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RESEARCH ARTICLE

Gonadal development and reproductive period of the characin *Astyanax* aff. *bimaculatus* (Characiformes: Characidae) in a tropical reservoir in southeastern Brazil

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ABSTRACT. The gonadal development of the characin *Astyanax* aff. *bimaculatus* from Funil Reservoir, an impoundment in the middle reaches of the Paraiba do Sul River in southeastern Brazil was analysed through histological, histochemical and histometric techniques, and the spawning season was determined. Unbalanced sex ratio was found, with females outnumbering and reaching larger sizes than males. The following stages of oogenic cell development were identified for females: primary growth, pre-vitellogenic (cortical alveolar) and vitellogenic. In males, the following stages were determined: spermatogonia primary and secondary, spermatocyte primary and secondary, spermatid and spermatozoa. The spawning occurs most of the year, peaking in January-February, as indicated by the gonadosomatic index. In the peak of the spawning period, the condition factor and hepatosomatic indices decreased suggesting use of stored body energy reserves during the reproduction. Spawning in batches, small-sized oocytes and a long reproductive season suggest that *Astyanax* aff. *bimaculatus* fits to an opportunist strategy. Such strategy may facilitate the use of a wide range of lentic and lotic body waters in the Neotropical region.

KEY WORDS. Freshwater fish, histology, oocyte, spawning type, spermatocyte.

INTRODUCTION

The life cycle patterns of fish are the most varied among the vertebrates and different reproductive strategies have enabled them to obtain success in different environments (Stearns 1992, Winemiller and Rose 1992, Blanck et al. 2007, Belova 2008). The reproductive strategies used by different fish species vary markedly (Dala-Corte and Azevedo 2010, Lowerre-Barbieri et al. 2011) and the success achieved in different environments can have ecological and evolutionary implications (Rizzo et al. 2002, Jamieson 2009). There is a need of studies on different fish groups, which is crucial to understand their reproductive strategies and tactics (West 1990, Parenti and Grier 2004, Belova 2008).

The gonadal development is cyclical and seasonal in most Teleostei. Germ cell renewal, differentiation, development and the release of sperm/oocytes throughout each reproductive cycle result in gonadal alterations that characterize different reproductive phases. Variations in the gonadal fish morphology reflect important ecological and behavioural adaptations during reproduction (Coward et al. 2002, Fishelson and Gon 2008, Martins et al. 2012). In addition, the phenotypic plasticity of gonadal morphology reflects the adaptability to environmental changes (Defalco and Capel 2009, Galvão et al. 2016). The histological stages of oocyte and spermatogenesis development together with macroscopic aspects of the gonads are used to characterize reproductive phases (Brown-Peterson et al. 2011).

The migratory behaviour and reproductive period are among the major life-history traits in the reproductive process. Such traits generate trade-offs in the development of any reproductive strategy (Trujillo-Jiménez et al. 2013). The spawning season can be assessed by changes in ovaries/testes maturation along the annual cycle (Brown-Peterson et al. 2011). The biological indices related to reproduction can indicate the way in which fish use environmental and energetic resources. The gonadosomatic index (GSI) is a good indicator of reproductive activity that is also determined by the stages of gonadal maturation (Le Cren 1951, Hojo et al. 2004, Hismayasari et al. 2015). Other indices such as the hepatosomatic index (HSI) and the condition factor (K) are also used to assess the reproductive period (Bolger and Connolly 1989, Jayasankar and Alagarswami 1994, Barbieri et al. 1996, Giosa et al. 2014). The HSI is usually used in fisheries science as an indicator of energy reserves in the liver (Cerda et al. 1996, Hismayasari et al. 2015). Variations in the HSI are related to the capacity of the liver to store glycogen, reflecting the physiological conditions and the availability of energy (mainly lipids) for the reproduction activity (Carvalho et al. 2009). The condition factor (K) can indicate the degree of fitness of the individuals and can be related to the reproductive process by reflecting the allocation of energy (mainly proteins) during the spawning period, in which the physiological state of the individual changes (Carvalho et al. 2009). Therefore, fish nutritional status and/or spending of reserves are a result of their relationship with the environment reflecting in these indices (Medeiros and Maltchik 2001, Lizama and Ambrosio 2002, Silva et al. 2018).

Environmental factors such as water temperature and rainfall are important drivers of the reproductive process for several fish species in the Neotropical Region (Hokanson 1977, Jonsson 1991, Bailly et al. 2008). Water temperature and rainfall can trigger the spawning process of many fish species and, therefore, their reproductive success may be related to increases in temperature and in water volume that is associated to increases in rainfall (Abrial et al. 2014). Seasonal variations in water level also have direct influence on the reproductive process because they cause changes in several water characteristics (e.g., transparency, temperature, and input of allochthonous materials), increasing habitat availability (mainly in the riverine zone) and food resources (Agostinho et al. 2004, Bailly et al. 2008, Espínola et al. 2016).

