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Histological description of the early gonadal development of *Arapaima gigas*, paiche

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Abstract

Arapaima gigas, paiche, is the largest freshwater fish species in the South American Amazon. Despite the importance of an accurate understanding of the species' gonadal sexual differentiation mechanisms, the gonadogenesis process is still not well studied. The aim of this study was to determine the histological characteristics of the early gonadal development of paiche fry, belonging to the same "levante" ("levante" is the word used to describe the removal of fry from the male paiche). The fry was sampled during the month of September 2016; from the 5-day post "levante" (dpl) to the 51 dpl with a minimum size of 2.7 cm and a maximum size of 16.0 cm in total length. The histology showed a large concentration of cells in the dorsal part of the abdominal cavity in 5 dpl fry, being greater in the left side. In addition, the gonadal ridges appeared at 6 dpl and the gonadal primordia appeared at 7 dpl. The two gonadal primordia were observed with different sizes, where the left primordia were larger and more basophilic than the right primordia. Finally, gonad differentiation between presumable females and males were observed with some structural differentiation between presumable females and presumable males since 43 post hatching day.

KEYWORDS
early gonadal development, gonadal primordia, levante, paiche

1 | INTRODUCTION

Gonadogenesis is the development of gonads, which is divided in steps, including the specification of gonad precursor cells, formation of a gonad primordium, and establishment of gonad polarity (Siegfried & Kimble, 2002). The origin and history of the germ line has been widely studied in the model organism *Danio rerio*, zebra fish (Maack & Segner, 2003) or *Cyprinus carpio*, carp (Parmentier & Timmermans, 1985); with special emphasis on the mechanism of sex differentiation. Despite the importance of determining the period in which the early determination and sex differentiation occurs, studies in fish reproduction have been mainly focused on the maturation of germ cells in adult fish, while early gonadal development has received relatively little attention (van Winkoop, Booms, Dulos, & Timmermans, 1992).

Among the freshwater fish considered with high importance for aquaculture, *Arapaima gigas*, paiche in Perú and pirarucu in Brazil, which belongs to Osteoglossomorpha, one of the three lineages of teleost (Vialle et al., 2018) is one of the largest freshwater fish in South America, with some adults reaching three meters in length and weight of more than 200 kg (Imbiriba, 2001; Lima et al., 2015; Moura Carvalho & Nascimento, 1992; Nelson, 1994; Oliveira et al., 2012). It is native to the Amazon basin, but also found in fish farms in Thailand and Malaysia (Froese & Pauly, 2018; Pereira, 1954). The commercial importance of paiche has been fairly emphasized with publications since the mid-18th century (Godinho, Santos, Formagio, & Guimarães-Cruz, 2005), placing emphasis on sex determination, molecular differentiation and gonadal activity (Almeida, Ianella, Faria, Paiva, & Caetano, 2013; Du et al., 2019; Prado-Lima & Val, 2015; Torati et al., 2017; Torati, Lima, Kirschnik, & Migaud, 2019; Watanabe et al., 2018).

According to fisheries records, until the 1960s, paiche was abundant near the large cities of the continental Amazon and its tributaries (Goulding, 1980), where it is found essentially in lakes and channels of floodplain areas (Arantes, Castello, Cetra, & Schilling, 2011). Reports of fishing landings by the Regional Production Agency of Loreto and Ucayali, Peru, show that the largest extraction of paiche was recorded in the Peruvian Amazon (around 200 tons in each region) from 1995 to 1999. However, records from 2000 reveal a sharp decrease in Peruvian landings, due to one of the most drastic decreases in natural populations, due to many years of strong fishing pressure and overexploitation. For this reason, the generation of knowledge related to the paiche biology is crucial (García-Dávila & Renno, 2016). Hence, the histological study of early gonadal development reaches notoriety as the basis for the study of its reproductive biology that will allow, in the future, the development of culture biotechnologies among which we can mention chromosome manipulation and hormonal treatments, which can be used to produce triploid, tetraploid, haploid, gynogenetic, and androgenetic fish (Foresti, 2000).

New techniques applied to aquaculture, such as monosex culture production by sexual reversal or gymnogenesis and androgenesis; require knowledge of the sexual differentiation timing. Careful histological observations of the gonadogenesis process are of primary importance for an accurate understanding of gonadal sexual differentiation mechanisms (Nakamura, Kobayashi, Chang, & Nagahama, 1998) and could help to develop efficient methods to direct sexual development in aquaculture species. Further, they can provide a guide to determine the hormone sensitive period in cases of sexual manipulation by exogenous steroid treatment (Foyle, 1993; Hunter & Donaldson, 1983; Strüssmann, Takashima, & Toda, 1996). Here, we performed histological cuts of the early gonadal development of *A. gigas*, in order to provide insights in the gonadal sexual differentiation mechanisms.

