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Reproductive biology of the Amazonian amphibian fish the splash tetra *Copella arnoldi* with emphasis to histological characterization

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Abstract

This study describes important components of the reproductive biology of the Amazonian amphibian fish *Copella arnoldi*. The species sex ratio biased to female 1.8 to 1 male and the length-weight relationship also shown differences between sexes (b value = 1.854 for females and 2.235 for males), although both presented allometric growth ($b < 3$), "being lighter for its length". The results also show the importance of the histological analysis to describe the gametogenesis and, consequently, define the reproductive cycle of a fish species. It showed both, males and females, spawning capable during a whole year, whereas the gonadosomatic index pointed spawning capable females during the winter and summer, and spawning capable males only in the autumn. The species presents the rare type of semicystic spermatogenesis and the spermatids complete their differentiation into the tubular lumen, forming a Type I spermatozoa. Both the characteristics are described for the first time for the Lebiasinidae family. Such data can contribute to the discussions about the position of the Lebiasinidae family within the Characiform order, which still does not have a conclusion. Moreover, understand the reproductive biology of a fish species helps its preservation in its habitat and may be applied in restoration programs.

KEYWORDS

fish reproduction, Lebiasinidae, oogenesis, reproductive cycle, spermatogenesis

1 | INTRODUCTION

The Amazon region stands out by its big natural diversity (Alho et al., 2015), including a unique and differentiated characterization in the terrestrial and aquatic habitats. The aquatic environment, for example, faces natural changes along the year, influenced by alternated rainy and dry periods, causing small streams emergence and/or disappearance (Junk et al., 2010). Those cyclical alternations may generate instability to the endemic species, which must, in response, develop new strategies that allow their reproduction and, consequently, survival.

The high adaptability of Amazonian fish species is unquestionable, which reflect in the elevated number of described species in the Amazonian basin (2.406 validated species), the highest freshwater biodiversity on earth, corresponding to 15% of all identified species (Jézéquel et al., 2020). However, the increasing anthropogenic threats

in this environment, such as the constant logging, and the catastrophic predictions of the global climate changes for a near future (IPCC, 2021), make the knowledge about the reproductive biology of fish species an urgent matter, in special those whose basic biology are poorly studied. Tagliacollo et al. (2021) show the number of threatened Neotropical freshwater fish species increased in 59%, emphasizing the need for such type of studies.

An Amazonian species with highlighted reproductive strategy is the splash tetra *Copella arnoldi* (Regan, 1912). Belonging to the Lebiasinidae family, *C. arnoldi* is a freshwater fish species that can be find in the Amazon basin, in Pará and Amapá states, in the Guianas (French Guiana, Guiana and Suriname) and Venezuela, in the Orinoco River mouth and in the Sucre and Monagas coastal drainages (Marinho & Menezes, 2017). It is an ornamental species with economic importance for the aquarium trade, presenting vibrating and exuberating colours (Marinho & Menezes, 2017), and the individuals have sexual dichromatism, being the males more colourful with bigger fins than females.

The specie's reproductive behaviour draws the attention: At the moment of reproduction, the *C. arnoldi* couple aligned in the water and jump outside to leaves that are above the water. Then, the female spawns the eggs in the leaves inferior part and the male fertilize them with its sperm (Krekorian & Dunham, 1972; Krekorian & Dunham, 1973; Marinho & Menezes, 2017; Nelson & Krekorian, 1976). This process repeats several times until the ideal number of eggs be placed in the leave. Following, the male splash water in the developing embryos to keep them wet and oxygenated until the eclosion time, when the larvae fall in the water (Mol, 2012; Nelson & Krekorian, 1976). This kind of water-air interaction is adopted in only 1% of the known fish species, which are, for this reason, called amphibian fishes, since they can stay any period of their lives outside the water. This adaptation has no phylogenetic relation, once it is a characteristic that appeared independently and can occur for different reasons, among them a best oxygenation and offspring protection (Espirito-Santo et al., 2018; Ishimatsu et al., 2018).

In spite of some studies existence about *C. arnoldi* reproductive behaviour, data regarding its reproductive biology are still rare. Considering, spawners and embryos are predicted as the most temperature-sensitive stages of the fish life cycle (Dahlke et al., 2020) and their primordial importance for *C. arnoldi* reproduction, its reproductive biology study can help to clarify its reproductive and populational dynamic, serving as a tool for management and conservation strategies and its habitat preservation (Dala-Corte & Azevedo, 2010). Thus, the present study aimed to describe the reproductive biology of *C. arnoldi* in small streams from Tocantins basin inside the Amazon Forest, using components as the gonadosomatic index (GSI), hepatosomatic index (HSI), sex ratio, length-weight relationship and the reproductive cycle performed by histology tool.

2 | MATERIAL AND METHODS

2.1 | Ethical statements and sample place

The specimens of *C. arnoldi* were collected in small freshwater streams that opened inside the Amazon Forest at the Fundação Zoobotânica de Marabá, PA, which occupies an area of 15,650 m^2 (05°23′31.6″S and 049°03′12.3″W) (See Figure 1, Cordeiro et al., 2019). The Amazonian streams characteristics included depth around 0.5 and 1.5 meters, solar penetration between 1.25 and 1.45 meter (Secch disc), average temperature of 25.8 ± 0.87°C, dissolved oxygen 3.11 ± 1.37 ppm, nitrite 0.125 ± 0.3 ppm and water pH of 7.06 ± 0.5. Moreover, as an additional characteristic, many of those streams totally dry up during winter due to this region water system that fully interrupts the rains between the middle of April and the end of October.

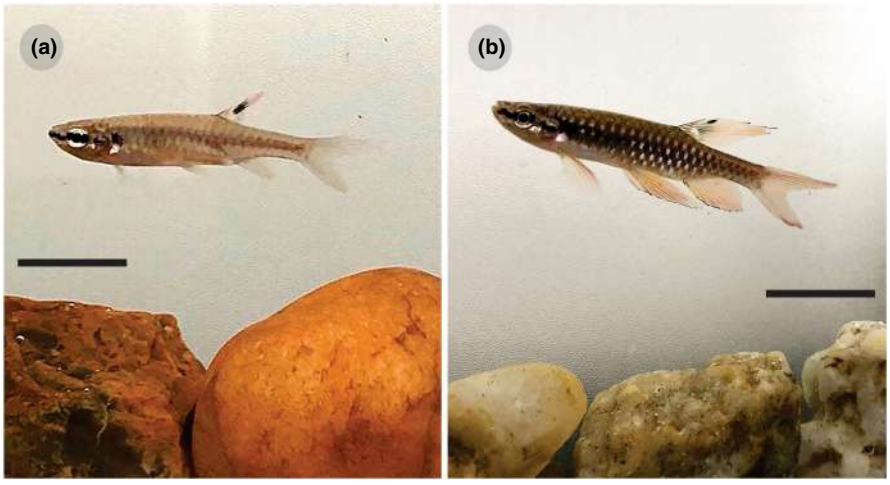

FIGURE 1 Specimens of *C. arnoldi*. (a) Female. (b) Male. Scale bar: 1 cm

The animals' collections were authorized by the Chico Mendes Institute for Biodiversity Protection (ICMBio), the Brazilian protected areas agency through protocol SISBIO 62027–1, and all experimental procedures imposed on animals were approved by the Animal Use Ethics Committee of Federal University of South and Southeast of Pará, (CEUA-UNIFESSPA, project number 23479.010692/2021–16), and all procedures used were consistent with the established guidelines of the National Council for the Control of Animal Experimentation (CONCEA). Voucher specimens of *C. arnoldi* (voucher number: 25619) were deposited at the Collection of the Fish Biology and Genetics Laboratory at the Institute of Biosciences, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

2.2 | Data collection

The collections were performed monthly from August 2017 to July 2018, with the aid of two nets (4.75 × 1.10 m) with a mesh of 1.0 × 1.0 mm that were used for trawling and waiting. Sampled specimens were taken to the laboratory, where they were euthanized by overdose of eugenol anaesthetic solution (20 ml of Eugenol in 100 ml absolute alcohol). Biometric data (total length [TL, cm] and standard length [SL, cm] using a digital pachymeter, and body total weight [W, g] in analytical balance) were taken.

Following, a ventro-longitudinal incision was performed in the animals and their gonads and livers were removed. Livers and gonads were weighed to evaluate the hepatosomatic index (HSI) using the formula HSI = WL/WT × 100 (WL = weight of the liver; WT = total weight of the fish), and the gonadosomatic index (GSI) using the formula GSI = WG/WT × 100 (WG: weight of the gonad; WT: total weight of the fish), respectively.

