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GAMETOGÊNESE E EMBRIOGÊNESE DO PEIXE-SAPO *Pseudopimelodus charus* (PSEUDOPIMELODIDAE) DA BACIA DO RIO SÃO FRANCISCO.

GAMETOGENESIS AND EMBRYOGENESIS OF THE FROGFISH *Pseudopimelodus charus* (PSEUDOPIMELODIDAE) FROM THE SÃO FRANCISCO RIVER BASIN.

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Resumo - Este estudo teve como objetivo analisar a gametogênese e a embriogênese de *Pseudopimelodus charus*. Foram coletados fragmentos de gônadas em diferentes estágios de maturação de peixes capturados no Rio São Francisco e submetidos a técnicas histológicas de rotina e histoquímicas clássicas. Para analisar o desenvolvimento embrionário, peixes em cativeiro foram submetidos à reprodução induzida. Machos e fêmeas receberam uma dose única de extrato hipofisário (2,5 mg/kg de peso corporal para machos e 6,0 mg/kg de peso corporal para fêmeas). A incubação dos ovos foi realizada em incubadoras em forma de funil com circulação constante de água a 24°C e as amostras foram coletadas a cada 10 minutos. A foliculogênese iniciou-se com a proliferação de ovogônias, originando os folículos, e a espermatogênese iniciou-se com a proliferação de espermatogônias, originando as células da linhagem espermática. As reações histoquímicas foram realizadas e glicoproteínas neutras foram detectadas na zona pelúcida e nas células foliculares, ácidos carboxílicos glicoconjugados foram detectados nos alvéolos corticais e glicoproteínas neutras e lipídios foram detectados nos glóbulos de vitelo. As principais fases da embriogênese foram identificadas, juntamente com suas respectivas durações após a fertilização: fechamento do blastóporo às 4h50, formação dos somitos às 9h40 e eclosão às 18h50 após a fertilização. Os resultados do presente estudo fornecem informações sobre a biologia reprodutiva dessa espécie pouco conhecida.

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Palavras-chave: Fechamento do blastóporo, Foliculogênese, Histologia, Reprodução induzida, Espermatogênese.

Abstract - This study aimed to analyze the gametogenesis and embryogenesis of *P. charus* captured from the São Francisco River. Gonad fragments at different stages of maturation were subjected to routine histological and classical histochemical techniques. To analyze the embryonic development, fish were submitted to induced reproduction. Males and females received a single dose of pituitary extract (2.5 mg/kg of body weight for males and 6.0 mg/kg of body weight for females). Egg incubation was carried out in funnel-shaped incubators with constant water circulation at 24°C and samples were collected every 10 minutes. Folliculogenesis began with the proliferation of oogonia originating follicles and spermatogenesis began with the proliferation of spermatogonia originating cells of the sperm lineage. Histochemical reactions were performed, and neutral glycoproteins were detected in the zona pellucida and follicular cells, glycoconjugated carboxylated acids were detected in the cortical alveoli, and neutral glycoproteins and lipids were detected in the globules of yolk. The main stages of embryogenesis were identified alongside their respective durations after fertilization: blastopore closure at 4:50h, formation of somites at 9:40h, and hatching at 18:50h after fertilization. The results of the present study provide insight into the reproductive biology of this little-known species.

Keywords: Blastopore closure, Folliculogenesis, Histology, Induced reproduction, Spermatogenesis.

INTRODUCTION

Brazil has great aquaculture potential due to the abundance of aquatic species, the natural conditions, and the favorable climate (Rocha et al., 2013). However, the successful production of aquatic species in intensive farming systems depends on prior knowledge of fish physiology. Therefore, it is important to understand the natural reproductive biology of relevant species to avoid damage and optimize farming systems (Urbinati and Carneiro, 2004).

The toadfish, cat-toadfish, or bee-catfish, *Pseudopimelodus charus* (Valenciennes, 1840), is endemic to the São Francisco River basin and can reach up to 38 cm in length and 2 kg in body weight. It is a species of commercial interest for fish farming due to its tasty meat and its good acceptance of pellet feed in captivity (Sampaio and Sato, 2006). As a pelagic species, it releases non-adhesive eggs into the water column and exhibits a high fertility rate (Teletchea et al., 2009). Furthermore, the eggs of this species are greenish in color, which is unusual among Siluriformes (Sato et al., 2003). Despite its importance, there are no studies describing the reproductive biology and embryogenesis of this species.