2 | MATERIAL AND METHODS

2.1 | Sampling

A total of 99 paiche fry were collected after the 5 day of post "levante" (dpl) ("levante" is the word used to describe the removal of fry from the male paiche) with a minimum size of 2.7 cm, up to 51 dpl with a maximum size of 16 cm from the Amazon Fish Products Company SA, located at kilometer 10 of the Jorge Basadre highway, Pucallpa-Ucayaly, Peru. During the first 15 days, the sampling was daily, to subsequently change to an interdaily sampling of three fry per day, recording

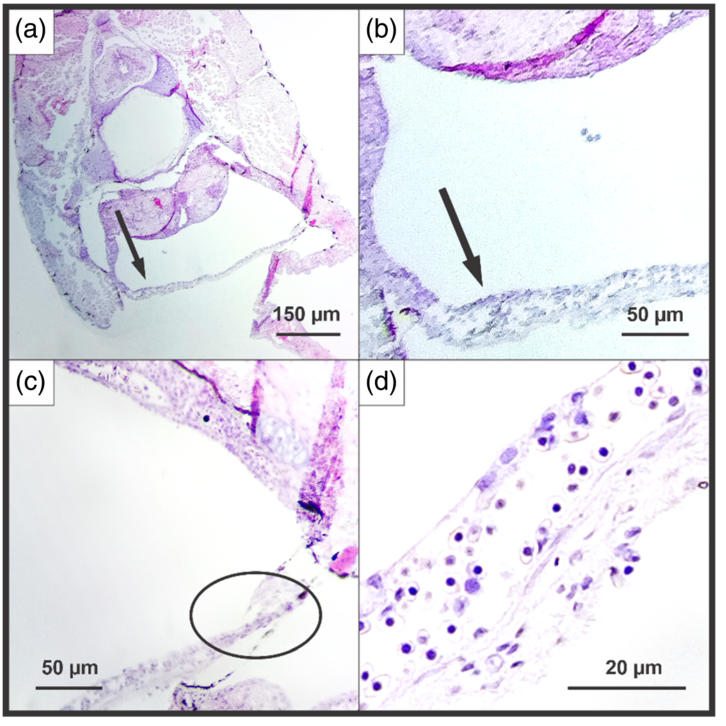

FIGURE 1 Photomicrography of *Arapaima gigas*, paiche. Histological section of 2.7 cm fry, at 5 dpl (21 phd): (a) panoramic view where a high cellular concentration is observed, with the detail that this is greater on the left side of the fry (b) and (d) and minor on the right side (c). There is no presence of ridges or gonadal primordia. Note in (d), the shape of the spiked cells, suggesting their migration through the mesodermal tissue by means of amoeboid movements. Staining: hematoxylin and eosin (HE)

the size of each individual. Fry were fixed and preserved in 10% formalin solution until their subsequent transportation to the Laboratory of Physiology, Genetics, and Reproduction of the National University of Santa, in Ancash, Peru.

It is worth mentioning that all the organisms were handled and slaughtered according to the state-of-the-art humane killing (American Veterinary Medical Association, Canadian Council of Animal Care in Science). The experiments will be carried out in accordance with the guidelines of the Ethics Committee for the use of animals in research in Peru. Guaranteeing at all times the animal welfare of the experimental organisms.

2.2 | Histology

A cross section of the fry body between the dorsal fin and the proximal area of the caudal fin was taken, accordingly to routine techniques. Cross sections were dehydrated in an automatic tissue dehydrator Thermo SCIENTIFIC—Microm STP120 in an ascending series of ethanol and embedded in paraffin. For the paraffin "blocks" preparation, a Thermo Scientific integrated inclusion center—Microm EC 350-2 was used. Six-micron cuts were made on a Thermo SCIENTIFIC Rotation Microtome—HM 325 Micron and mounted on slide holders for hematoxylin and eosin (HE) staining.