2.3 | Histological characterization

2.3.1 | Optical microscopy

The gonads were fixed in glutaraldehyde 2.5% (Êxodo Cientifica) for 24 h and then preserved in 70% ethanol. All material was processed according to standard protocols for light microscopy analysis (dx.doi.org/10.17504/protocols.io.ygvftw6, (Cordeiro et al., 2019). Briefly, they were dehydrated in increase ethanol series, diaphanized in xylol and submitted to the inclusion procedure using methacrylate glycol (Historesin, Technovit, 7100). The material was then sectioned in 5 μm slices in a microtome (Leica, 2200) equipped with a glass razor. Sections were caught in glass slides and stained with haematoxylin and eosin (H.E.) and Periodic Acid Schiff (P.A.S.) dyes, and then analysed under an optical microscope (Leica CTR4000). Photodocumentation was performed using a camera (Leica DFC310 FX) assisted by the Leica Application Suite 3.0.0 build 8134 program.

For the stereological analysis of spermatogenesis, 50 nuclei of each cell stage (spermatogonia, spermatocyte, spermatid and spermatozoa [Rodrigues et al., 2015]) were captured in optical microscope and measures were performed using the software ImageJ (Version 1.8.0. 64-Bit, National Institutes of Health, USA) (https://imagej.nih.gov/ij/download.html).

2.3.2 | Transmission electron microscopy

Gonad fragments from mature fish were fixed overnight 2.5% glutaraldehyde in 0.1 M Sorensen phosphate buffer, pH. 7.4 to serve for the transmission electron microscopy (T.E.M.). The material was post-fixed in the dark for 2 h in 1% osmium tetroxide in the same buffer, stained with a 0.5% aqueous solution of uranyl acetate for 2 h, dehydrated in acetone, embedded in Araldite®, and sectioned and stained with a saturated solution of uranyl acetate in 50% alcohol and lead citrate. Electron micrographs were obtained using a Philips – CM 100 transmission electron microscope.

2.4 | Statistics

Length-weight relationship calculation was performed for each sex applying linear equations on logarithmized data, following the formula W = 1.5*SL^0.249, obtained by the nonlinear regression fit by least squares method.

The GSI differences through the seasons were evaluated using the software "R" (R studio Version 1.2.5042, 2009–2020). For comparisons, Bartlett and Shapiro–Wilk tests were applied. Since the data presented nor homoscedasticity neither homogeneity the non-parametric Kruskal–Wallis test ($p < .005$) was performed.

The sex ratio of males and females, for the whole study, were compared using the chi-squared test (χ^2) ($p < .005$), hypothesizing a 1:1 ratio.

3 | RESULTS

3.1 | Biometric data, length-weight relationship, GSI and HSI data

It was sampled 64 specimens, being 41 females (Figure 1a), presenting an average standard length of 2.619 ± 0.558 cm and average weight of 0.221 ± 0.180 g,

and 23 males (Figure 1b), with 2.977 ± 0.633 cm of standard length and 0.265 ± 0.163 g of weight on average (Figures 2 and 3). The length-weight logarithmic presentation is plotted on Figure 4 and the allometric growth coefficient for the specie was 1.854 (R^2 = .8779) and 2.235 (R^2 = .9281), for females and males, respectively. According to the chi-squared test (χ^2 = 5.0625; p = .05), the sex ratio was 1 male to 1.8 female. The ratio of female specimens was higher than males in the majority of months, except from September and February (Figure 5). No animal was sampled neither in December nor April months.

The GSI analysis revealed two similar reproductive peaks for females, in the Winter and Summer seasons (Figure 6). In males the GSI peak was observed in the Autumn season (Figure 7). The HSI was similar for both, females and males during almost all the collection period, except for males in the Summer, which was higher.

3.2 | Morphological analysis

In *C. arnoldi* the gonads are paired and filiform organs, suspended by a mesovarium and mesorchium, which join caudally in an oviduct and a spermatic duct, for females and males, respectively. The testes are of the anastomosing tubular type (Figure 8a) with spermatogonia scattered along the entire epithelium (Figure 8c) and the ovaries are of the cyst ovarian type (Figure 9a).

3.3 | Oogenesis

The oogenesis in *C. arnoldi* was characterized as asynchronous, since the ovaries present oocyte batches in different developmental stages at the same time (Figure 9a). As in other Teleostei, the oogenesis begins with undifferentiated oogonia, following for oocytes in primary growth, secondary growth and spawning capable mature oocytes. In spite to be a conserved event, the oogenesis assumes some peculiarities that are specie-specific and thus on we describe here the oogenesis of *C. arnoldi*.

3.3.1 | Folliculogenesis

The first germ cell of oogenesis, the A undifferentiated oogonia, are found scattered along the epithelium (Figure 10a). After mitotic proliferation these cells originate new undifferentiated oogonia, forming nests (Figure 10b). They present an elongated nucleus containing one or more nucleoli and are observed in the ovary periphery and never bordering the ovarian lumen (Figure 10b). With the oogenesis progress these cells are

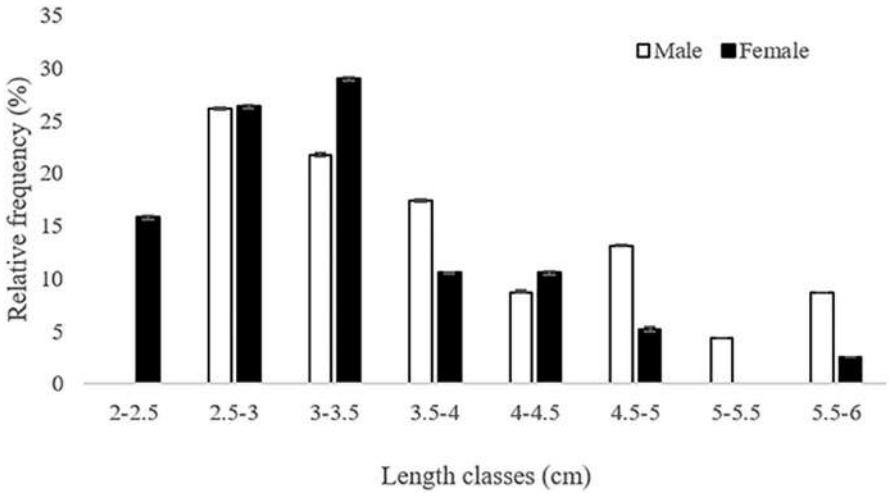

FIGURE 2 Distribution of the relative frequency of males and females of *C. arnoldi* by length classes

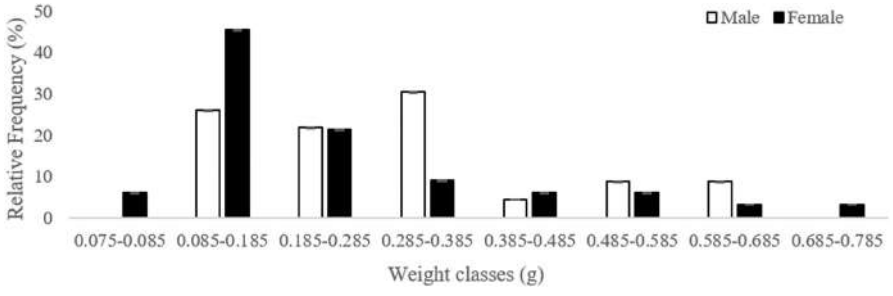

FIGURE 3 Distribution of the relative frequency of males and females of *C. arnoldi* by weight classes

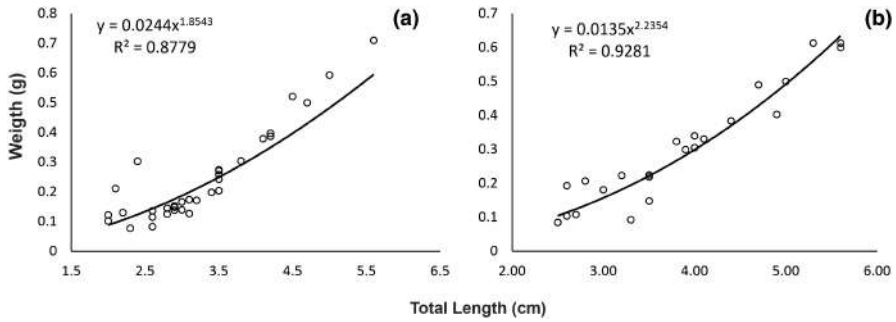

FIGURE 4 Allometric coefficient for *C. arnoldi* females (a) and males (b)

