Received: 3 August 2018 | Revised: 23 January 2019 | Accepted: 25 January 2019

DOI: 10.1111/jai.13879

ORIGINAL ARTICLE

WILEY Journal of Applied Ichthyology DWK

Testicular differentiation and development in South American catfish, surubim, *Pseudoplatystoma fasciatum*

Fernanda N. Valentin[1,2] | Laura S. O. Nakaghi[1] | Sérgio R. Batlouni[1] | Nivaldo F. do Nascimento[1] | Maria do Carmo Faria Paes[1] | Regiane Cristina da Silva[1] | Breno Manzini[1] | Sheryll Yohana Corchuelo Chavarro[1] | Marcelo Henrique Correa Assunção[1]

[1]Centro de Aquicultura da Universidade Estadual Paulista (CAUNESP), Jaboticabal, Brazil

[2]Faculdade de Medicina – FAMED, Universidade Federal do Pará, Altamira, Brazil

Correspondence
Fernanda Nogueira Valentin, Faculdade de Medicina – FAMED, Universidade Federal do Pará, Altamira, Brazil.
Emails: fervalentin@ufpa.br; fer_valentin@yahoo.com.br

Funding information
Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: 2009/15392-6 and 2010/16775-3

Abstract

In the species *Pseudoplatystoma fasciatum* the males are smaller than females. Thus, the objective of this study was to describe the process of testicular differentiation and provide tools for sex manipulation techniques aimed at improving the productivity of farming of sexually-reversed females. Correlation between the length, age, sex rate and the stage of gonadal development were analyzed in this species from samples collected between 0 and 240 days post-hatching. Testicular differentiation was divided into eight stages, based on the cellular and morphological characteristics of the gonad. The results showed that sexual differentiation has a greater correlation to the size than the age in this species. This study provides knowledge on the proliferation and distinctive arrangement of somatic cells, which enabled the early identification of the testis due to the presence of future fringes in the specimens. Testicular differentiation in *P. fasciatum* was histologically different from other species due to the proliferation and distribution of somatic cells in the regions that would originate the testicular tubules and sperm ducts. Meiosis began at a later stage in comparison with other species, therefore, it was not considered a criterion for early sexual characterization. It can be concluded that testicular differentiation in *P. fasciatum* occurs relatively early and this data can be used to improve sex inversion protocols and increase productivity in this species.

KEYWORDS
gonads, histology, somatic cells, testicular tubules

1 | INTRODUCTION

Among the great Pimelodidae catfish, the *Pseudoplatystoma fasciatum* (*P. fasciatum*), popularly known as "South American catfish" or "surubim," has a distinctive sexual dimorphism characteristic: the males are smaller than females. This feature could be exploited by using sexual manipulation techniques with the potential to increase the productivity of monosex batches of sex-reversed females (Devlin & Nagahama, 2002).

In many teleost species, sex determination can be influenced by environmental factors. The phenotype is the result of the genotype associated with the epigenetic information that will be transferred to the progeny, resulting in changes to the morphological development of undifferentiated gonadal primordia and thus altering the sex ratio of a population (Baroiller, D'Cotta, & Saillant, 2009; Labbé, Robles, & Herraez, 2017; Piferrer, Ribas, & Diaz, 2012). Successful sexual manipulation requires understanding of early gonadal ontogenesis,

Numbers of samples	Days post hatching	Testicular tissue	Ovarian tissue	Undifferentiated gonad
100	39–45	20.0	17.0	63.0
100	60–70	25.0	37.5	37.5
50	100–113	60.0	40.0	–
50	120–150	64.4	35.6	–
30	180	33.3	66.7	–
30	240	40.0	60.0	–

TABLE 1 Sexual rate (%) of gonads during sex differentiation in *P. fasciatum*

which is the period most susceptible to phenotype sex inversion in fish (Piferrer, 2001). Therefore, detailed knowledge of this period is essential to better comprehend gonadal differentiation and the cellular structures involved, as well as to develop successful and precise sex inversion protocols (Abozaid, Wessels, & Hoerstgen-Schwark, 2012; Athauda, Anderson, & Nys, 2012; Devlin & Nagahama, 2002).

This study examined the process of testicular development, aiming to determine the period of testicular differentiation, the sex rate, the correlation between size and age and the stage of gonadal development, as well as the proliferation of somatic and germ cells, which would originate the testicular tubules and sperm ducts in *P. fasciatum*.

2 | MATERIALS AND METHODS

2.1 | Animals

Induced reproduction of *P. fasciatum* adults was carried out using a commercial pituitary hormone. The spawning obtained were kept at Mar & Terra fish farm in raceway system with continuous water flow at 700 L/hr and density of 6 fish/L. Each raceway (n = 4) was divided into three sections (considered as triplicates). The physical and chemical characteristics of the water were monitored daily. The larvae were fed with *Artemia* nauplii until the animals achieved the mean of total length 10.5 ± 4.3 mm when they were subjected to conditional feeding, which consisted of partial and progressive replacement of semi-dry feed with commercial fish food. After this period of food transition, the juveniles with mean of total length 35.7 ± 4.3 mm were fed an extruded commercial fish food, containing 40% crude protein, six times a day.

2.2 | Sampling

Samples were collected from 3 to 3 hr until 24 hr post-hatching, each 6 hr until 48 hr post-hatching, once a day up to 6 days post-hatching (dph) and subsequently at 13, 20, 27, 39, 45, 57, 70, 100, 113, 120,

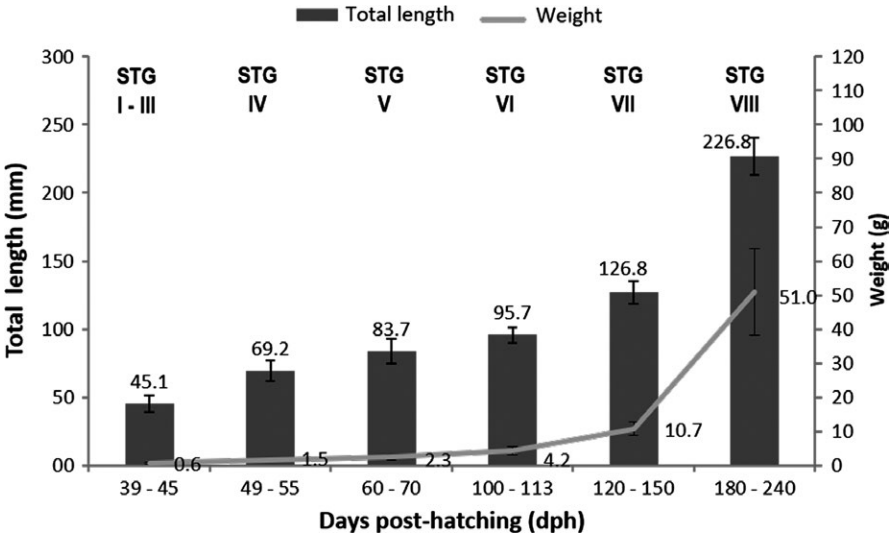

FIGURE 1 Total length (mm) and weight (g) of *P. fasciatum* animals during testicular differentiation and development. Stage (STG)

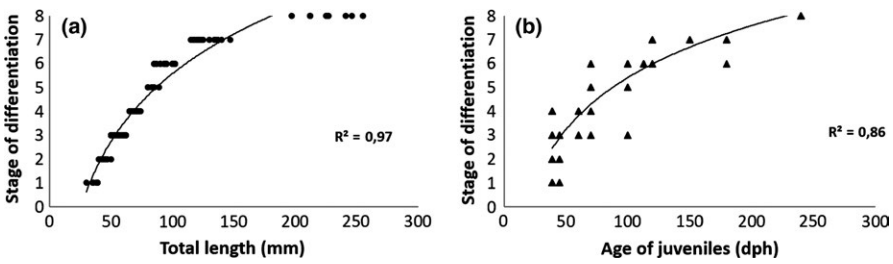

FIGURE 2 Correlation between size (a) and age (b) during the stages of testicular differentiation and development of *P. fasciatum* specimens. $p < 0.001$