3 | RESULTS

Histological sections in paiche fry, show a large agglomeration of cells in the dorsal part of the abdomen, specifically at the kidney region, in samples taken at the first day of sampling (5 dpl or 21 post hatching day, phd; Figure 1), with

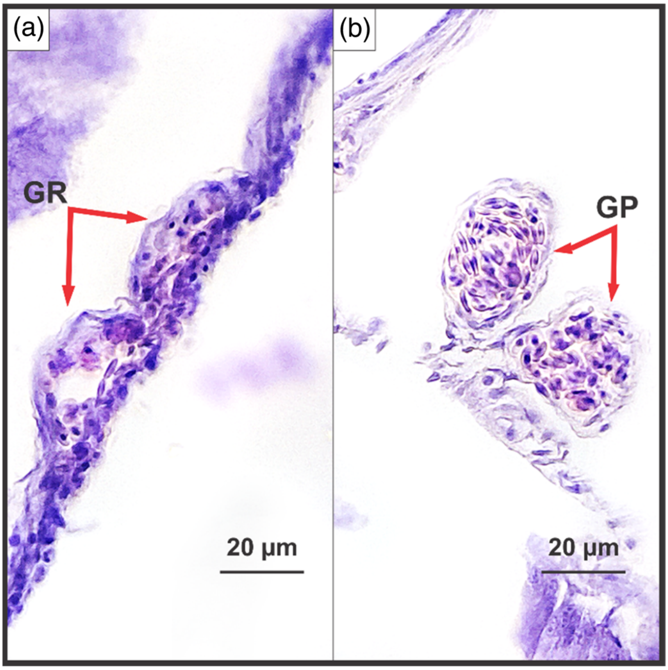

FIGURE 2 Photomicrography of *Arapaima gigas*, paiche. (a) Histological cross section of a 3.0 cm fry in total length and at 6 dpl (22 phd) in which the appearance of presumed gonadal ridges (GR) in the dorsal coelomic cavity. (b) Histological cross section of a 3.0 cm fry in total length and at 7 dpl (23 phd), showing the gonadal primordia (GP) indicated with red arrows. Staining: hematoxylin and eosin (HE)

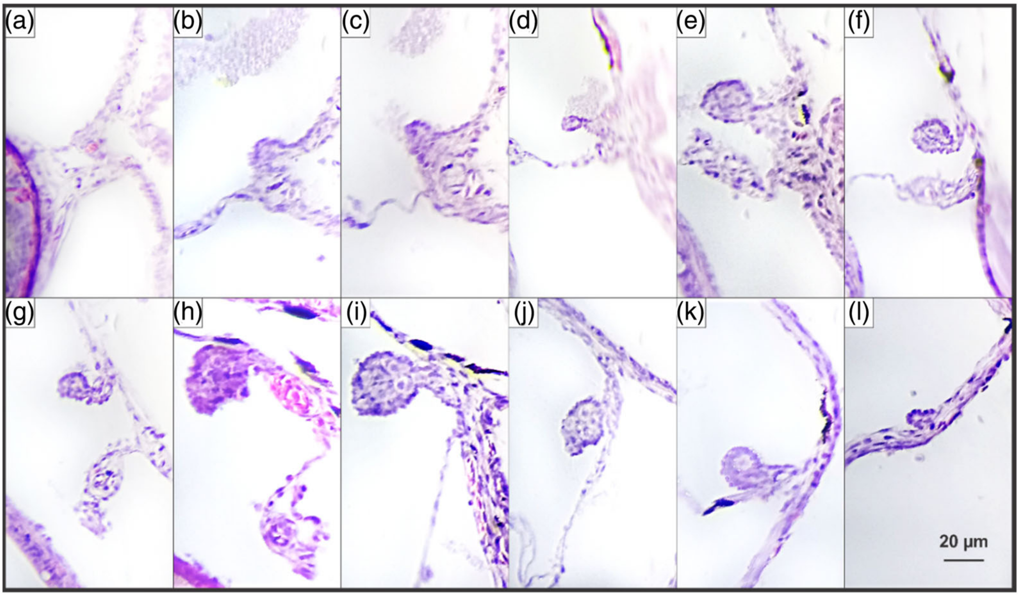

FIGURE 3 Photomicrography of *Arapaima gigas*, paiche. Cross-sectional histological description of a 3.1 cm full-length fingerling at 7 dpl (23 phd) from the posterior part of the primordium to the anterior part (a→k): Note the gradual appearance of the left gonadal primordium from the posterior part toward the previous one, in (e) the presence of a second less basophilic primordium is noted, which disappears and is completely lost in (i), while the left one remains until (l). Staining: hematoxylin and eosin (HE)