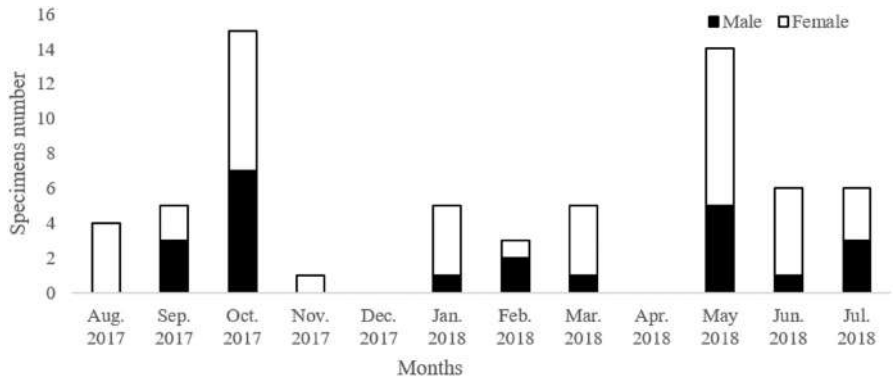

FIGURE 5 Proportion of male (black bar) and female (white bar) specimens collected along the sampling period

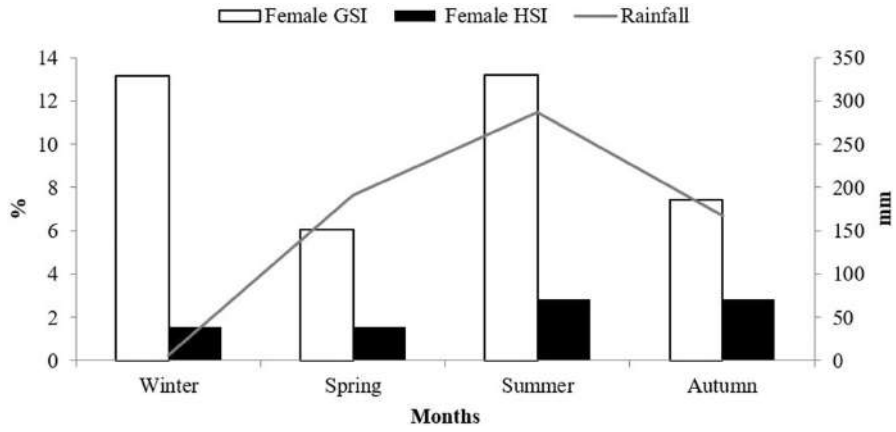

FIGURE 6 Gonadosomatic index (GSI) and hepatosomatic index (HIS) for females in the seasons

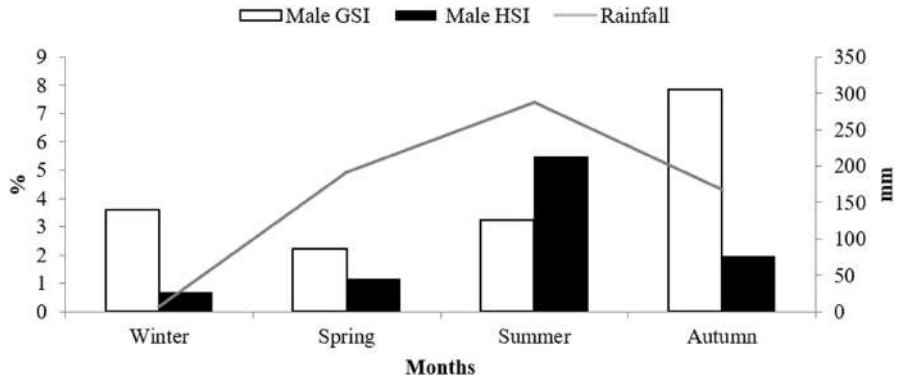

FIGURE 7 Gonadosomatic index (GSI) and hepatosomatic index (HIS) for males in the seasons

individually surrounded by pre follicular cells, differentiating in A differentiated oogonia. In this stage, the cells have a more spherical nucleus, containing a single and more evident nucleolus (Figure 10c).

The A differentiated oogonia proliferate, originating the Type B oogonia (Figure 10d). They keep inside cysts formed by the pre-follicle cells and have a rounder shape; the nucleus is more basophilic in relation to the cytoplasm and presents a single nucleolus (Figure 10d). The type B oogonia differentiate in oocyte, which initiate the meiosis I. The prophase I starts by the leptotene, zygotene, pachytene and diplotene stages, which

are mainly, distinguished by the meiotic figures formed in the nucleus by the chromosome arrangement. The leptotene and zygotene stages occur inside cysts formed by follicle cells (Figure 10e). From the pachytene stage the follicle cells start to surround the germ cells individually (Figure 10f), finishing in the diplotene stage (Figure 10g), moment in which occurs the ovarian follicle formation (Figure 10h).

3.3.2 | Primary growth

After the formation of the ovarian follicle, the oocyte enters in primary growth. The oocyte gradually develops, beginning with the Perinucleolar oocyte (Figure 10i), which is characterized by a cell with a low basophilic nucleus, presenting several nucleoli positioned in the periphery. This oocyte keeps developing until the cortical alveoli start to be synthetize in the cytoplasm periphery, which is considered the final part of the primary growth (Figure 9a).

The cortical alveolus stage oocyte is characterized by the cortical alveoli appearance in the cytoplasm periphery and consequently accumulation on its entire area. This oocyte increases in size during the alveoli accumulation (Figure 9a–c). During this stage, a narrow zona pellucida is also formed. Concomitantly the theca internal and external layers appear as the follicle complex is formed (Figure 11b). The follicle layer cells are squamous (Figure 9b).

3.3.3 | Secondary growth

The oocytes secondary growth is initially characterized by oocytes in initial vitellogenesis. In this stage, the oocytes' cytoplasm is full of cortical alveoli (Figure 9c). With the oocyte development, the yolk granules are being depositing in the cytoplasm, through the zona pellucida pores. They flow in straight to the central part of the oocyte cytoplasm (Figure 9c), filling the space until almost completely occupy the cytoplasm (Figure 9e), sharing the space with cortical alveoli still observed in the cytoplasm periphery (Figure 9d–e). The follicular cell layer is now formed by cuboid cells (Figure 9d).

In this stage, it also occurs the micropyle formation, which is seen as a zona pellucida invagination (Figure 9f). The zona pellucida is thicker and can be subdivided in intern, mid and extern layers (Figures 9d and 11c).

The end of secondary growth is considered at the moment the oocyte is full of yolk. However, the oocyte is considered mature only at the moment the nucleus migrates in direction to the micropyle, characterizing the oocyte as able to fertilizing.

3.4 | Spermatogenesis

Following the same principle of oogenesis, the stages of germ cells during *C. arnoldi* spermatogenesis were also described and the specie-specific peculiarities pointed as follows.

3.4.1 | Primordial germ cells (PGCs)

The first observed germ cells in *C. arnoldi* testes were the primordial germ cells (PGCs) (Figure 8b). They present a round to elliptic and low basophilic nucleus with a central nucleolus (Figure 8b). The PGC differentiate in the A undifferentiated* spermatogonia (Aund*).

3.4.2 | Spermatogonia

The spermatogonia were characterized in four types according to their morphology, such as nuclear shape and size and if they are isolated or inside cysts: A undifferentiated* (Aund*), A undifferentiated (Aund), A differentiated (Adiff) and type B spermatogonia (SgB). The type Aund* present a slightly elongated and more basophilic nucleus, showing an average nuclear diameter of $5.01 \pm 2.51\,\mu m$. In the cytoplasm some nuages are identified (Figure 8b,c). The Aund is generated from the Aund* mitotic divisions, being smaller ($4.24 \pm 1.48\,\mu m$ in nuclear diameter) with a rounder and less basophilic nucleus (Figure 8b,c). Both of them present a single and centralized nucleolus and are scattered along all the testicular germinal epithelium (Figure 8b,c).

The spermatogonia Adiff is grouped inside cysts formed by Sertoli cells. These cells have a smaller ($3.27 \pm 1.34\,\mu m$) and more basophilic nucleus presenting a well centralized nucleolus (Figure 8d,e). After some mitotic divisions the Adiff spermatogonia differentiate in type B spermatogonia that are the smallest spermatogonia type ($2.76 \pm 1.15\,\mu m$ in nuclear diameter). Type B spermatogonia still present a nucleolus (Figure 8d).