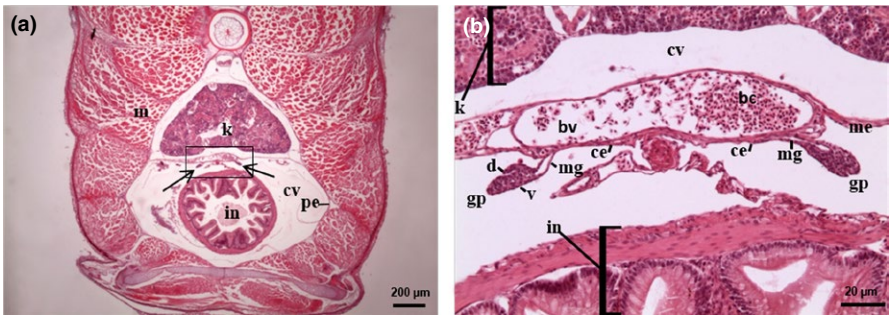

FIGURE 3 Photomicrography of the middle region of the body of *P. fasciatum* featuring undifferentiated gonads at 39 dph (stage I: 35.9 ± 4.3 mm). (a) Pair of gonadal primordia (arrows) inside the coelomic cavity (cv), peritoneum (pe), intestine (in), kidney (k) and muscle (m). (b) Detail: coelomic epithelium (ce), mesentery (me), gonadal primordium (gp), mesogonadium (mg), blood vessel (bv), blood cells (bc), dorsal region (d) and ventral region (v) of the developing gonad. Staining A–B: Haematoxylin Floxin (HF)

150, 180 and 240 dph. Entire larvae were collected from hatching up to 6 dph, the middle of the body was collected in 13 up to 39 dph and the gonad tissue in 45 until 240 dph. The fish were euthanized with 0.1% benzocaine solution (2 g dissolved in 150 ml of 96°GL alcohol and diluted in 20 L of water).

2.3 | Light microscopy

For light microscopy, samples were preserved in Karnovsky solution (glutaraldehyde + paraformaldehyde solution in Sorensen buffer, 0.1 M, pH 7.2) for at least 24 hr (Karnovsky, 1965). Some tissues were embedded in histosec® blocks (Merk Millipore, Germany), sectioned (5 µm) and stained with Haematoxylin and Eosin. The remaining samples were embedded in resin (Leica HistoResin Kit; Leica Biosystems, Germany) and the sections (3 µm) stained with Haematoxylin-Floxin and Periodic Acid Schiff + Iron Haematoxylin + Metanil Yellow complex (Quintero-Hunter, Grier, & Muscato, 1991). The slides were analysed using a Leica DM 2,500 photomicroscope equipped with a DFC 295 digital camera.

2.4 | Transmission electron microscopy

The samples were preserved in 2.5% glutaraldehyde and then in 1% osmium for 2 hr in the dark, dehydrated, embedded in Araldite, sectioned (70 nm) and contrasted in a concentrated solution of uranyl acetate in 50% ethanol and 0.2% lead citrate in NaOH (1 N). Electronmicrographies were obtained with a transmission electron microscope (JEOL 100 CX II E JEOL 1010).

2.5 | Statistics

Data are shown as mean ± standard deviation (*SD*). The Lilliefors test was used to verify normal distribution. The correlation between length, age and the stage of development was analysed by logarithmic regression equation and the Spearman non-parametric test. Statistical analysis was performed using the software SAS (Version 9.0, SAS Institute, Inc., Cary, NC). Significance was considered at 5% ($p < 0.05$).

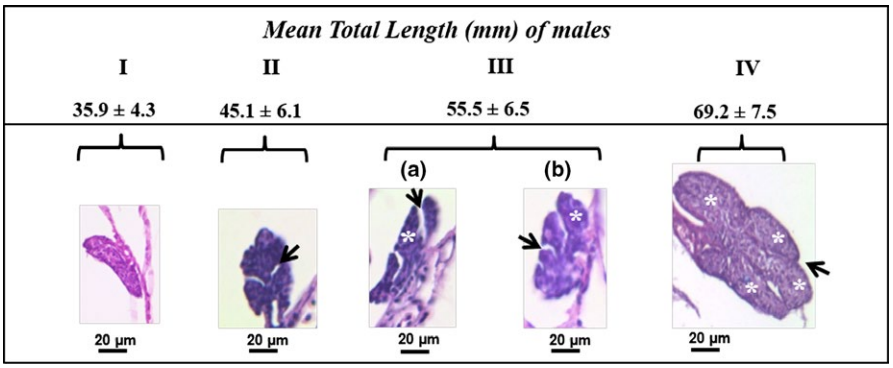

FIGURE 4 Photomicrography of the gonads of *P. fasciatum*. Main differences in the basic organization regarding the external features of the gonads during the beginning of testicular differentiation. (Stages I): 35.9 ± 4.3 mm, (stages II and III): 45.1 ± 6.1 and 55.5 ± 6.5 mm, (stage IV): 69.2 ± 7.5 mm. Featuring external histological aspects and the differentiation of a gonadal primordium into a presumptive testicle, transversal section. I: Elongated and straight shape. II: Gonadal primordium with grooves. III [(a) e (b)]: increase in the number of grooves and the presence of small projections on the male gonad. IV: evident projections, characteristic of testis. Arrows indicate the grooves and asterisks the projections. Staining I–IV: Haematoxylin-Floxin (HF)