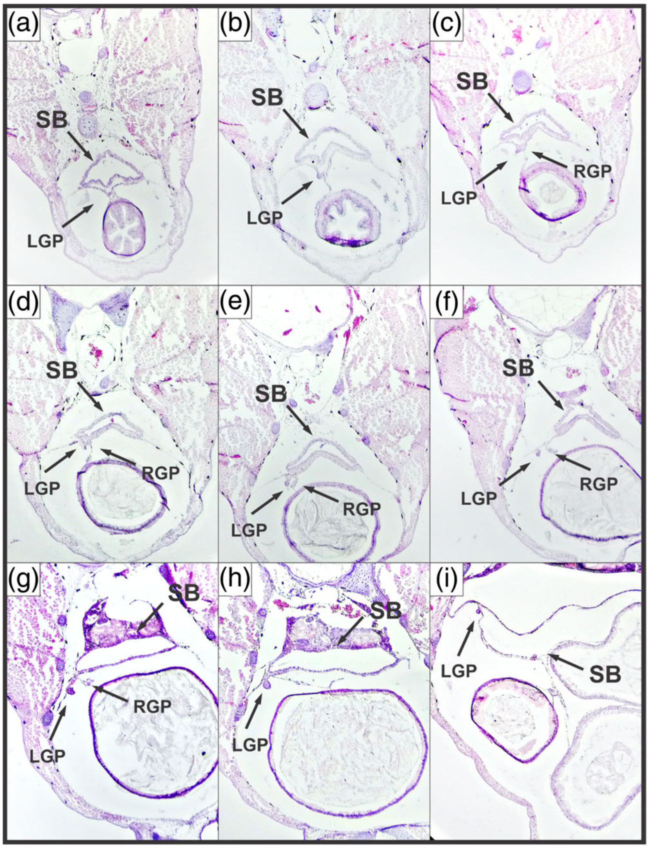

FIGURE 4 Photomicrography of *Arapaima gigas*, paiche. Cross-sectional histological description of a 3.1 cm full-length fingerling at 7 dpl (23 phd) from the posterior part of the coelomic to the anterior part (a→f): Note the gradual appearance of the left gonadal primordium (LGP) from the posterior part (a and b) to the middle (c, d e, f, g) and the front part of the abdomen (h and i). Note that both, LGP and RGP are on the left side of the coelomic. The swimming bladder (SB) looks small from (a) to (e), but increases its size in the anterior part of the abdomen (g, h, and i). Staining: hematoxylin and eosin (HE)

special emphasis on the left side of the fry. Samples that were taken 6 dpl or 22 phd, present some protuberances like genital crests in the dorsal part of the abdomen (Figure 2a). The presence of gonadal primordia (45.5 μm) is already observed in individuals at the 7 dpl or 23 phd, with an average of 3.1 cm in total length (TL), but positioning to the left side of the dorsal part of the coelomic cavity (Figure 2b).

Serial cuts from the posterior to the anterior part of the gonad in individuals with even gonadal primordia, revealed that the left gonadal primordium appears from the most dorsal part of the individual and increases in size as advances to the front of it (Figure 3). In cuts where the size of the left gonadal bud reaches its maximum (60 μm), the right gonadal bud is less basophilic, both the left and the right gonadal bud positioned toward the left side of the fry, and an increase in the size of the swim bladder is noted (Figure 4). Toward the anterior part of the primordia, the right gonadal primordium disappears, while the left one decreases in size as it approaches its anterior part. It has a strong positioning to the left of the coelomic cavity, in a position in which it seems to be "cornered" by the swim bladder (Figures 3 and 4). The gradual process of disappearance of the right gonadal primordium can be seen more clearly in a 24 days old individual (8 dpl or 24 phd; Figure 5), whose cuts correspond to the middle part of the left gonadal primordium.