There are two distinct phases during follicular growth: primary growth and secondary growth (Melo et al., 2016). Primary growth is characterized by cytoplasmic and nuclear changes in perinucleolar follicles in addition to the rapid accumulation of cortical alveoli in initial perinucleolar and advanced perinucleolar oocytes. Secondary growth is characterized by the accumulation of yolk globules in the ooplasm of pre-vitellogenic and vitellogenic oocytes (Patiño and Sullivan, 2002). During primary and secondary growth, follicles are dependent on gonadotropins (Lubzens et al., 2009).

During the ovarian development of teleosts, the proliferation and differentiation of primordial germ cells, which originate from oogonia, occurs; these become mature oocytes after meiosis (Patiño and Sullivan, 2002). The oocytes, together with the zona pellucida, follicular cells (layer granulosa), and theca, form the ovarian follicles (Selman and Wallace, 1989; Tyler and Sumpter, 1996). The zona pellucida is an acellular envelope that surrounds the oocyte (Modig et al., 2006). After fertilization, it differentiates into the chorion (Lubzens et al., 2009). Pelagic eggs normally have a thin chorion, while demersal eggs have a thick

chorion and other complex chorionic membranes, probably because they are often subjected to abrasive forces from the environment (Stehr and Hawkes, 1979; Gomes et al., 2007).

The male reproductive systems of various Siluriformes species have several characteristics in common (Loir et al., 1989). The testes of Siluriformes are usually paired, elongated, and contain several fringes or lobules (Santos et al., 2001; Brito and Bazzoli, 2003; Batlouni et al., 2006). Moreover, the testicular structure of vertebrates is generally divided into two compartments, the tubular and interstitial compartments, separated by a basement membrane (Grier et al., 2016).

There are six main phases of cells during spermatogenesis: primary spermatogonia, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. Spermatogonia are observed during the proliferative phase of sperm cells and can be classified into two types: primary and secondary spermatogonia. Primary spermatogonia are characterized by an increased cell size and vesicular nucleus, while secondary spermatogonia are smaller and exhibit clustering of cells. After successive mitotic divisions, secondary spermatogonia differentiate into primary spermatocytes. Primary spermatocytes, in turn, differentiate into secondary spermatocytes shortly after their first meiosis division. Spermatids originate from secondary spermatocytes and, in turn, differentiate into spermatozoa, which are released into the lumen of the seminiferous tubules (Grier, 1981; Gonçalves et al., 2006).

Embryogenesis is the period between the fertilization of the oocyte and hatching. During this period, blastopore closure is considered an important milestone in aquaculture, indicating successful fertilization. It is used to estimate fertilization rates (Abdo, 2016). While the duration of embryonic development can be influenced by several factors, it is mainly impacted by temperature. Higher temperatures tend to decrease the duration of embryogenesis (Borçato et al., 2004; Arashiro et al., 2018; Perroti et al., 2018). Additionally, biotic and abiotic factors can influence the survival rate of embryos, including salinity, pH, ammonia, and the presence of pollutants. This highlights the importance of a comprehensive understanding of the biology and physiology of a species in order to obtain a satisfactory survival rate and thus, ensure the economic viability of fish farming (Arashiro et al., 2018; Coelho et al., 2021).

The application of induced reproduction methods is common for commercial species found naturally in Brazil, since most of these species do not reproduce in captivity naturally. One of the most commonly used methods in fish farming is hypophysation, in which the

crude pituitary extract of common carp (*Cyprinus carpio*) is injected into the coelomic cavity of the farmed fish (Mylonas and Zohar, 2000; Sato et al., 2003).

The success of hypophysation in new fish species, such as *P. charus*, depends on an understanding of the gametogenesis and embryogenesis of the species, which are the most critical and restrictive phases in fish farming (Rizzo and Bazzoli, 2020). Due to a lack of studies on the gametogenesis and initial development of *P. charus*, the present study analyzed for the first time the folliculogenesis, spermatogenesis, and embryogenesis in this species with histological tools, with the aim of contributing to the conservation of the species and improvement in induced reproduction and cultivation techniques for this species.