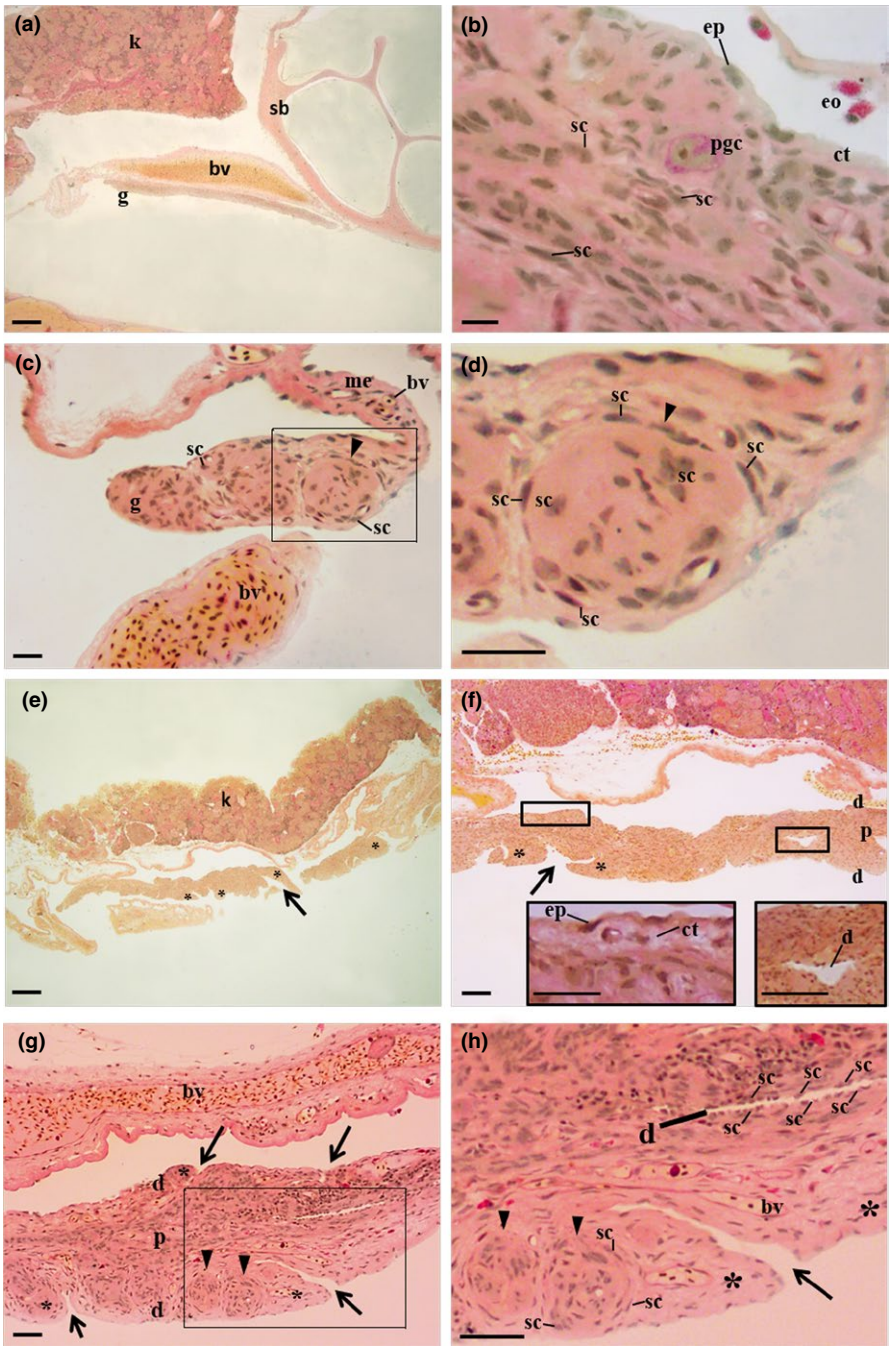

FIGURE 5 Photomicrography of the gonads of *P. fasciatum*. Stages I: (a) and (b) undifferentiated gonads. Stage II–IV: (c)–(h) start of testicular differentiation. (a) Panoramic view of a parasagittal section of an undifferentiated gonad (g) connected to a blood vessel (bv), positioned ventrolaterally to the swim bladder (sb) and ventrally to the kidney (k). Scale bar (200 μm). (b) Detail of Fig. A: Primordial germ cell (pgc), somatic cells (sc), eosinophils (eo) and lining epithelial tissue composed of stratified epithelium (ep) and dense connective tissue (ct). Scale bar (10 μm). (c) Presumptive testicle with somatic cells (sc), mesorchyum (me) connected to the mesentery (me) and blood vessels (bv). Scale bar (20 μm). (d) Detail featuring the proliferation of fusiform or flattened somatic cells (sc) and the circular clusters (arrowhead). Scale bar (20 μm). (e) Panoramic view of a Presumptive testicle with small grooves (arrows) and small projections (asterisks). Scale bar (200 μm). (f) Grooves and projections. Scale bar (80 μm). Detail: Lining epithelium (ep) of the gonad, dense connective tissue (ct) and a small testicular duct (d). Scale bar (20 μm). Distal region (d), proximal region (p). (g) The grooves (arrow) and projections increased in size and are now denominated future fringes (asterisks). Blood vessel (bv). Scale bar (20 μm). (h) Detail of the location of cellular clusters (arrow head) composed mainly of somatic cells in the distal region and of a testicular duct (d) in the proximal region. Future fringes (asterisks), blood vessel (bv). Scale bar (20 μm). Staining (a)–(h): Periodic Acid Schiff + Iron Haematoxylin + Metanil Yellow (PAS/HF/MY)

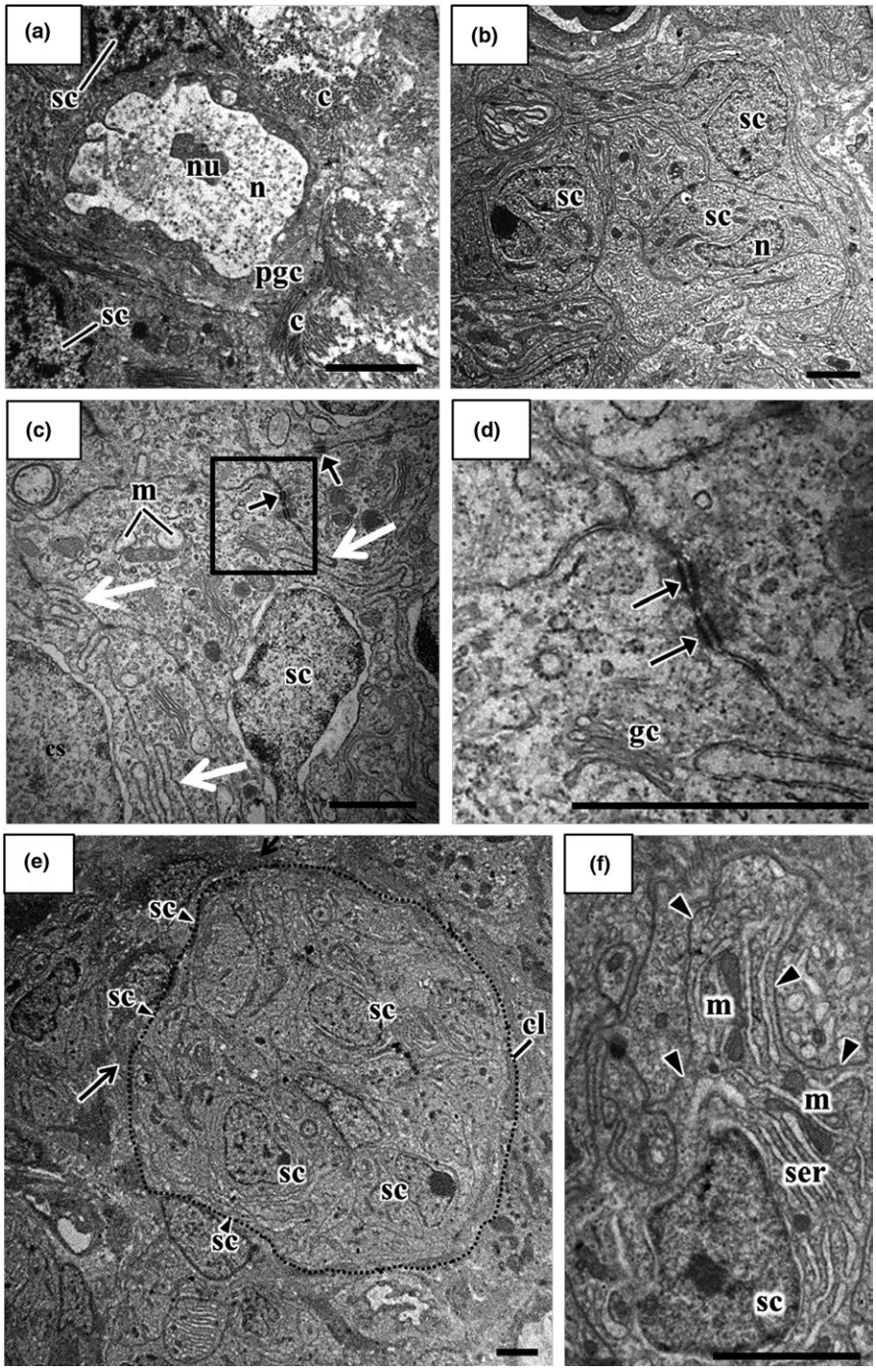

FIGURE 6 Transmission electronmicrography of the gonads of *P. fasciatum*. Stages I: (a) and (b) undifferentiated gonads. Stages II to IV: (c)–(f) start of testicular differentiation. (a) Primordial germ cell (pgc) surrounded by somatic cells (sc) with elliptical and irregular nuclei (n) and central nucleoli (nu). Presence of collagen (c) between the adjacent tissue. (b) Somatic cells had different shapes depending on how their nuclei (n) had been sectioned. (c) Desmosome cell junctions (black arrow) and interdigitations (white arrow) of the plasma membrane connecting the somatic cells. Mitochondria (m). (d) Detail of Fig. C featuring the union of these cells by desmosomes (black arrow). Golgi complex (gc). (e) Inside each cell cluster (cl)—delineated by the dotted line, several somatic cells (sc) are surrounded by other somatic cells (sc—arrowhead) and connected by cytoplasmic projections (black arrow). (f) Mitochondria with tubular cristae and a large quantity of smooth endoplasmic reticulum (ser) in the cytoplasm of somatic cells (sc). Cell border (arrowhead). Scale bar =2 μm [(a)–(f)]

3 | RESULTS

3.1 | Physical and chemical characteristics of the water

The parameters obtained were: Oxygen (7 mg/L), pH (6.0) Ammonia (0.25 mg/L) and temperature (25°C/morning and 30°C/afternoon).

3.2 | Physical and biological characteristics of specimens

The first indications of gonadal differentiation were observed in individuals of 39–45 dph. The rate of undifferentiated gonad, testicular and ovarian tissue, is detailed in Table 1 in the period of 39–240 dph evidencing a sexual ratio of 1:1 for males and females.