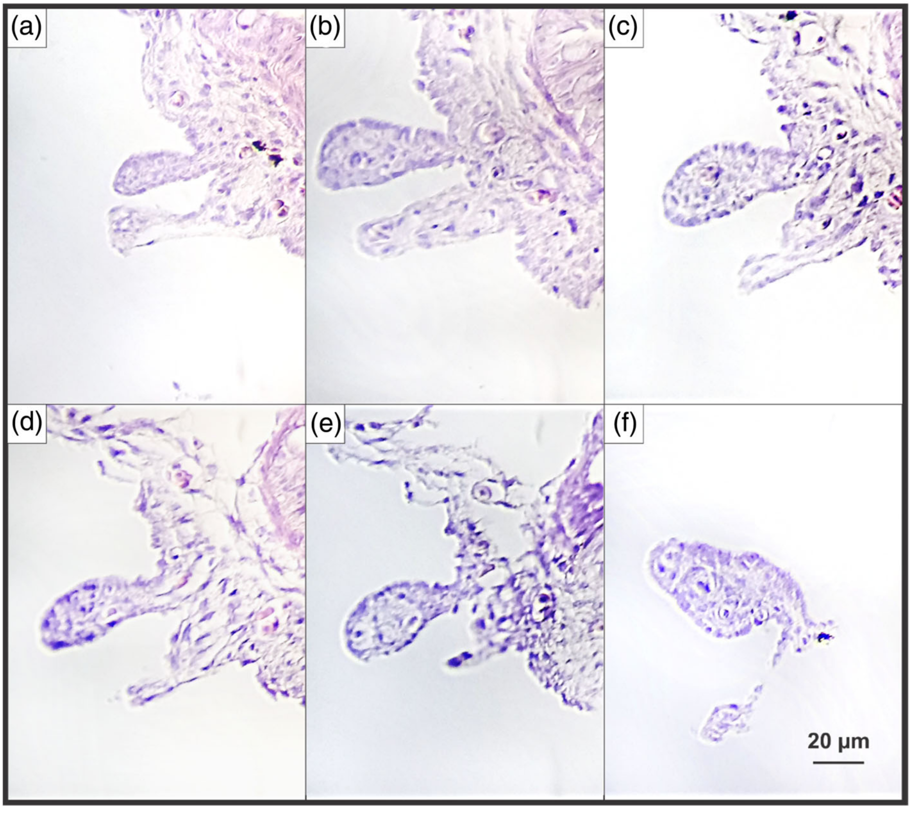

FIGURE 5 Photomicrography of *Arapaima gigas*, paiche. Transverse histological description of a 3.2 cm full-length fingerling at 8 dpl (24 phd), from the middle part of the primordia to the anterior part (a→f): Note the gradual disappearance of the right gonadal primordium. Staining: hematoxylin and eosin (HE)

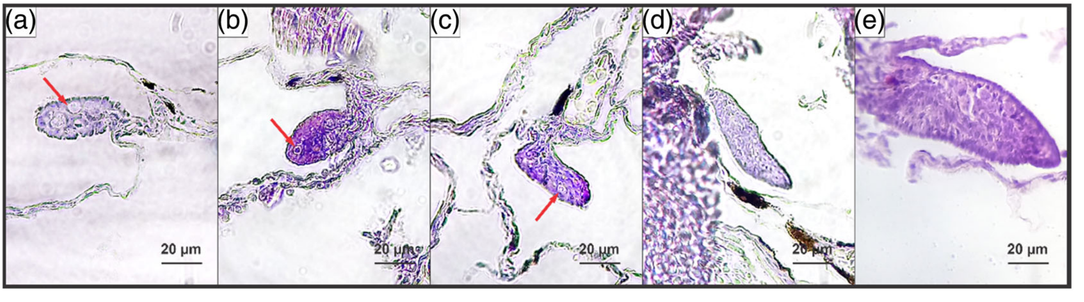

FIGURE 6 Photomicrography of *Arapaima gigas*, paiche. (a) Cross section of undifferentiated gonadal primordium of a 4.9 cm in total length fingerling (14 dpl or 30 phd). (b) From a 4.9 cm in total length fingerling (15 dpl or 31 phd). (c) of a 4.9 cm full-length fingerling (15 dpl or 31 phd). Note the clear presence of a gonia (red arrow) in a, b and c (d): a fingerling of 5.0 cm in total length (12 dpl or 32 phd). (e): of a 5.7 cm total length fingerling (19 dpl or 35 phd). Note the lengthening of the undifferentiated gonad in both (d) and (e). Staining: hematoxylin and eosin (HE)

Undifferentiated gonads are observed with an oval shape in fry that are up to 31 phd. The undifferentiated gonads then grow but undergo elongation when fry are up to 41 phd (Figure 6a: 60 μm, 6b: 40 μm, 6c: 44 μm, 6d: 68 μm and 6e: 84 μm). However, not all gonads maintain this undifferentiated