3.4.3 | Spermatocytes

The type B spermatogonia differentiate in spermatocyte, which enter in meiosis I (Figure 8e,f). Most of the spermatocyte's cysts observed in *C. arnoldi* testes were in leptotene and zygotene phases, with their nucleus measuring $3.01 \pm 1.31\,\mu m$ (Figure 8e,f) on average. We also observed spermatocytes in pachytene (Figure 8f), and secondary spermatocytes ($2.32 \pm 0.97\,\mu m$ in nuclear diameter) (Figure 8e). After meiosis II, the secondary spermatocytes originate the spermatids.

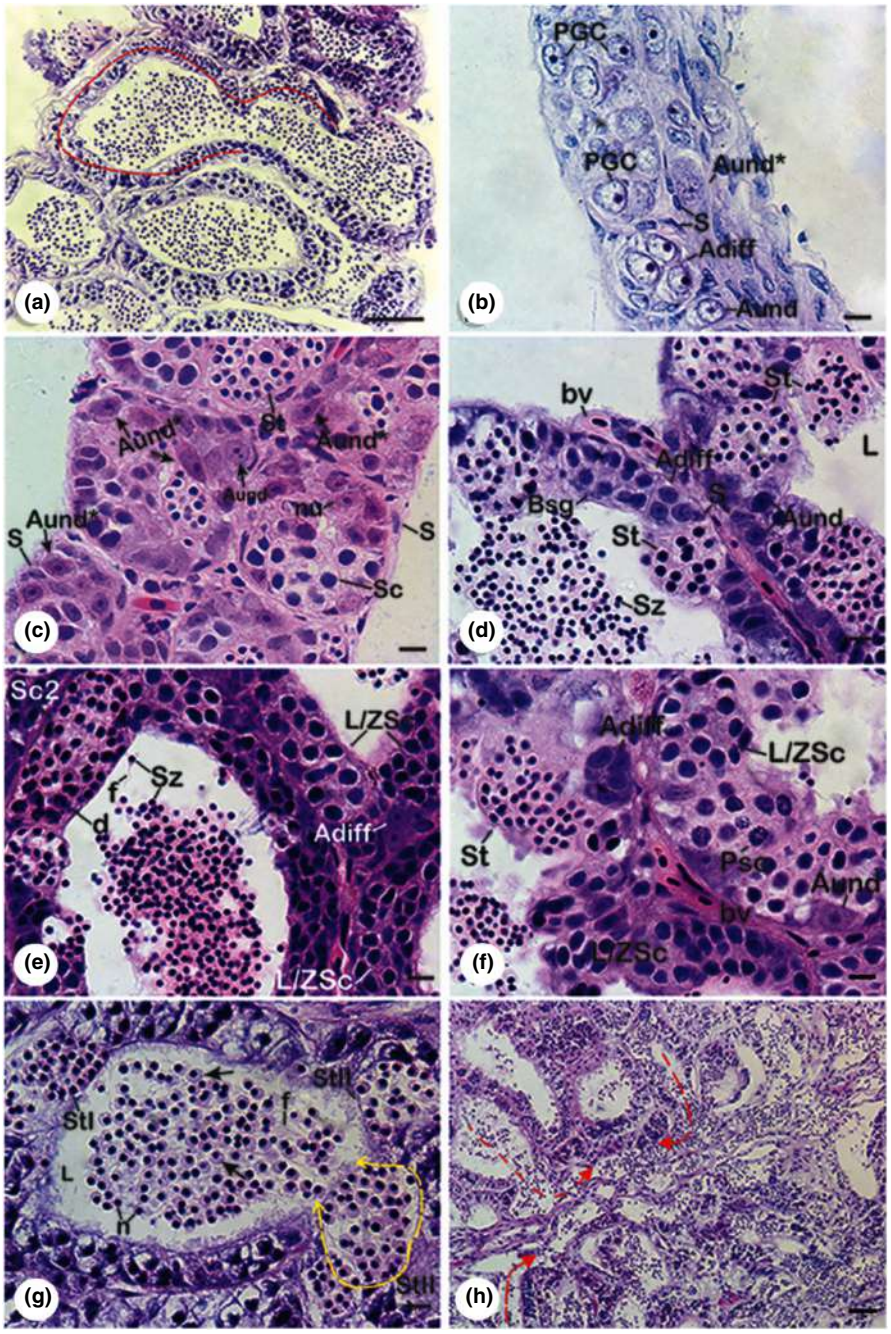

FIGURE 8 Histological analysis of *C. arnoldi* testes. (a) Dashed red line indicating the anastomosed tubular testis type; (b) immature testes showing the presence of primordial germ cells (PGCs), A undifferentiated spermatogonia (Aund* and Aund), A differentiated spermatogonia (Adiff); (c) testes in initial maturation (in developing) containing different types of spermatogonia scattered along the epithelium, besides spermatocytes (Sc) and spermatid (St); (d–f) testes in developing/spawning capable, showing the presence of cysts of A undifferentiated spermatogonia (Aund), A differentiated spermatogonia (Adiff) and B spermatogonia (Bspg), spermatocytes (leptotene/zygotene [L/ZSc]), pachytene spermatocytes [PSc] and secondary spermatocytes [Sc2]) and spermatid (St), and spermatid and spermatozoa (Sz) in the testicular lumen (L); (g) the semi cystic spermatogenic type, with a cyst of spermatid opening and releasing spermatids (double dashed yellow arrows) into the lumen (L), where they complete the differentiation in spermatozoa. Note the presence of sperm flagella (f). (h) Dashed red arrow indicate the opening of the germinal tubules into the main tubular duct. Arrow, spermatid cytoplasm; bv, blood vessel; d, diplotene spermatocyte; n, nucleus; nu, nuage; S, Sertoli cell; StI, spermatid 1; StII, spermatid II. Labeling = a-f,h: Haematoxylin and eosin; g: Periodic acid Schiff; Scale bars = a: 100 mm; b-g: 10 mm; h: 100 mm

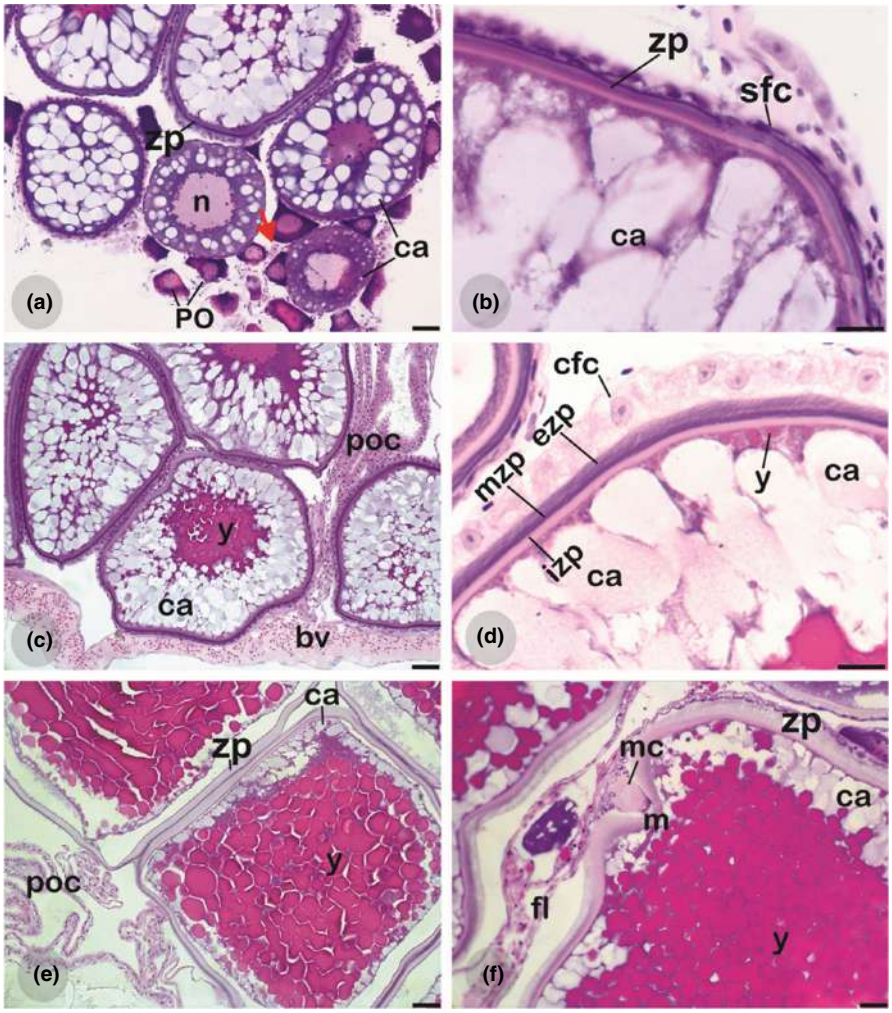

FIGURE 9 Histological characteristics of *C. arnoldi* oogenesis during primary and second growth. (a) Asynchronous development of the oogenesis, with perinucleolar oocytes (PO), cortical alveolar oocytes (ca) and oogonia (arrow); (b) highlight of the squamous follicular layer (sfc) in a cortical alveolar oocyte (ca); (c) ovary with cortical alveolar oocytes (ca), highlighting the begging of yolk deposition (y) and a post-ovulatory follicular complex (poc); (d) Vitellogenic oocyte, highlighting the cuboidal cells forming the follicular layer (cfc) and the internal (izp), mid (mzp) and external (ezp) layers from the zona pellucida; (e) advanced vitellogenic oocyte, showing the yolk (y) almost filling cell cytoplasm; (f) Vitellogenic oocyte exemplifying the micropyle (m) formation. Bv, blood vessel; fl, follicular layer; mc, micropylar cell; n, nucleus; OL, ovarian lumen; zp, zona pellucida. Labeling = Haematoxylin and eosin. Scale bars = a: 5 mm; b, d: 20 mm; c, e: 50 mm; f: 25 mm