The L_T (mm) and weight (g) of male of *P. fasciatum* were correlated during this period (Figure 1) and a regression equation obtained ($y = 0.2744e^{0.2771x}$), which indicated exponential growth and a significantly positive ($p < 0.0001$) correlation ($R^2 = 0.95$) during the whole period. Logarithmic regression analysis was performed (Figure 2) to understand the correlation between size (L_T) and age (dph) of animals and the stages of testicular differentiation and development. Although both parameters showed a positive correlation ($p < 0.001$) during this period, size had the most evident coefficient

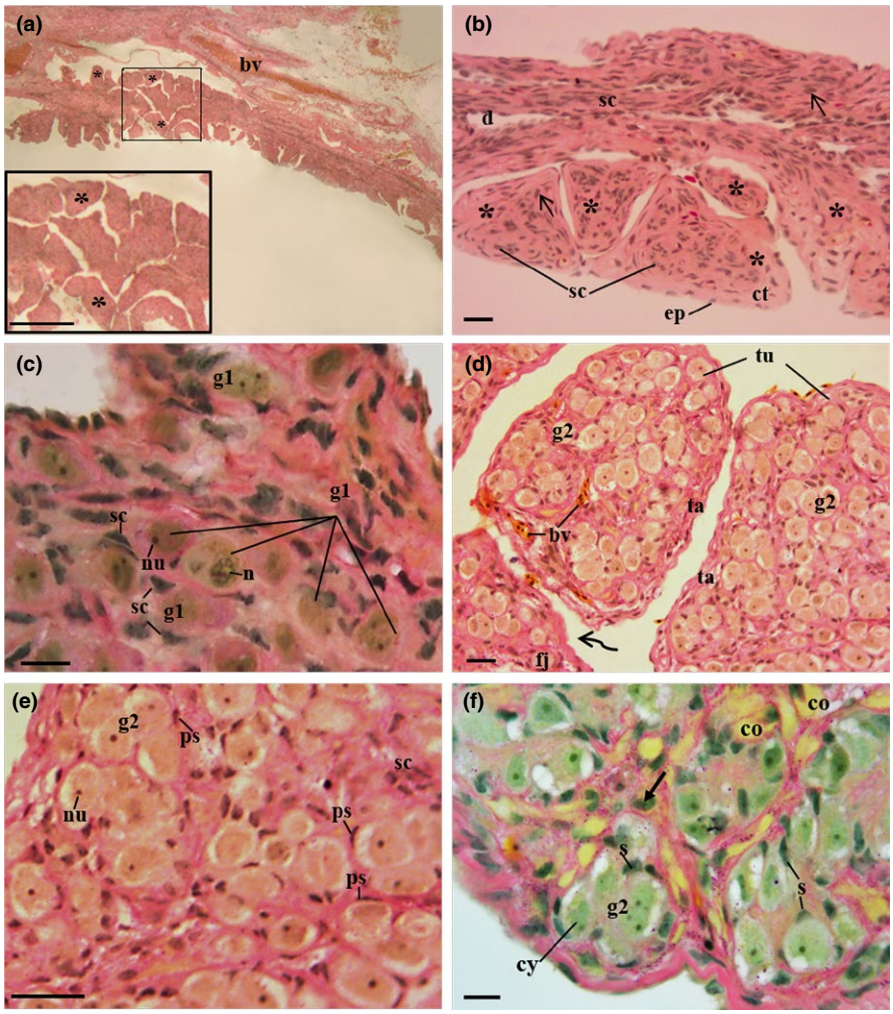

FIGURE 7 Photomicrography of the gonads of *P. fasciatum* at the start of testicular differentiation. Stage V and VI: (a)–(f). (a) Panoramic view of the fringed presumptive testicle showing grooves and small fringes (asterisks). Blood vessels (bv). Scale bar (200 μm). (b) Detail of the deferent duct (d) and small fringes (asterisks) that remain filled predominantly by somatic cells (sc). Lining epithelial (ep) and dense connective tissue (ct). Scale bar (20 μm). (c) Presence of primary spermatogonia (g1) individualized and surrounded by somatic cells (sc) throughout the gonad tissue. Nucleus (n) and nucleolus (nu). Scale bar (10 μm). (d) Testicular tubules with septum (wavy arrow) projecting inwards and originating the invaginations in the small fringes (fj). Presence of several secondary spermatogonia (g2), blood vessels (bv) and tunica albuginea (ta). Scale bar (20 μm). (e) Detail of Fig. D featuring a cluster of secondary spermatogonia (g2) with voluminous nuclei and centralized nucleoli (nu), surrounded by pre-Sertoli cells (ps). Scale bar (20 μm). (f): Developing cyst (cy) of secondary spermatogonia (g2) surrounded by Sertoli cells (s). Collagen (co), somatic cells (arrow). Scale bar (10 μm). Staining (a)–(f): Periodic Acid Schiff + Haematoxylin Ferrous + Metanil Yellow (PAS/HF/MY)

of correlation. Thus, the VIII stages of testicular development in this study are described in relation to the size, and not the age in this species.

3.3 | Undifferentiated gonads

In 63% of animals, with mean L_T = 35.9 ± 4.3 mm (stage I), the gonadal primordia presented as simple, paired structures and connected to a blood vessel by a mesogonadium through the coelomic epithelium, which in turn was connected on both sides of the mesentery. The mesentery consisted of connective tissue, epithelium (structure denominated peritoneum) and lined the whole coelomic cavity. The gonadal primordia were located ventrolaterally to the swim bladder, dorsally to the intestine and ventrally to the kidney (Figure 3).

On larger fish of the same age, the undifferentiated gonads were elongated, straight and thin (Figure 5a). The external region of the gonad was covered by squamous epithelium supported by dense connective tissue (Figure 5b). The gonads were composed of few primordial germ cells (PGCs) and several squamous somatic cells randomly distributed. The PGCs were larger than the somatic cells, elliptic, with voluminous nuclei containing condensed chromatin and prominent nucleoli (Figures 5b and 6a,b).

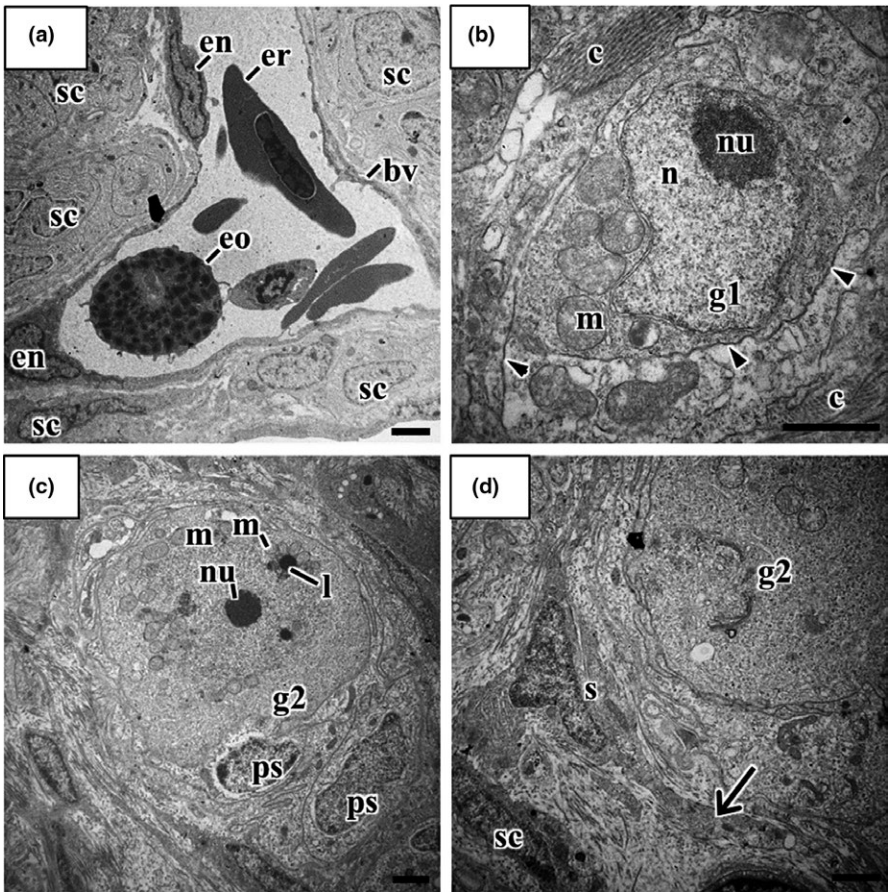

FIGURE 8 Transmission electronmicrography of the gonads of *P. fasciatum* at the start of testicular differentiation. Stage II–VI: (a)–(d). (a) Somatic cells (sc) near blood vessels (bv). Endothelium (en), eosinophil (eo) and erythrocyte (er). (b) Primary spermatogonia (g1) with elliptical nucleus (n), decentralized nucleolus (nu) and cytoplasm containing round mitochondria (m). Collagen (c), cell border (arrowhead). (c) Secondary spermatogonia (g2) with centralized nucleus (nu) and cytoplasm containing mainly round mitochondria (m) often associated with lysosomes (l), surrounded by pre-Sertoli cells (ps). (d) Sertoli cells (s) emitting cytoplasm projections (arrow) surrounding a spermatogonium (g2). Scale bar =2 μm [(a)–(d)]