MATERIALS AND METHODS

Female (n=37) and male (n=19) *P. charus* at different stages of gonadal maturation were captured from the São Francisco River, Três Marias region (18º07'59"S, 45º14'01"W) using a gillnet between January 2015 and December 2017. The fish, when still alive, were euthanized by immersion in eugenol (250 mg. L-1), according to the ethical principles established by the National Council for the Control of Animal Experimentation (CONCEA, 2013). This research was approved by the Committee on Ethics in the Use of Animals (CEUA PUC Minas protocol No. 021/2015).

Fragments of the ovaries and testes were fixed in Bouin's liquid for 12 h and subjected to routine histological techniques: paraffin embedding, 5-µm thick microtomy, hematoxylin-eosin (HE) staining. Fragments of the ovaries were also submitted to classical histochemical techniques: periodic acid-Schiff (PAS), Alcian blue (AB) pH 2.5, Alcian blue (AB) pH 0.5, and Sudan black B.

Induced reproduction was performed using captive-bred fish female (n=10) and male (n=10) raised at the Integrated Center for Fishing and Aquaculture Resources of Três Marias - CODEVASF, Minas Gerais, Brazil (18º11'58" S, 45º15'07" W). Specimens of *P. charus* breeders were kept in 200 m² tanks with an average depth of 1 m. The fish were fed a commercial pelleted feed with 36% crude protein at a rate of 1.5-2.0% of live weight per day (Sampaio and Sato, 2006). The selected breeders were kept in hypophysation tanks containing water at a temperature of 26°C and were submitted to induced reproduction.

The process was performed by injection of crude pituitary extract from the common carp *Cyprinus carpio* (CCPE) into the coelomic cavity of the breeders. Males and females

received a single dose of CCPE (2.5 mg/kg of body weight in males and 6.0 mg/kg of body weight in females). Oocyte extrusion and semen collection were performed manually, and fertilization was performed by the dry method. The eggs were incubated in 20-L funnel-shaped incubators with constant water circulation at 24°C. To monitor embryogenesis, egg samples were collected every 10 min. The samples were analyzed and photographed under a stereoscopic microscope. To calculate the fertilization rate, only embryos that reached blastopore closure (viable eggs) were used. The fertilization rate was calculated according to the following formula: Fertilization rate (TF) = (number of viable eggs / total number of eggs) x 100.

RESULTS

Folliculogenesis of *P. charus* began with the proliferation of germ cells or oogonia that had little cytoplasm and a vesicular nucleus with a central nucleolus. The oogonia gave rise to oocytes which were classified into four developmental stages based on the characteristics of the ooplasma, nucleus, and envelopes (zona pellucida, follicular cells, and connective theca): initial perinucleolar, advanced perinucleolar, pre-vitellogenic, and vitellogenic. Initial perinucleolar oocytes (O1) had a strongly basophilic cytoplasm and a nucleus with several nucleoli (Fig. 1A). Advanced perinucleolar oocytes (O2) had a finely granular basophilic cytoplasm and a nucleus with several nucleoli attached to the nuclear envelope (Fig. 1A). Pre-vitellogenic oocytes (O3) were characterized by the presence of clear vesicles called cortical alveoli in the peripheral ooplasm, zona pellucida, and cubic follicular cells (Fig. 1B). Vitellogenic oocytes (O4) contained ooplasm filled with acidophilic calf globules, thin zona pellucida, and prismatic follicular cells (Fig. 1C).

Histochemical reactions revealed that the yolk globules, zona pellucida, and follicular cells exhibited positive reactions to the PAS technique, indicating the presence of neutral glycoproteins (Fig. 1D). Carboxylated acid glycoconjugates were detected in the cortical alveoli, as reflected by the positive reaction to the AB technique at pH 0.5 and 2.5 (Fig. 1E). Lipids were also detected in the yolk globules, as reflected by the positive reaction to the Sudan black B technique (Fig. 1F).