3.4.4 | Spermatids

The spermatids pass through a differentiation process called spermiogenesis. It was possible to identify two types of spermatids in *C. arnoldi* spermiogenesis. The spermatid I, whose nuclear diameter is 1.66 ± 0.84 μm on average, are placed together inside the cysts and no spaces are observed among them (Figure 8g). On the other hand, the spermatids II are scattered into the cysts and spaces can be observed among them (Figure 8g). This morphological change occurs due to the flagellum emergence on the spermatids and the release of cytoplasm wastes. Before the end of the differentiation process, the cysts broke and release the spermatids II to the tubular lumen where they complete the differentiation process to form the spermatozoa (Figure 8e–g).

3.4.5 | Spermatozoa

The *C. arnoldi* spermatozoon exhibits a round head, midpiece and single flagellum (Figures 8e,g and 11f). The head is formed by a round nucleus with condensed chromatin surrounded by a narrow strip of cytoplasm with no organelles neither acrosomal vesicle (Figure 11f). Its diameter is approximately 1.7 ± 0.46 μm.

3.5 | Reproductive cycle

Based on the analyses of the germ cells frequency in the gonads and the changes of the germinal epithelium for both, males and females, the *C. arnoldi* gonadal maturation phases during the seasons were described, according to Brown-Peterson et al. (2011) classification, and its reproductive cycle throughout the year was defined (Figure 12).

Males were described as Immature, when their gonads presented only PGCs and undifferentiated spermatogonia (Aund* and Aund) and A differentiated spermatogonia (Figure 8b). No gonadal lumen is visible in this phase (Figure 8b). As the testes develops and enter in the Developing phase/Initial maturation, cysts of spermatocytes and spermatids appears and the germinal epithelium assumes a different form, being possible to observe the delimitations of seminal tubules (Figure 8c). With

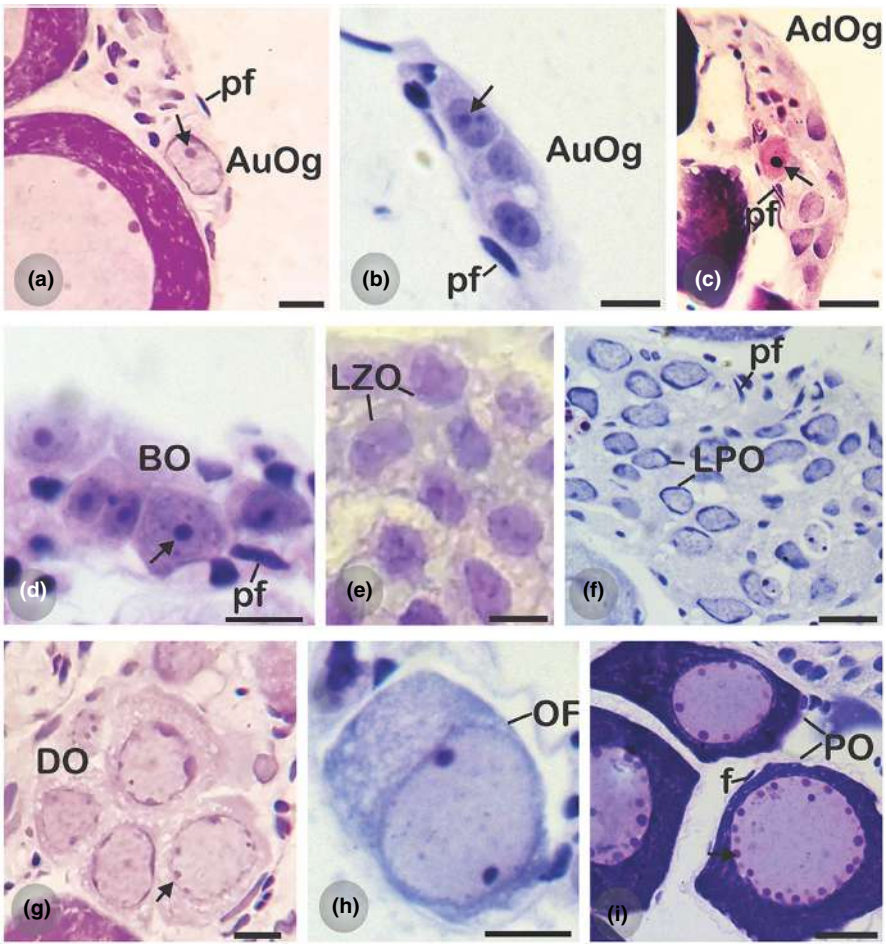

FIGURE 10 Histological analysis of *C. arnoldi* folliculogenesis. (a) A undifferentiated oogonia (AuOg); (b) nest of a undifferentiated oogonia (AuOg); (c) A differentiated oogonia AdOg; (d) B oogonia (BO); (e) leptotene/zygotene oocyte (LZO); (f) late pachytene oocyte (LPO); (g) diplotene oocyte (DO); (h) ovarian follicle (OF); (i) Perinucleolar oocytes (PO). Arrow, nucleolus; f, Follicular cell; pf, pre follicular cell; labeling = Haematoxylin and eosin; scale bars = a: 5 mm; b-d: 10 mm; e-i: 20 mm

the Developing phase/Mid maturation advancement is already possible to observe spermatids and spermatozoa in the lumen even with low volume of cysts in the testes (Figure 8d–g). The most advanced configuration of the testes (Developing/Spawning Capable) occurs with a big volume of spermatids and sperm in the lumen with several spermatogenic cysts, and the junction of the germinal epithelium, directing the sperm for the main testicular duct in the centre of the gonad (Figure 8h).

Both, Developing and Spawning capable females were sampled throughout the entire year, and two reproductive peaks were observed, a slow one in the Winter and a big one during the Autumn (Figure 12). In the ovaries of the females in Spawning capable phase both, vitellogenic oocytes and post-ovulatory follicular complex (POC) were always observed (Figure 9e).

4 | DISCUSSION

Both, the length-weight relationship (2.5 ≥ allometric growing coefficient ≤ 3.5; Froese, 2006) and the sex ratio (1 male: 1 female; [Vazzoler, 1996]) parameters for *C. arnoldi* differed from what is the general expected range for fishes. The gonadosomatic index (GSI) did not differ statistically among the seasons for both the sexes. However, it showed a contradictory tendency in relation to the reproductive peak in the species, since females presented two GSI peaks, one in Winter and another in Summer, while the males presented a single GSI peak, in the Autumn season.

This inconsistency was solved through histological analyses that showed male and female specimens spawning capable in all seasons, with males presenting ready sperm and females with mature oocytes during the entire collection period. This reproductive strategy seems to be not a rare condition among the Amazonian fish species. Cordeiro et al. (2020) and Mendes et al. (2017), for example, described mature specimens of *Astyanax bimaculatus* and *Boulengerella cuvieri,* along the entire sampling year in Amazonian rivers. *Copella arnoldi* males also presented a rare type of semicystic spermatogenesis and the Type I spermiogenesis. The present study shows the importance of the morphological analyses to understand the fish reproduction, since this definition using only the GSI can bias the conclusion, inducing the study to a wrong interpretation and description.