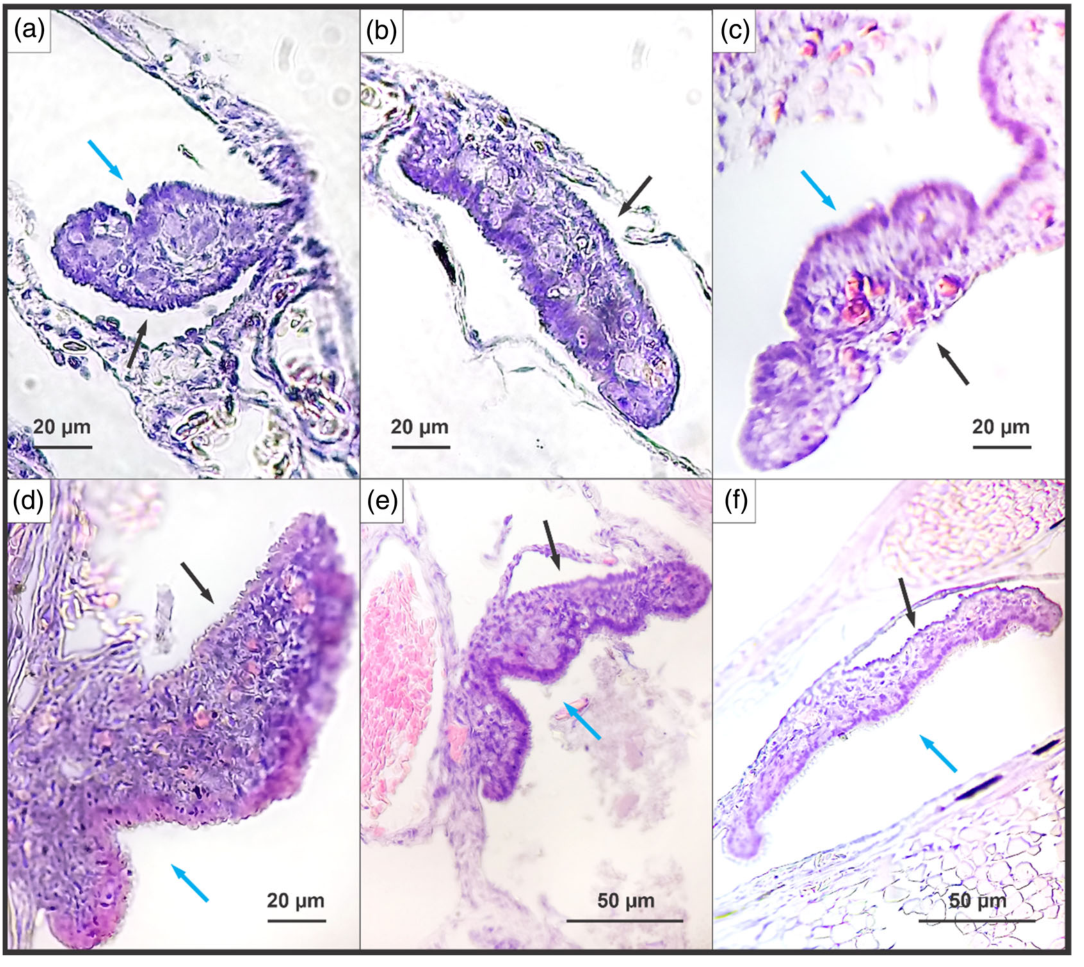

FIGURE 7 Photomicrography of *Arapaima gigas*, paiche. Cut of presumably female gonads of *A. gigas*, paiche. (a): of a 6.3 cm total length fingerling (23 dpl or 39 phd); (b): a 6.9 cm total length fingerling (25 dpl or 41 phd); (c): a 8.0 cm of total length fingerling (29 dpl or 45 phd); (d): of a fingerling of 10.5 cm in total length (35 dpl or 51 phd); (e): from a fingerling 11.6 cm in total length (39 dpl or 55 phd) and (f): from a fingerling 9.8 cm in total length (41 dpl or 57 phd). Observe the dorsal part with regular edges (black arrow) and the abdominal barde with completely irregular edges with invagination(s) (blue arrow) suggesting possible females (presumptive ovaries). Staining: hematoxylin and eosin (HE)

condition. Fry at 39 phd contain gonads in which a dorsal ventral arrangement can be observed with the presence of an invagination, giving the appearance of lamellas formation, which would correspond to presumable females. This condition becomes more evident after 45 phd, where a smooth dorsal part is clearly observed, contrasting with the quite irregular ventral part and with invaginations, which would correspond to future ovarian lamellae (Figure 7). On the other hand, it is not possible to differentiate the dorsal side of the ventral side in males. In contrast to females, males present regular edges and the shape of an "elongated boxer's pear" is observed with one thin end attached to the coelomic wall and the other rounded end. This can be observed at 43 phd (Figure 8).