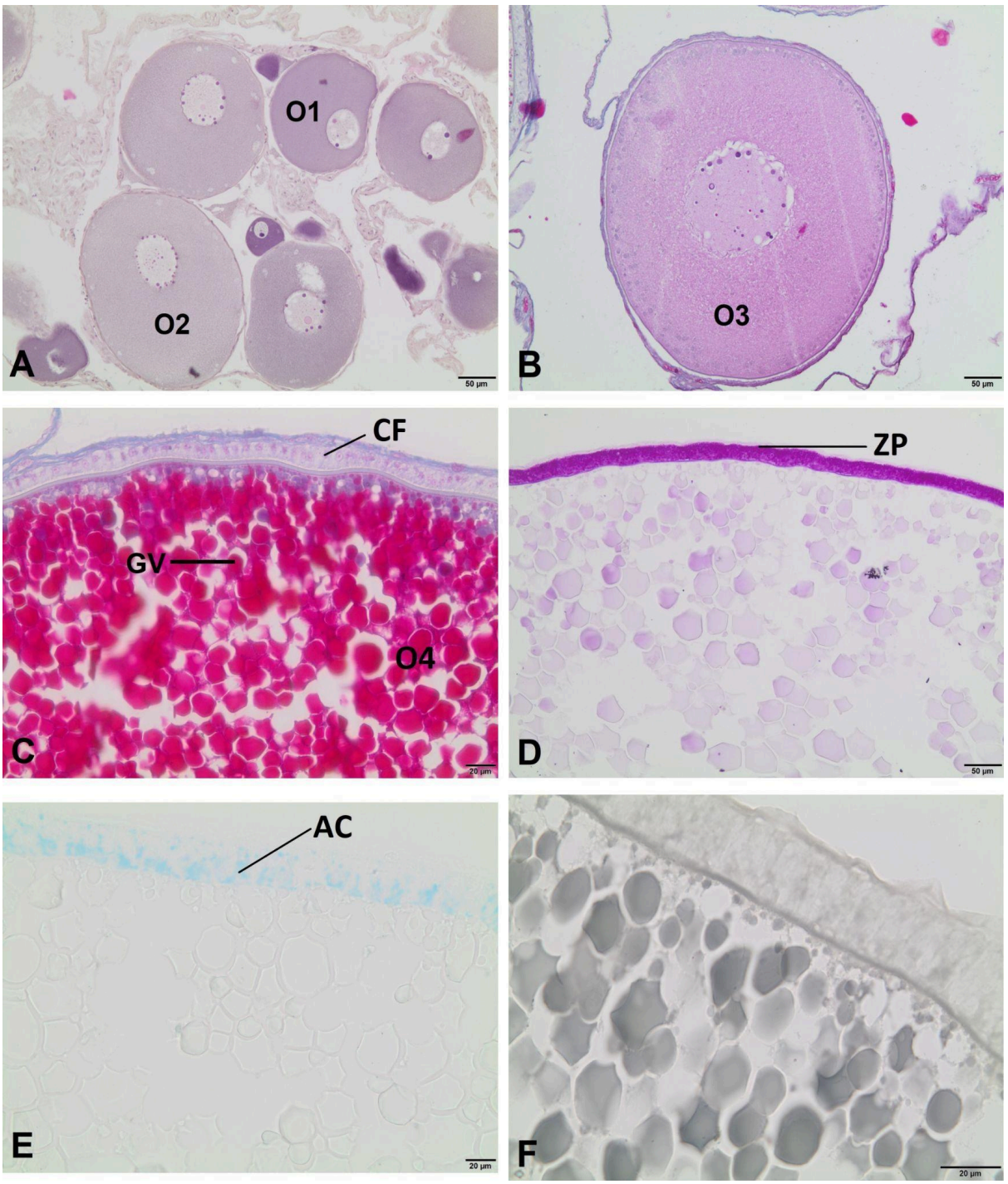

FIG 1. Histological sections of *P. charus* ovaries, showing different stages of gonadal maturation and histochemical contents of vitellogenic follicles. **A**. rest ovary containing only initial (O1) and advanced (O2) perinucleolar follicles – (Scale bar= 50 µm); **B.** initial maturation containing perinucleolar follicles and pre-vitellogenic follicle (O3) – (Scale bar= 50 µm); **C.** advanced maturation showing vitellogenic follicle (O4) containing yolk globules (GV) – (Scale bar= 20 µm); **D.** PAS-positive calf cells, zona pellucida (ZP), and follicular cells (FC) – (Scale bar= 50 µm); **E.** Alcian Blue pH 2.5-positive cortical alveoli (AC) – (Scale bar= 20 µm); **F.** positive Sudan black calf blood cells – (Scale bar= 20 µm).

P. charus testes were divided into two regions: cranial or spermatogenic and caudal or secretory. In the cranial region, seminiferous tubules containing cysts (Fig. 2A) of different spermatogenic lineage cells were observed: primary spermatogonia, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa (Fig. 2B). In the caudal region, there were secretory cells and seminiferous tubules filled with acidophilic secretion (Fig. 2C). In spermatozoa, the lumen of the seminiferous tubules was empty or contained residual spermatozoa (Fig. 2D).