According to Tesch (1986), the length-weight relationship is used for estimating the weight corresponding to a

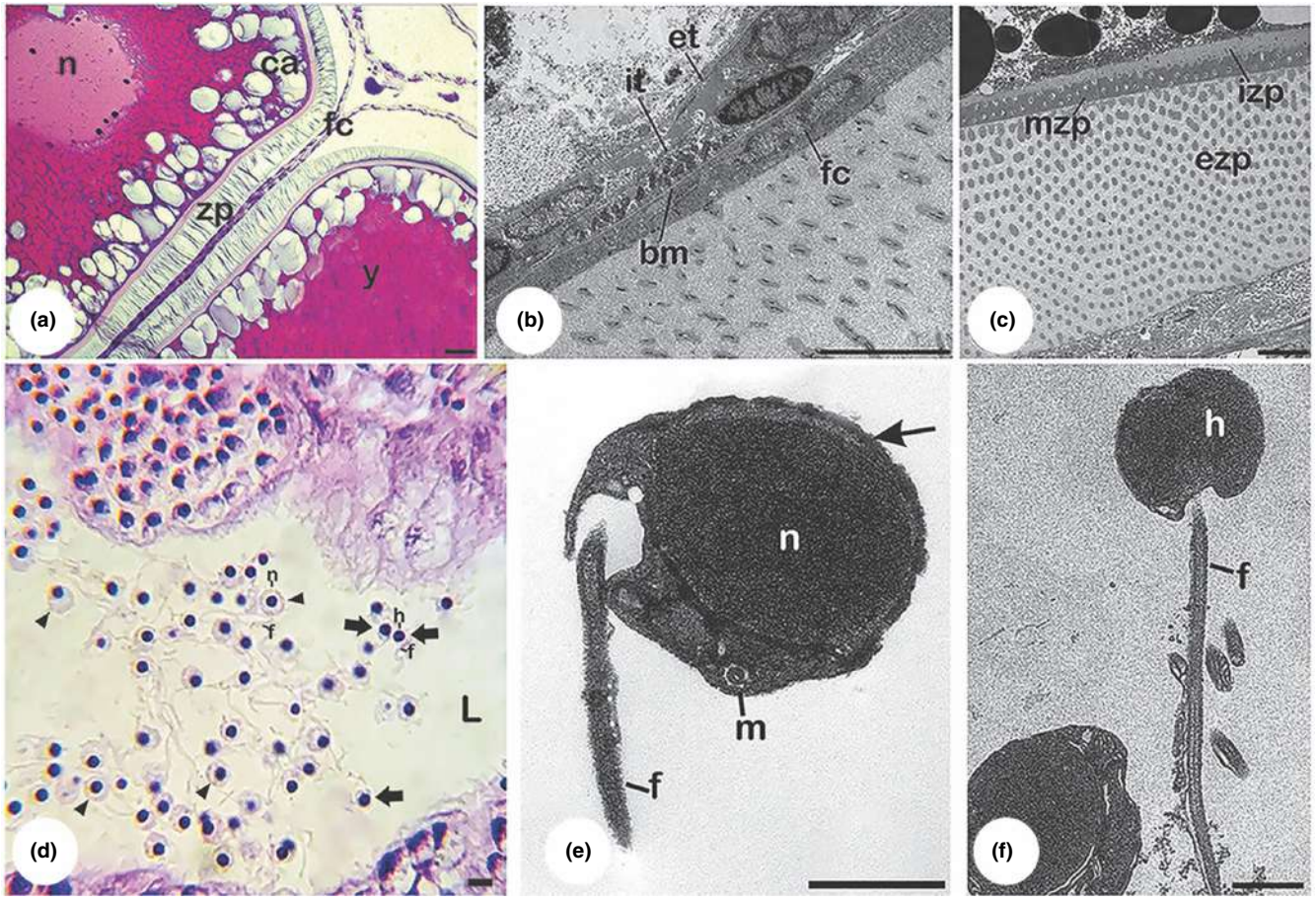

FIGURE 11 Highlight of the zona pellucida and spermatid/spermatozoa in optical and Transmition electron microscopy. (a) Thick zona pellucida (zp) of an oocyte in secondary growth; (b) highlight of external theca (et) and internal theca (it) layers, basement membrane (bm) and squamous follicular cell (fc); (c) highlight of the zona pellucida, showing the internal (izp), mid (mzp) and external zona pellucida (ezp) layers; (d) spermatids (arrow head) and spermatozoon (arrow) in the testicular lumen (L); (e) highlight of the spermatid and spermatozoa (f) found in the testicular lumen. Thin arrow, spermatid cytoplasm; cortical alveoli (ca); follicular cell layer (fc); f, flagellum; h, head; m, mitochondria; n, nucleus; yolk (y). Scale bars: A: 25 μm; b-c: 5 μm; d: 10 μm; e: 2 μm; f: 1 μm

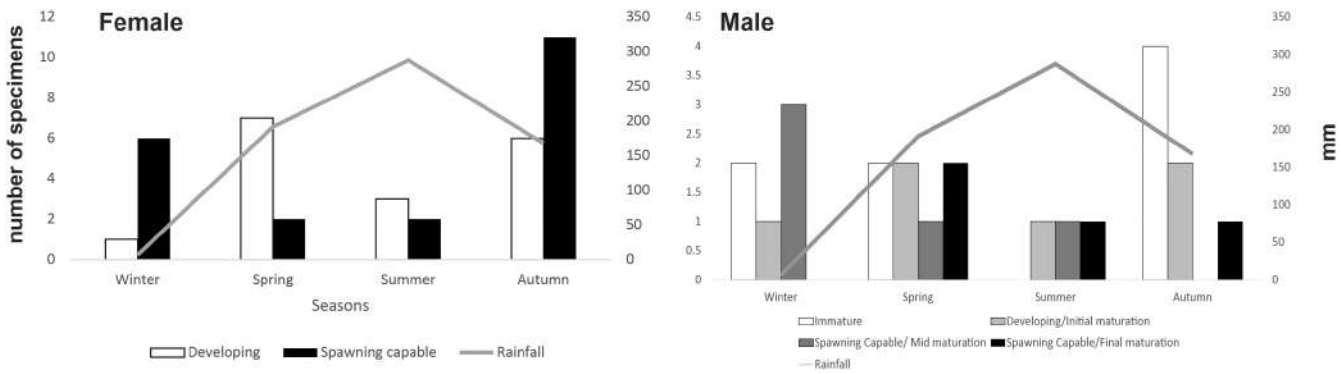

FIGURE 12 Reproductive cycle of *C. arnoldi* female and male throughout the seasons

given length, based on the assumption the heavier a fish is for a given length, in better condition it is. Additionally, Froese (2006) stated that when the allometric growing coefficient in fish species is under 3, it can suggest either that the large specimens have changed their body shape to become more elongated or the small specimens were in better nutritional condition. In *C. arnoldi* case, we believe that this analysis result reflects its sharp body shape as can be seen in Figure 1.

In a previous study with the same species, Machado et al. (2020) got a different result and stated its allometric coefficient was within the general range for any fish

species. This difference can resulted from the different methodologies applied in the studies, since Machado et al. (2020) only weighted and measured the *C. arnoldi* exemplars 1 year after their preservation in alcohol, which may cause their retraction by dehydration, once none of the individuals from that study achieved 4 cm length, and in our collection fish ranged from 2 to 5.6 cm. Indeed, in that study the authors suggest future studies to estimate this parameter using fresh and preserved specimens to determine the effects of preservation. Another hypothesis is that, in spite of the big number of collected individuals, the researchers did not succeed sampling the biggest size ones. On the other hand, it can be an indicative of the high habitat conservation where the animals from our study were sampled, since a great investment in weight may represent a species population strategy to optimize physiological activities to resist to constant environmental changes in consequence of human actions (Froese, 2006).

The observed dominance of *C. arnoldi* female specimens along the collection period was also described in other species. Carvalho et al. (2021), for example, showed this sex ratio unbalance for six out of seven species reported in their study. In some other species, however, the population sex ratio tends to male predominance. In *Brycon orbignyanus*, it is related to sex inversion of female specimens, forming secondary males, culminating in final sex ratio of 2♂:1♀ (Quirino et al., 2022). Independently of the scenario, the sex ratio unbalance may threat a species conservation status, as in *B. orbgnyanus* classified as Endangered (ICMBio, 2018).

Another possible explanation involves fishes' reproductive behaviour. Once the reproductive success in males generally depends of the number of fertilized partners, they usually are the most active sex in the search for partner and, consequently, are more prone to meet predators. On the contrary, the female reproductive success is correlated to the number of produced oocytes, leaving them heavier and slower and, therefore, more prone to be catch (Magnhagen, 1991).

