al.*, 2009). This process of folliculogenesis was completed in *P. fasciatum* by 180 dpf, and by 240 dpf the germinal epithelium of the ovaries was completely filled with lamellae, which in turn were full of follicles containing perinuclear oocytes.

This study has shown that ovarian differentiation occurred with the intense proliferation of somatic cells and subsequent formation of the ovarian cavity at an early stage of development (juveniles with $L_T = 51{\cdot}5 \pm 8{\cdot}3$ mm, stages I–V) and that sex inversion protocols could thus be successfully applied before this period. Furthermore, the results show that both size and age can influence gonad differentiation and development in *P. fasciatum*. It was also shown that the start of meiosis and folliculogenesis took place at a later stage when compared to other species of the order Siluriforme and that the ovary showed characteristics of ovarian cysts. This research provides important information

on the gonad development and biology of *P. fasciatum* and can be used as a reference for future basic and applied studies.

The experimental protocol was approved by the Animal Welfare and Ethics Committee (Comite de Ética e Bem-Estar Animal) of the Faculty of Agrarian and Veterinary Sciences, Univ Estadual Paulista – UNESP, Jaboticabal-SP, Brazil (Protocol: 016263/09). The authors would like to thank the CAUNESP (Reproduction Research Group and Nutrition Laboratory) and Mar & Terra Ltd for providing the biological material and technical assistance and also FAPESP for the PhD scholarship granted (2009/15392-6) and for financial assistance (2010/16775-3). We would also like to thank I. Quagio-Grassiotto and T. S. Manzzoni from the Department of Morphology at the Institute of Biosciences – UNESP/Botucatu, O. Matheus from Department of Animal Morphology and Physiology, M. D. S. Ferreira from the Electron Microscopy Multi-user Laboratory at the Department of Molecular Cell Biology and Pathogenic Bio-agents, J. A. Senhorini for the technical assistance and B. G. S. Favaretto for the services provided with the schematic drawings.

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