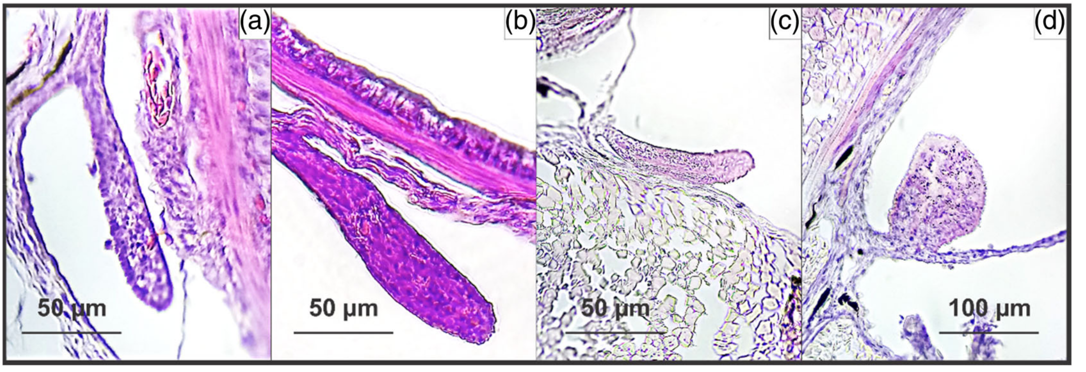

FIGURE 8 Photomicrography of *Arapaima gigas*, paiche. Cut of presumably male gonads. (a): of a 7.4 cm total length fingerling (27 dpl or 43 phd); (b): of a 9.3 cm total length fingerling (31 dpl or 47 phd); (c): of a fry 11.5 cm in total length (43 dpl or 59 phd); (d): from the same fingerling as in (c), but toward the anterior part of the primordium. Note the presence of channels inside the primordium that look like spermatic ducts suggesting a possible male (presumptive testicle). Staining: hematoxylin and eosin (HE)

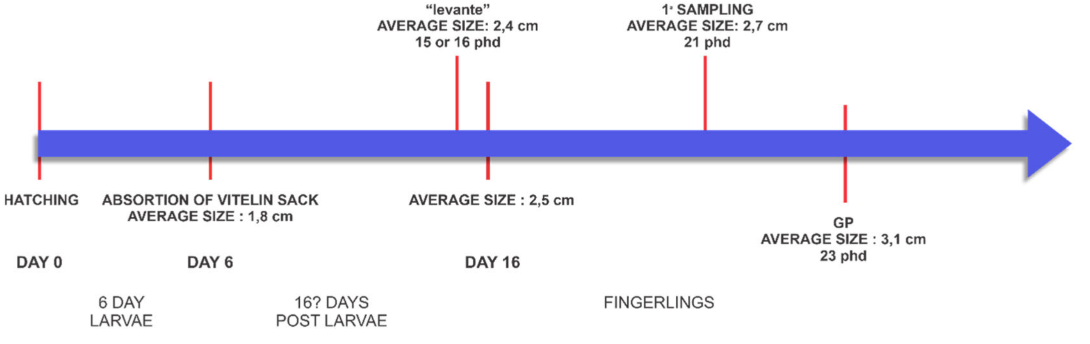

FIGURE 9 Temporal schematic representation of the development of the larvae, post larvae, and fingerlings of *Arapaima gigas*, paiche specifying the time of raising, first sampling, and appearance of gonadal primordia (GP)

4 | DISCUSSION

Due to the characteristics of the species reproduction, the fry used for this study were not collected on the day they hatched, but rather the "levante" was carried out at a later period of time not determined, so it cannot be assured with certainty the age of the fry in which the gonadal primordia appear. However, according to the Fisheries Development Fund (FONDEPES, 2016), depending on the temperature of the water, handling and feeding, paiches go through a stage of larvae that includes from the moment of hatching until the moment the yolk sac is absorbed, which occurs at 6 phd with an average size of 1.8 cm in TL, from where they are considered as post larvae individuals. Without specifying the duration of this stage, fingerlings are already mentioned at 16 phd, when the paiches reach a 2.5 cm of length (Figure 9).