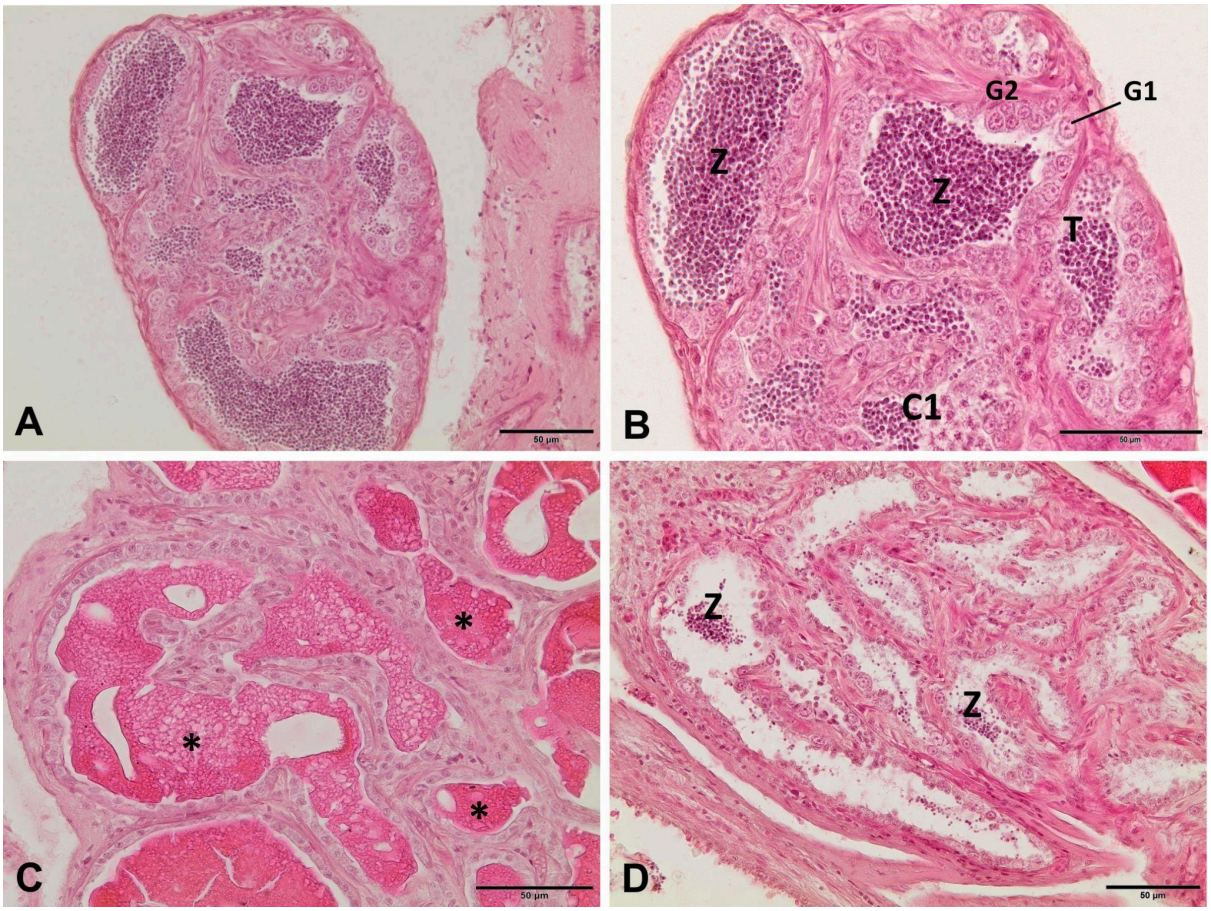

FIG 2. Cross-sections of *P. charus* testes stained with HE**. A.** Testis showing organization in cysts; **B.** Section of the cranial region of the testis showing the testicular organization in cysts, containing primary spermatogonia (G1), secondary spermatogonia (G2), spermatocytes (C), spermatids (S), and spermatozoa (Z); **C.** Caudal region with seminiferous tubules filled with acidophilic secretion (*); **D.** Sperm testicle with empty seminiferous tubule lumen or containing residual spermatozoa (Z). (Scale bars= 50 µm).