3.4 | Beginning of testicular differentiation

3.4.1 | External morphology

Undifferentiated gonad presented elongated and straight shape in fish at mean L_T = 35.9 ± 4.3 (stages I) Figure 4. Grooves and small projections, evidence of the differentiation of a gonadal primordium into a presumptive testicle, were observed for the first time in animals with mean L_T = 45.1 ± 6.1 and 55.5 ± 6.5 mm (stages II and III) Figure 4. Most of the gonads were differentiated in fish at mean L_T = 69.2 ± 7.5 mm in length (stage IV) Figure 4. At this stage, the grooves became more pronounced and formed four very evident projections on the gonad giving it a cauliflower shape. Larger fish had more developed gonads, as illustrated in Figure 4.

3.4.2 | Internal morphology

At the beginning of testicular differentiation (animals with mean L_T = 45.1 ± 6.1 and 55.5 ± 6.5 mm, stages II and III), the undifferentiated gonads were developing into presumptive testicles connected to the coelomic cavity by the mesorchium, which was composed of a layer of dense connective tissue and blood vessels (Figure 5c). The testicles that were developing, showed a compact tissue with few elliptic germ cells, which were surrounded by somatic cells, at the ventral region (Figure 5c,d).

Even though somatic cells were randomly distributed throughout the gonad, some were arranged in distinctive patterns (Figure 5c,d), forming circular structures (cell clusters) (Figures 5d and 6e). The contacting surface of somatic cells contained interdigitations and desmosomes (Figure 6c,d). The cytoplasm of some somatic cells observed near blood vessels (Figure 8a) contained mitochondria with tubular cristae and smooth endoplasmic reticulum (Figure 6f), suggesting that these may be Leydig cells precursors.

In animals with mean L_T = 69.2 ± 7.5 mm (stage IV), digitiform projections appeared on the distal region of the presumptive testicle, transforming its external appearance from straight to irregular (Figure 5e and f). At the same time, the first evidence of small sperm ducts was observed in the proximal region (Figure 5f—detail). At this stage, the cell clusters became more evident and it is known that these structures originate the testicular tubules at later stages of development (Grier, 1981). In the proximal region, the somatic cells remained predominantly randomly distributed; however, at times they were observed surrounding the developing sperm duct (Figure 5g and h—detail).

3.5 | Proliferation of germ cells

In animals with mean L_T = 83.7 ± 9.1 mm (stage V) there was an increase in the width and length of the gonad and in the depth of the grooves, forming projections of fringed or digitiform aspect, future fringes (Figure 7a). Nevertheless, the presumptive

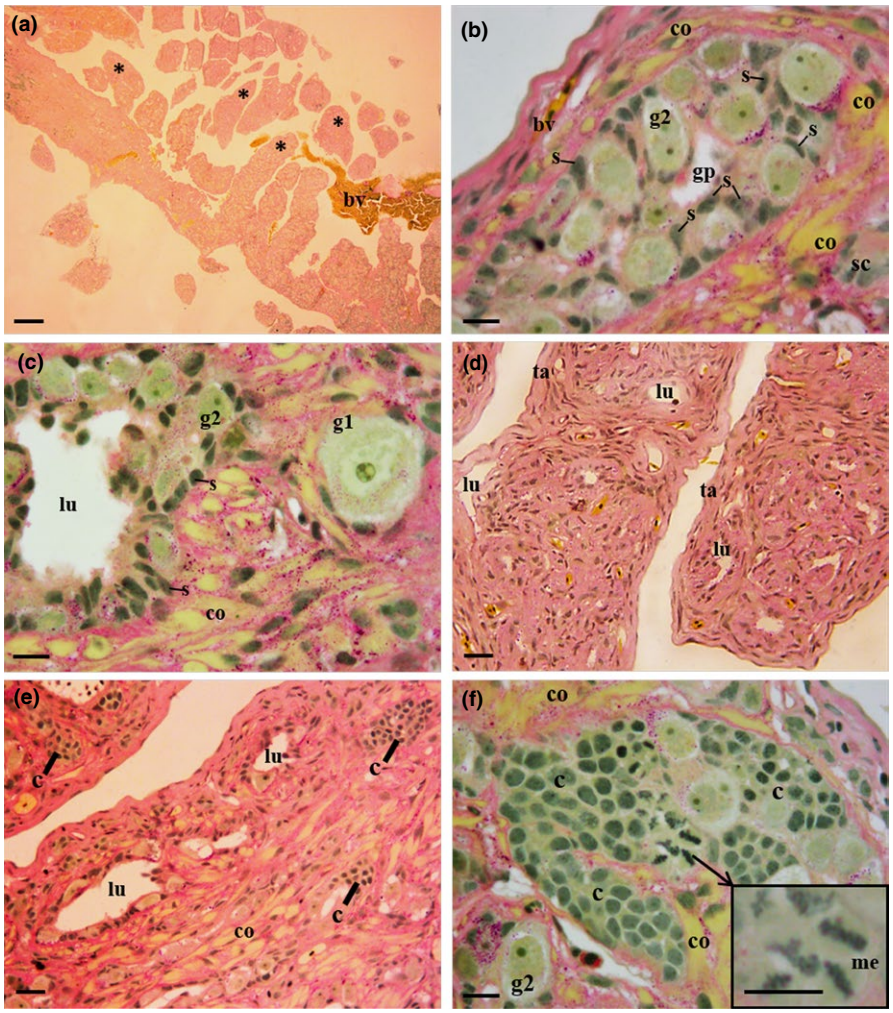

FIGURE 9 Photomicrography of anastomosing tubular unrestricted testis of *P. fasciatum*. Stage VII and VIII: (a)–(f). (a) Panoramic view of the larger testicle, with fringes (asterisks) of different shapes and sizes. Blood vessel (bv). Scale bar (200 μm). (b) Presence of a small gap (gp) between the spermatogonia (g2) that are individualized by Sertoli cells (s). Collagen (co) and somatic cells (sc). Scale bar (10 μm). (c) Increase in the gap between the spermatogonia originating the lumen (lu) of the testicular tubules (tu). (d) Anastomosing tubular testicle, featuring the lumen (lu) and tunica albuginea (ta). Scale bar (20 μm). (e) With the start of meiosis, cysts of spermatocytes (c) could be seen throughout the testicular parenchyma. Lumen (lu) and collagen (co). Scale bar (20 μm). (f) In the insert (arrow), the size and the basophilic nuclei of spermatocytes (c) can be seen. Isolated spermatogonia (g2) and collagen (co). Scale bar (10 μm). Insert: Presence of germ cells in meiosis (me). Scale bar (10 μm). Staining (a)–(f): Periodic Acid de Schiff + Iron Haematoxylin + Metanil Yellow (PAS/HF/MY)

testicles remained composed mainly of somatic cells and connective tissue (Figure 7b). As testicular development progressed, the germ cells or spermatogonia stem cells proliferated (primary spermatogonia stage) and were distributed throughout the gonad (Figure 7c). These cells were elliptic, had basophilic nuclei, decentralized nucleoli and cytoplasm containing mainly globular mitochondria (Figure 8b).