Moreover, in the studied specie sex unbalance ratio may reflect the strategy of parental care starred by males. After the reproduction, males stay near the eggs splashing water to oxygenate and keep them humidified (Krekorian & Dunham, 1972; Nelson & Krekorian, 1976). This reproductive behaviour maintain the males occupied in the stream banks proximity for at least 3 days (Krekorian & Dunham, 1972). Since our sampling method mainly involved to pass the nets throughout the riverbed, it may had favoured the collection of female specimens. As far as we know, this is the first study to present data on the sex ratio for this species on its natural environment and, as unbalances in sex ratio may reflect in a problem for the population restoration, the knowledge of this data is essential for the management of a such species in its habitat.

Another novelty for the study of the species reproductive biology is the description of its reproductive cycle using histology. These analyses enable understanding the reproductive strategy adopted by the species along an entire annual cycle. Once the specimens always present several vitellogenic oocytes and mature sperm in their ovaries and testes, respectively, the species seems to be able to reproduce along all the sampling period. Krekorian and Dunham (1972) observed during laboratory experimentation that a group of three *C. arnoldi* female spawned more than 29 times with 13 different males in 2 years. The males also spawned with more than one female, and still, according to the authors, they would probably have spawned more if they were allowed to remain in the spawning aquariums. This strategy might ensure the specie perpetuation, since they produce a very small offspring containing between 1 and 210 eggs (Krekorian & Dunham, 1972).

Copella arnoldi also presents the rare type of spermatogenesis, known as semicystic. In this type of spermatogenesis, the cysts broken themselves before the end of spermatogenesis and the spermatids and sometimes even the spermatocytes are released to the tubular lumen, where they complete their differentiation in spermatozoa. Although, the semicystic type of spermatogenesis been already observed in different taxonomic groups, such as Bleniidae, Corydoradinae, Opheliidae, Scorpaenidae, Soleidae (Schulz & Nóbrega, 2011), Characidae (Andrade 2001; Bazzoli & Godinho, 1991; Costa et al., 2014; Magalhães et al., 2011), Malapteruridae (Shahin, 2006), Fundulidae (Selman & Wallace, 1986) and Scorpaenidae (Muñoz et al., 2002), to the best of our knowledge, this is the first description of semicystic spermatogenesis for a species from Lebiasinidae family.

The *C. arnoldi* spermiogenesis process involves the rotation of the spermatid nucleus in 90° on the flagellum axis, ending with the spermatozoa head perpendicularly positioned in relation to the flagellum, which characterizes the *C. arnoldi* spermiogenesis as Type I (Jamieson, 1991; Mattei, 1970). Santana et al. (2013) described the spermiogenesis for nine species from the Lebiasinidae family and for all of those species, the spermatozoa presented a slight elongated head, lieing laterally to the flagellar axis, which defines the spermiogenesis on those species as Type II (Mattei, 1970). Although the monophyly of the Lebiasinidae family (Arcila et al., 2017), these results evidence a non-conserved spermatic structure of its members, which can reflect evolutionary divergence among the species. Sassi et al. (2020), stated the notorious evolutionary divergence between the Lebiasinidae subfamilies and

the chromosomal diversity among the members of the *C. arnoldi* subfamily Pyrrhulininae.

Moreover, it seems that only *C. arnoldi* among the species of its subfamily presents the uncommon reproductive behaviour of deposit eggs on leaves outside of the water. *Copella callolepis* and *C. nattereri*, for example, laid their eggs on submerged leaves (Marinho & Menezes, 2017). Therefore, altogether, those evolutive adaptations may reflect in the sperm morphology differences.

Lastly, this study also shows that the use of the GSI index must be used only as a complementary resource in studies describing the reproductive cycle of a certain species, mainly when species with partial spawning are studied, since it does not bring precise data and may, therefore, bias the analyses. In this regard, Siqueira-Silva et al. (2013), evaluated the GSI in each gonadal maturation phase of the partial spawner *Cichla kelberi,* and observed that the GSI for mid maturation and mature phases were similar and, consequently, the use of this metric only to define the reproductive cycle of that species could lead to wrong interpretation. Thus, in both the Siqueira-Silva et al. (2013) and in the present study, the importance of the histology for an accurate definition of the reproductive cycle in a fish species is evidenced.

Consequently, microscopy associated to histology tool have a greater importance than only scientific description of fish gonadal maturation phases throughout the year. Once the definition of a species reproductive cycle, emphasizing its spawning peak, is the first step to artificially reproduce the fishes in experiments and even in fishfarmings, its use is of paramount importance, because the broodstock hormonal induction before or after the gonadal maturation will result in the specimens' bad reproductive performance.

The present study brings some important components for understanding the reproductive biology of the amphibius Amazonian fish, the splash tetra *C. arnoldi* and for the first time describes its oogenesis and spermatogenesis morphological characterization, besides its sperm structure, with very interesting particularities, as the semicystic spermatogenesis. Furthermore, it evidences the histological analyses relevance to better define the reproductive cycle of any fish species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Alho, C. J. R., Reis, R. E., & Aquino, P. P. U. (2015). Amazonian freshwater habitats experiencing environmental and socioeconomic threats affecting subsistence fisheries. *Ambio*, *44*, 412–425. https://doi.org/10.1007/s13280-014-0610-z

Andrade, R. F., Bazzoli, N., Rizzo, E., & Sato, Y. (2001). Continuous gametogenesis in the neotropical freshwater teleost, Bryconops affinis (Pisces: Characidae). *Tissue and Cell*, *33*(5), 524–532. https://doi.org/10.1054/tice.2001.0206

Arcila, D., Ortí, G., Vari, R., Armbruster, J. W., Stiassny, M. L. J., Ko, K. D., Sabaj, M. H., Lundberg, J., Revell, L. J., & Betancur-R, R. (2017). Genome-wide interrogation advances resolution of recalcitrant groups in the tree of life. *Nature Ecology & Evolution*, *1*(2), 20. https://doi.org/10.1038/s41559-016-0020

Bazzoli, N., & Godinho, H. P. (1991). Reproductive biology of the Acestrorhynchus lacustris (Reinhardt, 1874)(Pisces: Characidae) from Três Marias reservoir, Brazil. *Zoologischer Anzeiger*, *226*(5–6), 285–297.

Brown-Peterson, N. J., Wyanski, D. M., Saborido-Rey, F., Macewicz, B. J., & Lowerre-Barbieri, S. K. (2011). A standardized terminology for describing reproductive development in fishes. *Marine and Coastal Fisheries*, *3*(1), 52–70. https://doi.org/10.1080/19425120.2011.555724

Carvalho, I. F. D. S., Cantanhêde, L. G., Diniz, A. L. C., Carvalho-Neta, R. N. F., & De Almeida, Z. D. S. (2021). Reproductive biology of seven fish species of commercial interest at the Ramsar site in the Baixada Maranhense, legal Amazon, Brazil. *Neotropical Ichthyology*, *19*(2), 1–18. https://doi.org/10.1590/1982-0224-2020-0067

Cordeiro, J. G., Maximino, C., & De Siqueira-silva, D. H. (2019). *Histological staining of fish gonadal tissue Jhennifer Gomes Cordeiro 1, Caio Maximino 1, Diógenes Henrique de Siqueira-Silva 1 1 Universidade Federal do Sul e Sudeste do Pará* (pp. 1–7). protocols.io. https://doi.org/10.17504/protocols.io.ygvftw6

Cordeiro, J. G., Rodrigues, J., Rodrigues, M. D. S., Bertolini, R. M., Nóbrega, R. H., Yasui, G. S., Maximino, C., & Siqueira-Silva, D. H. (2020). Reproductive cycle of the tetra *Astyanax bimaculatus* (Characiformes: Characidae) collected in Amazonian streams. *Zygote*, *28*(1), 37–44. https://doi.org/10.1017/S0967199419000601

Costa, F. G., Adolfi, M. C., Gomes, C. C., Jesus, L. W. O., Batlouni, S. R., & Borella, M. I. (2014). Testes of *Astyanax altiparanae*: The Sertoli cell functions in a semicystic spermatogenesis. *Micron*, *61*, 20–27. https://doi.org/10.1016/j.micron.2014.02.004

Dahlke, F. T., Wohlrab, S., Butzin, M., & Pörtner, H.-O. (2020). Thermal bottlenecks in the life cycle define climate vulnerability of fish. *Science*, *369*(6499), 65–70. https://doi.org/10.1126/science.aaz3658

Dala-Corte, R. B., & Azevedo, M. A. (2010). Biologia reprodutiva de Astyanax henseli (Teleostei, Characidae) do curso superior do Rio dos Sinos, RS, Brasil. *Iheringia. Série Zoologia*, *100*(3), 259–266. https://doi.org/10.1590/S0073-47212010000300012

Espirito-Santo, H. M. V., Sodré, J. G., & Zuanon, J. (2018). He leaps, she beats: The role of social interactions on the overland movements of an Amazonian amphibious killifish. *Ecology of Freshwater Fish*, *28*(3), 356–364. https://doi.org/10.1111/eff.12458

Froese, R. (2006). Cube law, condition factor and weight-length relationships: History, meta-analysis and recommendations. *Journal of Applied Ichthyology*, *22*(4), 241–253. https://doi.org/10.1111/j.1439-0426.2006.00805.x

ICMBio – Instituto Chico Mendes de Conservação da Biodiverdidade. (2018). *Livro Vermelho da Fauna Brasileira Ameaçada de Extinção*. ICMBio/MMA.