Approximately 75% of females and 100% of males exhibited a positive response to hypophysation, with the release of viable oocytes. The extrusion of oocytes occurred manually at 12 h and 30 min after the application of CCPE with the water temperature at 26ºC. The average fertilization rate (%) of oocytes was 75.9 ± 4.5. The newly extruded vitellogenic oocytes were spherical, green, and non-adhesive.

Embryonic development was completed with the eggs hatching at 18:50 h after fertilization of the oocytes, with the water temperature at 24ºC. During this period, the following main phases of embryogenesis were observed (with the respective duration times): cleavage with 32 blastomeres (1:30 h) resulting from the fourth mitotic division; after each division, the size of the blastomere decreased (Fig. 3A). High blastula (2:20 h): identified by the high disposition of the blastoderm and the reduced size of the blastomeres (Fig. 3B). Low blastula (2:40 h): beginning of epiboly movement in the blastoderm (Fig. 3C). Closing of the blastopore (4:50 h): the blastoderm had expanded over the yolk; in this phase, there was also an elongation of the embryo (Figs. 3D and 3E). Differentiation of the embryonic layers (6:50 h): with the end of the gastrula phase, the highest cells differentiated (Fig. 3F). Formation of somites and distinction of the cranial and caudal regions (9:40 h): the formation of somites, distinction of the cranial and caudal regions, and the beginning of the formation of the optic cup (Fig. 3G). Tail release (15:00 h): after the formation of the optic cup, the tail was released through movements produced by the embryo (Fig. 3H). Hatching (18:50 h): through the movements of the tail, the chorion ruptured (Fig. 3I).

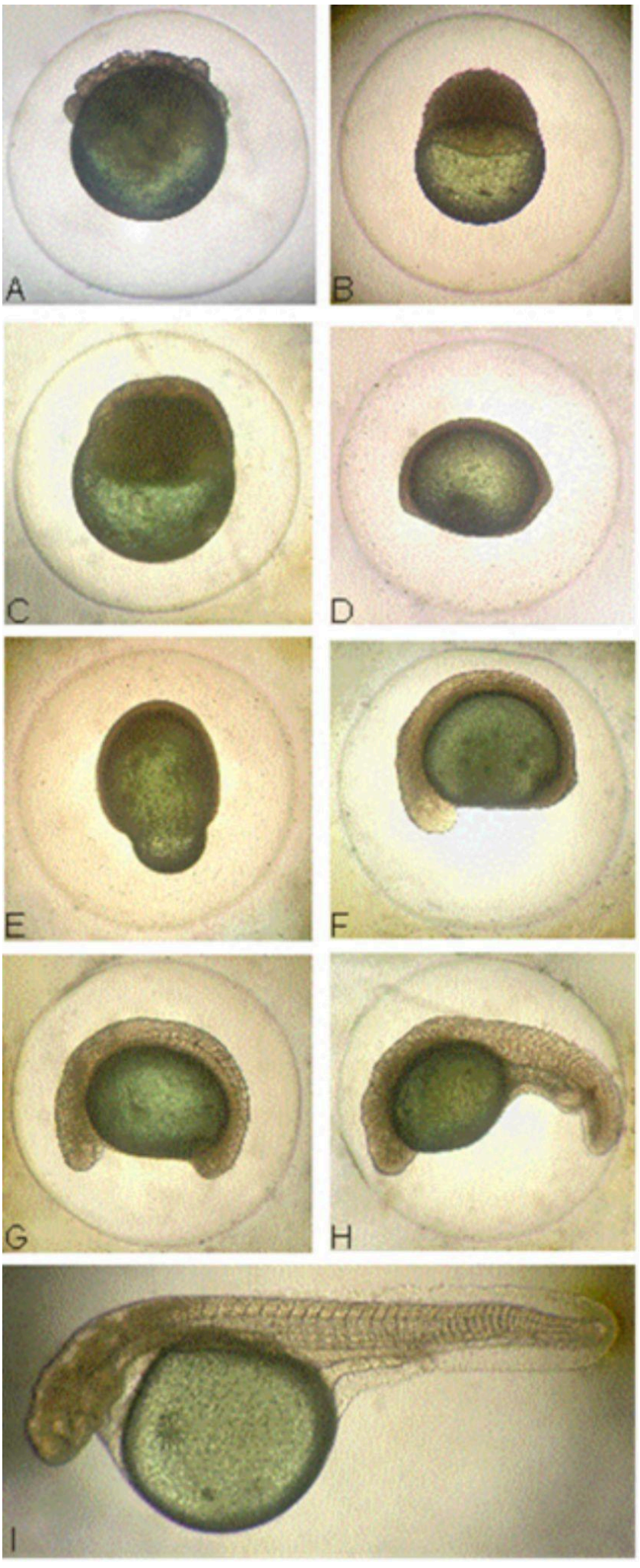
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FIG 3. Main stages of *P. charus* embryogenesis. **A.** cleavage with 32 blastomeres; **B.** high blastula; **C.** low blastula; **D-E**. blastopore closure; **F.** differentiation of the embryonic leaflets; **G.** formation of somites and distinction of cranial and caudal regions; **H.** tail release; **I.** hatching.