In animals with mean L_T = 95.7 ± 5.7 mm (stage VI), testicular development features were noteworthy, such as the invaginations in the small fringes of the testicles by septa from the tunica albuginea (Figure 7d) and the presence of secondary spermatogonia. In the small fringes had several clusters of spermatogonia surrounded by somatic cells, which from this stage onwards were denominated pre-Sertoli cells (Figure 7e). The spermatogonia were round with voluminous nuclei (Figure 7e), single central nucleoli and cytoplasm full of mitochondria connected to lysosomes (Figure 8c). Within every cluster, each spermatogonium was surrounded by Sertoli cells, forming cysts (Figure 7f). The Sertoli cells had triangular nuclei and scarce cytoplasm, similarly to pre-Sertoli cells; however, they emitted cytoplasmic projections and surrounded each spermatogonium individually (Figure 8d).

3.6 | Anastomosing tubular testis

The testicles of animals with mean L_T = 126.8 ± 8.5 mm (stage VII) were thicker and longer, with larger fringes of different sizes throughout the surface, characterizing it as fringes testis (Figure 9a). The tunica albuginea externally covered the whole testicle, enveloping each fringe (Figure 9d). Internally and within the cysts, the lumen was formed by the gradual distancing of the spermatogonia, Sertoli cells and other adjacent cells. Small spaces were formed and fused together creating a luminal compartment and cylindrical structures with a cavity, originating the testicular tubules and characterizing the testicle as of the anastomosing tubular unrestricted type (Figure 9b–d).

The testicular tubules were composed of secondary spermatogonia (Figure 9b,c), some of which were connected to neighbouring cells by interdigitations of the plasma membrane and desmosomes, ensuring greater adhesion and increasing the contacting surface between them (Figure 10a). Furthermore, small invaginations of the plasma membrane and communicating junctions connected spermatogonia to Sertoli cells (Figure 10b,c).

Spermatogenesis was evident in animals with mean L_T = 226.8 ± 13.6 mm (stage VIII), as in some areas of the testicular

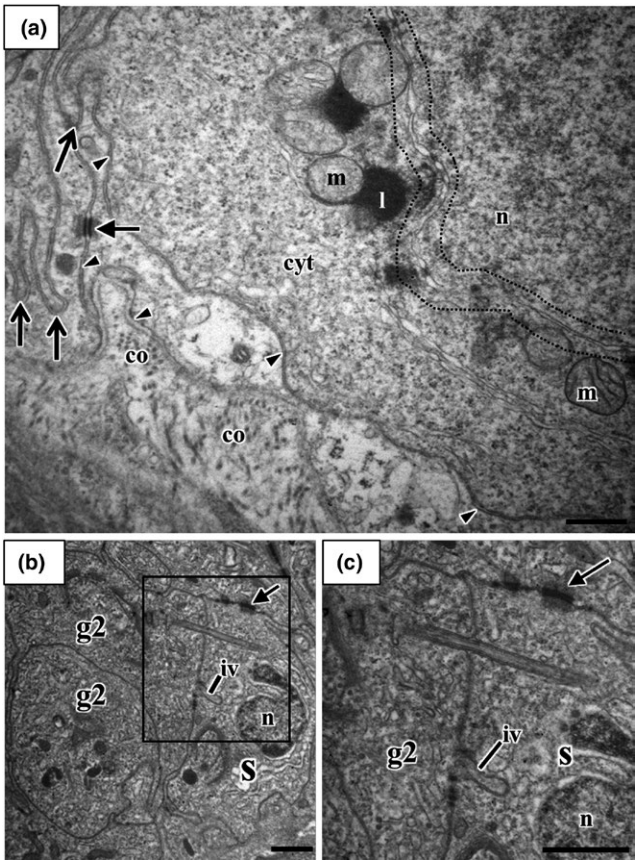

FIGURE 10 Transmission electronmicrography of anastomosing tubular unrestricted testis of *P. fasciatum*. Stage VII: (a)–(c). (a) Interdigitations (arrow) composed of invaginations and evaginations of the cell membranes. The barrier between the nucleus (n) and cytoplasm (cyt) was formed by the internal nuclear lining and by the smooth endoplasm reticulum of the cytoplasm (dotted line). Cell border (arrowhead), mitochondria (m), lysosomes (l), collagen (co) and desmosomes (arrow). [(b)–(c)] Secondary spermatogonia (g2) and Sertoli cells linked by desmosomes (arrow). Small invaginations of the plasma membrane (iv) communicating secondary spermatogonia (g2) to Sertoli cells (s). Scale bar =1 μm [(a)–(c)]

parenchyma the spermatogonia multiplied by mitosis and entered meiosis, differentiating into prophase I and originating spermatocytes (Figure 9e). The spermatocytes, with nuclei more basophilic, were smaller than spermatogonia, with absent nucleoli (Figure 9f). Inside the cysts, the spermatocytes were surrounded by Sertoli cells. The germ cells were at the same stage of development and the changes occurred in synchronization, characteristic of this phase.

Thus, the testicles were completely formed, however, the final stage of spermatogenesis (development of spermatids) had not been reached by the last sample collection (stage VIII), even though the testicles had begun cellular differentiation with germ cells entering meiosis. The main features of testicular differentiation and development mentioned in this study are detailed in Table 2 and Figure 11.

4 | DISCUSSION

It was observed that fish length had a stronger influence (R^2 = 0.97) in determining testicular development in *P. fasciatum* than age (R^2 = 0.86); therefore, it was the parameter used in associations with the stages of gonadal development as also observed by Blazquez, Felip, Zanuy, Carrillo, and Piferrer (2001) and Gao et al. (2009).

Sexual dimorphism is evident in most of the fish, internally and externally. It alters gonad morphology and affects growth rate and maturation age (Strussmann, Cota, Cota, Phonlor, Higuchi, & Takashima, 1996; Strussmann, Takashima, Takashima, & Toda, 1996); which are characteristics of commercial interest, especially in monosex farming (Marjani, Jamili, & Mostafavi, 2009). In the present study, the dimorphism between testicles and ovaries was observed at the start of the process of testicular differentiation. Early identification of the testis was possible due to the future fringes appearance of the gonad as a result of the proliferation of the stroma and also the early cellular arrangement (circular formation) of somatic cells in the distal region; which later originated the sperm ducts in *P. fasciatum* and which has been reported in several gonochoristic species (Kobayashi et al., 2011; Komatsu, Nakamura, & Nakamura, 2006; Meijide, Lo Nostro, & Guerrero, 2005). Therefore, the morphological indications of gonadal differentiation may be related to the different arrangements of somatic and germ cells during early development (Mazzoni, Grier, & Quagio-Grassiotto, 2010, 2014; Valentin et al., 2016), and may precede the formation of sperm ducts in males and the start of meiosis in females (Kobayashi et al., 2011; Strussmann, Takashima et al., 1996).

In *P. fasciatum*, the increase of frequency and the distribution of somatic cells were related to the earliest sexual discernment of the gonad, distinguishing an undifferentiated gonad from a presumptive testicle even when the germ cells were at a quiescent state. This aspect can be considered different from the earliest sexual discernment described in many species, as in these species it has been characterized by simultaneous proliferation and reorganization of germ and somatic cells (Guerrero-Estevez & Moreno-Mendoza, 2012; Mazzoni, Grier, & Quagio-Grassiotto, 2014; Meijide et al., 2005).

The morphologic characteristics observed in the testis of *P. fasciatum* at the start of testicular differentiation (presence of invaginations in the small fringes) were similar to the corresponding structures in the testicles of adults, which are characterized by the presence of testicular fringes that have been reported in families from the order Siluriformes (Santos, Bazzoli, Rizzo, & Santos, 2001).