IPCC. (2021). *Assessment Report 6 Climate Change 2021: The Physical Science Basis*. Retrieved from https://www.ipcc.ch/report/ar6/wg1/

Ishimatsu, A., Mai, H. Van, & Martin, K. L. M. (2018). Patterns of fish reproduction at the Interface between air and water. *Integrative and Comparative Biology*, *58*(6), 1064–1085. https://doi.org/10.1093/icb/icy108

Jamieson, B. G. M. (1991). *Fish evolution and systematics: Evidence from spermatozoa*. Cambridge.

Jézéquel, C., Tedesco, P. A., Bigorne, R., Maldonado-Ocampo, J. A., Ortega, H., Hidalgo, M., Martens, K., Torrente-Vilara, G., Zuanon, J., Acosta, A., Agudelo, E., Maure, S. B., Bastos, D. A., Gregory, J. B., Cabeceira, F. G., Canto, A. L. C., Carvajal-Vallejos, F. M., Carvalho, L. N., Cella-Ribeiro, A., … Oberdorff, T. (2020). A database of freshwater fish species of the Amazon Basin. *Scientific Data*, *7*(1), 96. https://doi.org/10.1038/s41597-020-0436-4

Junk, W. J., Soares, M. G. M., Junk, W. J., & Soares, M. G. M. (2010). *Aquatic Ecosystem Health & Management Freshwater Fish Habitats in Amazonia : State of Knowledge, Management, and Protection Freshwater Fish Habitats in Amazonia : State of Knowledge, Management, and Protection*. *4988*. https://doi.org/10.1080/146349801317276107

Krekorian, C. O. N., & Dunham, D. W. (1973). Visual discrimination by the spraying characid, Copeina arnoldi Regan. *Animal Behaviour*, *21*(4), 741–748. https://doi.org/10.1016/S0003-3472(73)80100-9

Krekorian, C. O. N., & Dunham, D. W. (1972). Preliminary observations on the reproductive and parental behavior of the spraying characid Copeina arnoldi Regan. *Zeitschrift Fur Tierpsychologie*, *31*(4), 419–437. https://doi.org/10.1111/j.1439-0310.1972.tb01778.x

Machado, A. F. D. V. N., Lobato, C. M. C., Gusmão, R. R., Montag, L. F. D. A., & Prudente, B. S. (2020). Length-weight relationships of eleven fish species captured in 18 streams of the Capim River basin, Brazil. *Journal of Applied Ichthyology*, *36*(5), 745–747. https://doi.org/10.1111/jai.14049

Magalhães, A. L. B., Andrade, R. F., Gomes, B. V. C., Perini, V. R., Rizzo, E., & Bazzoli, N. (2011). Ultrastructure of the semicystic spermatogenesis in the south American freshwater characid Hemigrammus marginatus (Teleostei, Characiformes). *Journal of Applied Ichthyology*, *27*(4), 1041–1046. https://doi.org/10.1111/j.1439-0426.2011.01747.x

Magnhagen, C. (1991). Predation risk as a cost of reproduction. *Trends in Ecology & Evolution*, *6*(6), 183–186. https://doi.org/10.1016/0169-5347(91)90210-O

Marinho, M. M. F., & Menezes, N. A. (2017). Taxonomic review of Copella (Characiformes: Lebiasinidae) with an identification key for the species. *PLoS One*, *12*(8), e0183069. https://doi.org/10.1371/journal.pone.0183069

Mattei, X. (1970). Spermiogene'se compare'e des poisson. In B. Baccetti (Ed.), *Comparative spermatology* (pp. 57–70). Academic Press.

Mendes, Y. A., Ferreira, M. A. P., Lobato, C. M. C., Silva, G. M. F., Montag, L. F. A., & Rocha, R. M. (2017). Reproductive characteristics of pike-characids Boulengerella cuvieri (Ctenoluciidae) in the middle Xingu River, eastern Amazon. *Journal of Fish Biology*, *91*(1), 346–353. https://doi.org/10.1111/jfb.13339

Mol, J. H. (2012). *The freshwater fishes of Suriname*. (Brill, ed.).

Muñoz, M., Sàbat, M., Mallol, S., & Casadevall, M. (2002). Gonadal structure and Gametogenesis of Trigla lyra (Pisces: Triglidae). *Zoological Studies*, *41*(4), 412–420.

Nelson, S. G., & Krekorian, C. O. N. (1976). The dynamics of parental care of Copeina arnoldi (pisces, characidae). *Behavioral Biology*, *17*(4), 507–518. https://doi.org/10.1016/S0091-6773(76)90919-6

Quirino, P. P., Delgado, M. L. R., Gomes-Silva, L., Benevente, C. F., Grigoli-Olivio, M. L., Bianchini, B. C., Ninhaus-Silveira, A., & Veríssimo-Silveira, R. (2022). Female sex inversion as a reason for an unbalanced sex ratio in the neotropical species Brycon orbignyanus. *Aquaculture Research*, *53*(5), 1706–1726. https://doi.org/10.1111/are.15701

Rodrigues, M., Silva, S.-s., De, D. H., Quirino, P., Ninhaus-silveira, A., & Veríssimo-, R. (2015). Spermatogenesis in the yellow-tail tetra *Astyanax altiparanae*: A histological analyses with emphasis to spermatogonial and spermatid types. *Boletim do Instituto de Pesca Sao Paulo*, *41*, 697–705.

Santana, J. C. D. O., Netto-Ferreira, A. L., Calcagnotto, D., & Quagio-Grassiotto, I. (2013). Sperm characteristics as additional evidence of close relationship between Lebiasina and Piabucina (Characiformes: Lebiasinidae: Lebiasininae). *Neotropical Ichthyology*, *11*(3), 573–579. https://doi.org/10.1590/S1679-62252013000300010

Sassi, F. D. M. C., Hatanaka, T., De Moraes, R. L. R., Toma, G. A., De Oliveira, E. A., Liehr, T., Rab, P., LAC, B., Viana, P. F., Feldberg, E., Nirchio, M., MMF, M., JFSE, S., & Cioffi, M. D. B. (2020). An insight into the chromosomal evolution of Lebiasinidae (Teleostei, Characiformes). *Genes*, *11*(4), 365. https://doi.org/10.3390/genes11040365

Schulz, R. W., & Nóbrega, R. H. (2011). Anatomy and histology of fish testis. *Encyclopedia of Fish Physiology: From Genome to Environment*, *1*, 616–626. doi:10.1016/B978-0-1237-4553-8.00246-X

Selman, K., & Wallace, R. A. (1986). Gametogenesis in *Fundulus heteroclitus*. *American Zoologist*, *26*(1), 173–192.

Shahin, A. A. B. (2006). Semicystic spermatogenesis and biflagellate spermatozoon ultrastructure in the Nile electric catfish *Malapterurus electricus* (Teleostei : Siluriformes : Malapteruridae). *Acta Zoologica*, *87*, 215–227.

De Siqueira-Silva, D. H., Vicentini, C. A., Ninhaus-Silveira, A., & Verissimo-Silveira, R. (2013). Reproductive cycle of the neotropical cichlid yellow peacock bass *Cichla kelberi*: A novel pattern of testicular development. *Neotropical Ichthyology*, *11*(3), 587–596. https://doi.org/10.1590/S1679-62252013000300012

Tagliacollo, V. A., Dagosta, F. C., Pinna, M. D., Reis, R. E., & Albert, J. S. (2021). Assessing extinction risk from geographic distribution data in Neotropical freshwater fishes. *Neotropical Ichthyology*, *19*(3), 1–26. https://doi.org/10.1590/1982-0224-2021-0079

Tesch, F. W. (1986). Age and growth. In W. E. Ricker (Ed.), *Methods for assessment of fish production in fresh waters* (pp. 93–123). Blackwell Scientific Publications.

Vazzoler, A. E. A. D. M. (1996). *Biologia Da Reprodução De Peixes Teleósteos: Teoria E Prática*. Editora da Universidade Estadual de & Maringá (Eds.), *EDUEM* (597.50416).

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