Taking these criteria into account, it can be established that the individuals used in this study, with an average length of 2.7 cm on the first day of sampling (5 days after "levante"), were already in the "fingerling" stage. Also considering that the "levante" was performed when the individuals were 2.4 cm in length (finishing the post larvae stage), they were already approximately 15–16 phd at the time of "lifting" and 20–21 phd at the beginning of sampling; that is, at an age of 3 weeks post hatch (wph). Therefore, it can be established that the approximate age of appearance of the gonadal primordia in paiche, with an average length of 3.1 cm in TL, is 23 phd; that is, at 3 weeks of age and at the beginning of the 4-week post hatching (Figure 3b).

An adult paiche matures between 4 and 5 years (Campos Baca, 2001), and our histological description revealed the appearance of the gonadal primordia in the paiche when it is 3 weeks old or 23 phd. This correlates to the different sturgeon species maturation, such as the primitive actinopterygian that has existed from at least 200 million years (Bemis, Findeis, & Grande, 1997), whose males mature at 4 years of age and females between six and 12 years (Doroshov, Moberg, & Van Eenennaam, 1997). In the Siberian sturgeon, *Acipenser baerii*, the primordium appears at 14 phd, in the Russian sturgeon, *Acipenser guldenstaedtii*, it appears at 18 phd (Rzepkowska & Ostaszewska, 2013), in the short-nosed sturgeon, *Acipenser brevirostrum*, it appears at 26 phd (Flynn & Benfey, 2007), and at 16 phd in the Adriatic sturgeon, *Acipenser naccarii* (Grandi & Chicca, 2008).

There is only one report of gonadal primordium in paiche in a fingerling with 2.7 cm of TL (Da Costa Amaral, Lima, Ganeco-kirschnik, & de Almeida, 2020); that is, with a smaller size than that reported by this work. However, it is not possible to determine the age with this size since important details such as size at the time of the "lift," time elapsed since the "lift" and the time of sampling are unknown, as well as the management given to the fingerlings in the different Brazilian farms from which the specimens used in that study were obtained.

In regard to the disappearance of the right primordium observed in Figures 3 and 4, this could be a consequence of the evolution of the paiche due to its dependence on atmospheric oxygen. Many reports mention that specimens of paiche, both female and male, have been found that not only have the left gonad, but have also traces of the right one (Campos Baca, 2001; Fontenele, 1955; Luling, 1969 & Sanchez, 1961). The same authors describe a particular case of a paiche male with 1.9 m of length, in which whereas the left testicle was 24 cm long, the right testicle was 2 cm long, meaning that the right testis was approximately 1/10 of the left one, which could represent an ancestor with two equal and functional gonads.

The paiche is a teleostean osteoglassidae that has modified its swim bladder into an organ responsible for air respiration (air breathing organs, ABO), turning it into the fish most dependent on atmospheric oxygen (Brauner, Matey, Wilson, Bernier, & Val, 2004; Val & Almeida-Val, 1995). Despite that paiche larvae are aquatic respirators, within 8–9 days post hatch (about 18 mm of length) they become aerial respirators (Brauner et al., 2004). At the same time that the change of breath is occurring, the appearance of the gonadal primordia is occurring as well, which allows us to propose that the evolution of paiche toward obligate and almost exclusive air-breathing including the modification of its swim bladder enlarging, is what would be causing the narrowing of the coelomic cavity and atrophy of the right gonad.

Although there is a coincidence in the appearance of the gonadal primordia between *A. gigas* and different sturgeon species, their sexual differentiation process is not the same, at least in their cut appearance. The sturgeon gonads remain totally undifferentiated at 80 phd, while those of *A. gigas* remain undifferentiated only up to 41 phd (Figure 6e,f). Regarding the differentiation between females and males, the presumable females appear earlier (39 phd), maintaining a group of undifferentiated individuals who cannot be classified as presumable males because they could be females whose ovarian development has been delayed by extrinsic and/or intrinsic factors. This distinction between sexes, at least in terms of its cut appearance, it is already possible since 43 phd (Figures 7 and 8) even when no cells entering meiosis are observed, which would definitively indicate a histological differentiation toward the ovary.

5 | CONCLUSIONS

The gonadal primordia in paiche appear at 23 phd, where the left is larger and basophilic than the right, hence, the right gonad is destined to atrophy from the moment of its appearance, which could be the product of the evolution of paiche toward obligate and almost exclusive air-breathing thanks to the modifications incurred upon its swim bladder.

The presumable females begin to differentiate from 39 phd, however, there are gonads that remain undifferentiated up to 41 phd, and later, two groups of gonads from 43 phd are distinguished that look very different in the histological cuts. The presumable females are those that have a fairly clear back ventral arrangement, while the presumable males lack it, sporting a gonad shaped like an "elongated punching bag."

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