DISCUSSION

The results of the present study demonstrate, for the first time, the histological and histochemical characteristics of folliculogenesis and spermatogenesis of *P. charus*. In addition, this study provides a description and duration of the main morphological events when this species is submitted to hypophysation-induced reproduction.

Four stages were identified during the development of ovarian follicles of *P. charus*. This is similar to the results of other studies carried out in other Siluriformes, such as *Pimelodus maculatus* (Quagio-Grassiotto et al., 2011), *Sorubim lima* (Quagio-Grassiotto et al., 2013), and *Iheringichthys labrosus* (Santos et al., 2019).

The morphology of the cortical alveoli is an important parameter for the grouping of fish (Belova, 2008). The content of the cortical alveoli is released into the peri-vitelline space at the time of fertilization; this blocks polyspermy and contributes to the hardening of the chorion (Hart, 1990; Arantes et al., 2017). In teleosts, the content of the cortical alveoli varies and in *P. charus*, carboxylated acid glycoconjugates similar to other Pseudopimelodus were detected in these structures (Bazzoli and Godinho, 1994). This indicates that the polyspermy-blocking mechanism may be similar between species of this genus.

The thickness and structure of the zona pellucida vary among species and reflect adaptations to different ecological conditions (Fausto et al., 2004). In our study, neutral glycoproteins were detected in the zona pellucida of vitellogenic oocytes. Neutral glycoproteins are commonly observed in the zona pellucida of vertebrates (Lubzens et al., 2009).

The follicular cells of teleost vitellogenic oocytes may have different morphologies and contents, depending on the species (Grier et al., 2017). The follicular cells of the vitellogenic follicles of *P. Charus* were prismatic and contained neutral glycoproteins, similar to the descriptions of Melo et al. (2011) and Santos et al. (2019) in relation to other Siluriformes. The prismatic follicular cells in the vitellogenic oocytes of Siluriformes such as *Iheringichthys labrosus*, *Franciscodoras marmoratus,* and *Lophiosilurus alexandri* may contain synthetic machinery capable of secreting mucous substances related to adhesiveness; these are transferred to the zona pellucida for adhesion of the eggs to the substrate (Rizzo et al., 2002; Santos et al., 2006). Although *P. charus* had prismatic follicular cells, this species

did not secrete mucus substances, only neutral glycoproteins, exhibiting free eggs. This is consistent with the observations of Sato et al. (2003).

The green color of the vitellogenic oocytes of *P. charus* is unusual for Siluriformes, which usually have oocytes with yellow and orange coloration (Sato et al., 2003; Perini et al., 2010). The color of vitellogenic oocytes varies among teleost species depending on the diet of the species; the color is important for the identification of offspring and the selection of healthy females (Lubzens, et al., 2009). The yolk color also plays an important role in sexual selection, parental care, and feeding behavior (Blount et al., 2000).

The testicles of *P. charus* were organized in cysts of cells of the spermatogenic lineage, a typical characteristic of teleosts, including fish of the Pseudopimelodidae family (Barros et al., 2007). The testicular structure of Neotropical teleosts consists of two compartments: the tubular and interstitial compartments (Santos et al., 2001; De Siqueira-Silva et al., 2019). In the tubular compartment, Sertoli cells and germ cells are observed, while in the interstitial compartment, Leydig cells and connective tissue are found (De Siqueira-Silva et al., 2019). The cranial or spermatogenic region and the caudal or secretory region were also identified in the testes of *P. charus*, consistent with observations in other Siluriformes such as *Trachelyopterus galeatus*, *Pimelodus maculatus*, *Lophiosilurus alexandri*, *Loricaria lentiginosa*, *Rhinelepis aspera*, and *Iheringichthys labrosus* (Santos et al., 2001; Barros et al., 2007; Guimarães-Cruz et al., 2005; Melo et al., 2011).