Another important aspect in discerning testicular differentiation is the distribution and arrangement of spermatogonia during the initial period of gonadal development (Mazzoni et al., 2014). In *P. fasciatum*, the spermatogonia were arranged into clusters that would originate the first testicular tubules, similarly to *Cyprinus Carpio* (Mazzoni et al., 2014). On the other hand, the location of the clusters of spermatogonia and sperm ducts varied between the species. These differences are often associated with the shape of the

TABLE 2 Main features of testicular differentiation and development in *P. fasciatum*

Gonad	Animals (L_T/mm)	Main events
Undifferentiated Gonad	35.9 ± 4.3 (Stage I)	Straight shape. Few PGCs and several squamous somatic cells randomly distributed
Start of Testicular differentiation	45.1 ± 6.1 (Stage II)	Grooves on the external region of the gonad and the presence of small projections on the male gonad
		Few elliptic germ cells, which were surrounded by somatic cells
	55.5 ± 6.5 (Stage III)	Increase in the number of grooves and projections
	69.2 ± 7.5 (Stage IV)	The first testicular tubules and ducts can be seen. Proliferation of somatic cells in circular pattern.
	83.7 ± 9.1 (Stage V)	Increase in the number of grooves
		Projections are more prominent and numerous
		Presence of primary spermatogonia
		Somatic cells (pre-Sertoli) can be seen
	95.7 ± 5.7 (Stage VI)	Tunica albuginea is formed
		Septa appear and form testicular tubules
		Cysts of secondary spermatogonia
Anastomosing tubular unrestricted testis	126.8 ± 8.5 (Stage VII)	Testicles increase in thickness and length
		Presence of fringes of various shapes and sizes
		Invagination of the tunica albuginea
		Anastomosing seminiferous tubules are formed
		Cysts of secondary spermatogonia surrounded by Sertoli cells
		Presence of collagen fibres around the cysts
		Luminal compartment starts to form
	226.8 ± 13.6 (Stage VIII)	Start of meiosis
		Presence of cysts containing spermatocytes

testicles in adults (Batlouni, Romagosa, & Borella, 2006; Mazzoni et al., 2014). Although these structures are present in different regions, both species have a similar unrestricted spermatogonial distribution (Grier, Linton, Leatherland, & Vlaming, 1980).

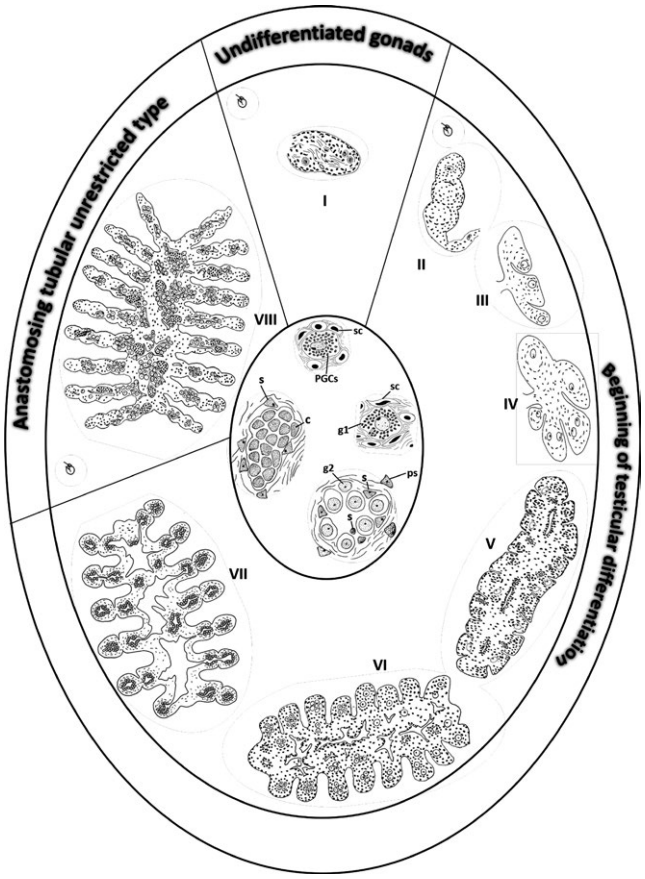

FIGURE 11 Summarized schematic representation of the VIII gonadal phases during the process of testicular differentiation and development of *P. fasciatum* and the main types of corresponding germ cells, according to the following numbers and abbreviations. I: (PGCs). II–V: (g1). VI–VII: (clusters of g2). VIII: (cysts of g2 and cysts of c). Abbreviations: PGCs (primordial germ cells), g1 (primary spermatogonia), g2 (secondary spermatogonia), c (spermatocyte), sc (somatic cells), ps (pre-Sertoli cells), s (Sertoli cells)

In the majority of teleost, including *P. fasciatum*, the spermatogonia and Sertoli cell proliferation occurs simultaneously. The appearance of the lumen in the testicular parenchyma indicates that the differentiation and proliferation of Sertoli cells occur during the ontogenetic process and before the meiosis onset (Cassel, Neves da Silva, & Ferreira, 2013; Mruk & Cheng, 2004). In catfish and Nile tilapia, this proliferation of Sertoli cells is greatly reduced when the germ cells enter meiosis and the stabilization of the number of Sertoli cells per cyst is probably due to the formation of the cellular barrier (Schulz et al., 2005). This fact is relationed to the levels of steroid hormones, mainly androgens (11-ketotestosterone, 11-KT) that vary during testicular maturation, suppressing the expression of AMH in Sertoli cells and allowing the onset of spermatogenesis (Schulz et al., 2010).

Spermatogenesis in fish is regulated by a neuroendocrine mechanism that involves the interaction between germ, Sertoli and Leydig cells (Miura, 1999; Schulz et al., 2010; Yaron, 1995). In the specie used in this study, interstitial cells such as Leydig cells precursor had cytological characteristics often associated with steroid secreting

cells; such as tubular cristae, endoplasmic reticulum and proximity to blood vessels, performing a critical role in regulating self-renewal and differentiation of spermatogonial stem cells (Nakamura & Nagahama, 1989; Potter & DeFalco, 2017).

In this way, it was possible to visualize Leydig cells with typical ultrastructural characteristics at the start of testicular differentiation and simultaneous to the formation of the small fringes and somatic cell clusters.

It can be concluded that length, and not age, is the main factor associated with the start of testicular ontogenesis in *P. fasciatum*. The findings from this study show that the formation of small fringes and the circular arrangement of the somatic cells within the presumptive testicles were the criteria that defined sexual dimorphism, and not the development of sperm ducts. The start of gonadal differentiation in *P. fasciatum* occurred at an early stage, in specimens with L_T = 45.1 ± 6.1 to 55.5 ± 6.5 mm/39–45 dph (stage II and III), suggesting that sex inversion protocols may be successfully applied prior this moment in this specie, because the physiological sexual differentiation (critical period) occurs before the morphological sexual differentiation (Nakamura, 2013).

ACKNOWLEDGMENTS

The experimental protocol was approved by the Ethics Committee on the Use of Animals (CEUA) of the School of Agricultural and Veterinary Sciences, Universidade Estadual Paulista—UNESP, Jaboticabal-SP, Brazil (Protocol: 016263/09). The authors would like to thanks the: CAUNESP, Mar & Terra Ltda, FAPESP for the PhD scholarship (2009/15392-6) and financial assistance granted (2010/16775-3), Department of Morphology at the Institute of Biosciences—UNESP/Botucatu—SP, Talita Sarah Mazzoni, Electron Microscopy Multi-user Laboratory at the Department of Molecular Cell Biology and Pathogenic Bio-agents at FMRP/USP/ Ribeirão Preto - SP and B. G. S. Favaretto for the services provided with the schematic drawings.

ORCID

Fernanda N. Valentin https://orcid.org/0000-0002-8279-3758
Nivaldo F. do Nascimento https://orcid.org/0000-0002-0515-3431

REFERENCES

Abozaid, H., Wessels, S., & Hoerstgen-Schwark, G. (2012). Elevated temperature applied during gonadal transformation leads to male bias in zebrafish (*Danio rerio*). *Sexual Development*, *6*(4), 201–209. https://doi.org/10.1159/000336297

Athauda, S., Anderson, T., & de Nys, R. (2012). Effect of rearing water temperature on protandrous sex inversion in cultured Asian Seabass (*Lates calcarifer*). *General and Comparative Endocrinology*, *175*(3), 416–423. https://doi.org/10.1016/j.ygcen.2011.11.040

Baroiller, J. F., D'Cotta, H., & Saillant, E. (2009). Environmental effects on fish sex determination and differentiation. *Sexual Development*, *3*(2–3), 118–135. https://doi.org/10.1159/000223077

Batlouni, S. R., Romagosa, E., & Borella, M. I. (2006). The reproductive cycle of male catfish *Pseudoplatystoma fasciatum* (Teleostei, Pimelodidae) revealed by changes of the germinal epithelium – An approach addressed to aquaculture. *Animal Reproduction Science*, *96*(1–2), 116–132. https://doi.org/10.1016/j.anireprosci.2005.11.012

Blazquez, M., Felip, A., Zanuy, S., Carrillo, M., & Piferrer, F. (2001). Critical period of androgen-inducible sex differentiation in a teleost fish, the European sea bass. *Journal of Fish Biology*, *58*(2), 342–358. https://doi.org/10.1006/jfbi.2000.1453

Cassel, M., Neves da Silva, D. F., & Ferreira, A. (2013). Cytoarchitectonical dynamic of Sertoli cells in *Melanorivulus punctatus* (Cyprinodontiformes: Rivulidae). *Micron*, *45*, 115–118. https://doi.org/10.1016/j.micron.2012.11.005

Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture*, *208*(3–4), 191–364. https://doi.org/10.1016/s0044-8486(02)00057-1

Gao, Z., Wang, H.-P., Rapp, D., O'Bryant, P., Wallat, G., Wang, W., … MacDonald, R. (2009). Gonadal sex differentiation in the bluegill sunfish *Lepomis macrochirus* and its relation to fish size and age. *Aquaculture*, *294*(1–2), 138–146. https://doi.org/10.1016/j.aquaculture.2009.05.024

Grier, H. J. (1981). Cellular-organization of the testis and spermatogenesis in fishes. *American Zoologist*, *21*(2), 345–357. https://doi.org/10.1093/icb/21.2.345

Grier, H. J., Linton, J. R., Leatherland, J. F., & De Vlaming, V. L. (1980). Structural evidence for two different testicular types in teleost fishes. *American Journal of Anatomy*, *159*(3), 331–345. https://doi.org/10.1002/aja.1001590307

Guerrero-Estevez, S., & Moreno-Mendoza, N. (2012). Gonadal morphogenesis and sex differentiation in the viviparous fish *Chapalichthys encaustus* (Teleostei, Cyprinodontiformes, Goodeidae). *Journal of Fish Biology*, *80*(3), 572–594. https://doi.org/10.1111/j.1095-8649.2011.03196.x

Karnovsky, M. J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *Journal of Cell Biology*, *27*, 137–138.

Kobayashi, T., Ishibashi, R., Yamamoto, S., Otani, S., Ueno, K., & Murata, O. (2011). Gonadal morphogenesis and sex differentiation in cultured chub mackerel, *Scomber Japonicus*. *Aquaculture Research*, *42*(2), 230–239. https://doi.org/10.1111/j.1365-2109.2010.02616.x

Komatsu, T., Nakamura, S., & Nakamura, M. (2006). A sex cord-like structure and some remarkable features in early gonadal sex differentiation in the marine teleost Siganus guttatus(-Bloch). *Journal of Fish Biology*, *68*(1), 236–250. https://doi.org/10.1111/j.1095-8649.2005.00897.x

Labbé, C., Robles, V., & Herraez, M. P. (2017). Epigenetics in fish gametes and early embryo. *Aquaculture*, *472*, 93–106. https://doi.org/10.1016/j.aquaculture.2016.07.026

Marjani, M., Jamili, S., & Mostafavi, P. G. (2009). Influence of 17-alpha methyl testosterone on masculinization and growth in tilapia (*Oreochromis mossambicus*). *Journal of Fisheries and Aquatic Science*, *4*(1), 71–74. https://doi.org/10.3923/jfas.2009.71.74

Mazzoni, T. S., Grier, H. J., & Quagio-Grassiotto, I. (2010). Germ line cysts and the formation of the germinal epithelium during the female gonadal morphogenesis in *Cyprinus Carpio* (Teleostei: Ostariophysi: Cypriniformes). *Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology*, *293*(9), 1581–1606. https://doi.org/10.1002/ar.21205

Mazzoni, T. S., Grier, H. J., & Quagio-Grassiotto, I. (2014). Male gonadal differentiation and the paedomorphic evolution of the testis in teleostei. *Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology*, *297*(6), 1137–1162. https://doi.org/10.1002/ar.22915

Meijide, F. J., Lo Nostro, F. L., & Guerrero, G. A. (2005). Gonadal development and sex differentiation in the cichlid fish *Cichlasoma dimerus* (Teleostei, Perciformes): A light- and electron-microscopic study. *Journal of Morphology*, *264*(2), 191–210. https://doi.org/10.1002/jmor.10329

Miura, T. (1999). Spermatogenetic cycle in fish. In E. Knobil & J. D. Neil (Eds.), *Encyclopedia of reproduction* (pp. 571–578). New York, NY: Academic Press.

Mruk, D. D., & Cheng, C. Y. (2004). Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocrine Reviews*, *25*(5), 747–806. https://doi.org/10.1210/er.2003-0022

Nakamura, M. (2013). Morphological and physiological studies on gonadal sex differentiation in teleost fish. *Aqua-BioScience Monographs*, *6*, 1–47.

Nakamura, M., & Nagahama, Y. (1989). Differentiation and development of leydig-cells, and changes of testosterone levels during testicular-differentiation in tilapia *Oreochromis-niloticus*. *Fish Physiology and Biochemistry*, *7*(1–6), 211–219. https://doi.org/10.1007/bf00004709

Piferrer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture*, *197*(1–4), 229–281. https://doi.org/10.1016/s0044-8486(01)00589-0

Piferrer, F., Ribas, L., & Diaz, N. (2012). Genomic approaches to study genetic and environmental influences on fish sex determination and differentiation. *Marine Biotechnology*, *14*(5), 591–604. https://doi.org/10.1007/s10126-012-9445-4

Potter, S. J., & DeFalco, T. (2017). Role of the testis interstitial compartment in spermatogonial stem cell function. *Reproduction*, *153*(4), R151–R162. https://doi.org/10.1530/REP-16-0588

Quintero-Hunter, I., Grier, H., & Muscato, M. (1991). Enhancement of histological detail using metanil yellow as counterstain in periodic acid/Schiff 's hematoxylin staining of glycol methacrylate tissue sections. *Biotechnic & Histochemistry*, *66*, 169–172. https://doi.org/10.3109/10520299109109964

Santos, J. E., Bazzoli, N., Rizzo, E., & Santos, G. B. (2001). Morphofunctional organization of the male reproductive system of the catfish *Iheringichthys labrosus* (Lutken, 1874) (Siluriformes: Pimelodidae). *Tissue & Cell*, *33*(5), 533–540. https://doi.org/10.1054/tice.2001.0207

Schulz, R. W., de Franca, L. R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R. H., & Miura, T. (2010). Spermatogenesis in fish. *General and Comparative Endocrinology*, *165*(3), 390–411. https://doi.org/10.1016/j.ygcen.2009.02.013

Schulz, R. W., Menting, S., Bogerd, J., Franca, L. R., Vilela, D. A. R., & Godinho, H. P. (2005). Sertoli cell proliferation in the adult testis – Evidence from two fish species belonging to different orders. *Biology of Reproduction*, *73*(5), 891–898. https://doi.org/10.1095/biolreprod.105.039891

Strussmann, C. A., Cota, J. C. C., Phonlor, G., Higuchi, H., & Takashima, F. (1996). Temperature effects on sex differentiation of two south American atherinids, *Odontesthes argentinensis* and *Patagonina hatcheri*. *Environmental Biology of Fishes*, *47*(2), 143–154. https://doi.org/10.1007/bf00005037

Strussmann, C. A., Takashima, F., & Toda, K. (1996). Sex differentiation and hormonal feminization in pejerrey *Odontesthes bonariensis*. *Aquaculture*, *139*(1–2), 31–45. https://doi.org/10.1016/0044-8486(95)01161-7

Valentin, F. N., Batlouni, S. R., Nascimento, N. F., Silva, R. C., Manzini, B., Hilbig, C. C., … Nakaghi, L. S. O. (2016). Ovarian differentiation and development in cachara Pseudoplatystoma fasciatum catfish. *Journal of Fish*, *Biology(1)*, 445–466. https://doi.org/10.1111/jfb.13034

Yaron, Z. (1995). Endocrine control of gametogenesis and spawning induction in the carp. *Aquaculture*, *129*(1–4), 49–73. https://doi.org/10.1016/0044-8486(94)00229-h

How to cite this article: Valentin FN, Nakaghi LSO, Batlouni SR, et al. Testicular differentiation and development in South American catfish, surubim, *Pseudoplatystoma fasciatum*. *J Appl Ichthyol*. 2019;00:1–12. https://doi.org/10.1111/jai.13879