Acidophilus secretion detected in the caudal region of the testes has also been observed in other Siluriformes species, such as *T. galeatus*, *P. maculatus*, *L. alexandri*, *R. aspera,* and *I. labrosus* (Santos et al., 2001; Melo et al., 2011). Moreover, this secretion has been observed in other orders of fish, such as *Zosterisessor ophiocephalus* (Perciformes) and *Mimagoniates barberi* (Characiformes) (Lahnsteiner et al., 1992; Pecio et al., 2001). The function of this secretion is related to motility and maturation, increasing sperm viability and integrity (Lahnsteiner, 2003; Chowdhury and Joy, 2007).

The pituitary method is applied in several farmed freshwater fish species and is commonly used in South America. In the current study, this method exhibited a satisfactory result, with a positive response rate of 75% for females and 100% for males. This result is similar to that observed in other species of Siluriformes, such as *Sorubim lima*, *Rhamdia quelen*, *Steindachneridion melanodermatum*, *Pimelodus maculatus*, and *Pseudoplatystoma corruscans* (Ludwig et al., 2005; Amorim et al., 2009. Shibatta et al., 2011, Arantes et al., 2013a; Arantes et al., 2013b). This finding suggests that *P. charus* may be viable for breeding in captivity (Sampaio and Sato, 2006). When the fertilization rate was low, histological

analyses showed that most oocytes were in different stages of follicular atresia. This suggests that induced reproduction may not have been carried out at the ideal moment (Ervilha et al., 2022).

Embryogenesis of *P. charus* was completed 18:50 h after fertilization of the eggs, with a water temperature of 24º C. This is similar to that observed in other Siluriformes such as *Pimelodus maculatus* (18 h) and *Pseudoplatystoma corruscans* (19 h) at a water temperature of 24ºC (Arantes et al., 2013a; Arantes et al., 2013b). In the present study, the temperature of 24ºC was efficient for egg incubation, embryo development, and hatching. Other early development studies of Neotropical fish have also successfully used a water temperature of 24ºC (Rodrigues-Galdino et al., 2010; Arantes et al., 2013b; Paschoalini et al., 2021).

Although the main morphological events of embryogenesis are similar among teleosts, variations in the duration of each phase are observed due to factors such as water temperature, egg size, and the reproductive strategy of the species. Increasing the water temperature, within certain limits, accelerates chorion rupture, larval hatching, and embryonic development, as demonstrated for other Siluriformes species, such as surubin hybrids (*Pseudoplatystoma corruscans* x *Pseudoplatystoma fasciatum*), *Pseudoplatystoma corruscans*, *Pimelodus maculatus*, and *Pseudopimelodus mangurus,* and in other orders such as *Carassius auratus auratus* (Cypriniformes) and *Leporinus piau* (Characiformes) (Borçato et al., 2004; Faustino et al., 2007; Marques et al., 2008; Arashiro et al., 2018; Urushibata et al., 2020). In addition, fish with adhesive eggs and parental care behavior, such as the traíras *Hoplias malabaricus* (48 h) and *Hoplias lacerdae* (50 h), have prolonged embryonic development periods at a temperature of 24ºC, as compared to species with free eggs and the absence of parental care, such as *P. charus* (Gomes et al., 2007).

CONCLUSION

The current study showed that induction of reproduction by the pituitary method is successful in *P. charus* in captivity. Reproducers responded positively to the induction, resulting in a good fertilization rate and satisfactory embryonic development. Taken together, the results of the present study contribute to our understanding of the reproductive biology of the frogfish. These findings serve as a basis for the conservation of the species, demonstrating that induced reproduction can be used for fish farming of this Neotropical native species. Mayer (2019) argued that induced reproduction is essential for the expansion of fish farming and the conservation of native species. Other methods of artificial induction, including

synthetic hormones, should be tested on different species in order to reduce operational costs and increase productivity and fertilization rates.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by F.P. Arantes. The first draft of the manuscript was written by B. Thuller and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Funding acquisition, resources and Supervision was performed by N. Bazzoli.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Animal Experimentation Guide established by the Brazilian College of Animal Experimentation.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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