Fish of the Characiformes order exhibit a wide variety of life strategies, with an adaptive divergence that does not match any other animal order (Fink and Fink 1981, Nelson 2006). *Astyanax* (Baird & Girard, 1854) is the most diversified genus of the Characidae with more than a hundred species widely distributed in the Brazilian watersheds (Reis et al. 2003, Hirt et al. 2011, Silva et al. 2012). This genus probably has great ecological importance and great adaptive plasticity (Gurgel 2004, Orsi et al. 2004, Abelha et al. 2006). The characin *Astyanax* aff. *bimaculatus* is widely distributed in southeastern Brazil. The studied species belongs to the *Astyanax bimaculatus* complex but still there is no available literature clarifying its taxonomic status. The studied species has a large orbital diameter (35.0–38.3% head length), great height (43.5–45.7% standard length, only pentacuspidated teeth in the dental bone, and two circular foramens in the specialized neural process.

This species seems to perform small movements for reproduction in both lentic and lotic environments (Godinho et al. 2010, Weber et al. 2012). Species of *Astyanax* are pelagic spawners normally reproducing in schools during upstream movements (Breder and Rosen 1966, Mazzoni and Iglesias-Rios 2004, Súarez et al. 2017). Garutti (1989) and Gennari-Filho and Braga (1996) reported *Astyanax bimaculatus* (Linnaeus, 1758) as total or batch spawner, depending on the environmental conditions. Winemiller (1989) classified *A. bimaculatus* as having a seasonal reproductive strategy, based on duration of the breeding season, average female reproductive bouts per year, oocyte diameter and mean generation time, among other features. The objective of this study was to assess macro and microscopic characteristics of the gonadal development of *Astyanax* aff. *bimaculatus* from Funil Reservoir, an impoundment in the middle reaches of the Paraiba do Sul River in southeastern Brazil, and to describe the gametogenesis through histological, histochemical and histometric techniques. We assessed the reproductive strategy and analysed changes in the reproductive endpoints of GSI, K and HSI to determine the reproductive period.

MATERIAL AND METHODS

The Funil Reservoir (22°30'–22°40'S; 44°30'–44°45'W, 440 m) is located in the middle reaches of the Paraíba do Sul River basin, in southeastern Brazil. The reservoir has an extension of approximately 20 km, area of 40 km^2, and mean depth of 22 m. The retention time is short (10–50 days), with great variation in water levels and substantial erosion of the banks. According to Branco et al. (2002), the Funil Reservoir is developing increasingly eutrophic conditions due to anthropogenic influences. There is little vegetation cover around the reservoir because of previous agricultural use for coffee plantation and pasture.

The reservoir was built in 1969 to generate hydroelectric power and to reduce the floods in the area downstream the dam. In addition, domestic and public drinking water supply, irrigation and aquaculture are among other uses of the reservoir. The climate is subtropical with monthly mean water temperatures of 18–24 °C, with maximum in January-February and minimum in July-August. Rainfall is at highest levels in the summer months (December–January; 200–250 mm per month) and at the lowest in the winter months (June-August), with less than 50 mm per month (Marengo and Alves 2005).

The fish were captured bimonthly by gill nets from September 2006 to October 2007. Three gill nets (50 x 3 m; stretch mesh 25, 50 and 75 mm) were set up at sunset and retrieved in the following morning at four sites randomly chosen across the reservoir area. All individuals were killed by immersion in water at 4 °C, identified and measured for total length (TL, nearest 1 mm), and weighted for total mass (TW, nearest 0.01 g). A ventral incision was made to expose gonads for determination of the sex and the gonadal development phases. Gonads were removed and weighed wet (GW, nearest 0·01 g). A portion of each gonad was preserved in Bouin's solution during eight hours for histological analyses following Vazzoler (1996). Then, gonads were transferred to 70% ethanol for preservation.

The gonads were subject to histological techniques and embedded in paraffin. Transversal sections (5 µm of thickness) were cut, mounted on glass slides and stained in haematoxylin and eosin (HE). To determine the histochemical content of the oocyte structures, the following techniques were used: Alcian Blue (AB) pH 2.5 and Schiff's acid + reactive acid (PAS) for the detection of acid and neutral glycoproteins, respectively (Myers et al. 2008).

Gonad sections collected in different regions (proximal, medium and distal) were examined. Microphotographs were taken with a Sony Cyber Shot DSC-W 230 digital camera coupled to an Olympus B941 (Tokyo, Japan). To characterize the reproductive phases, we used the terminology proposed by Quagio-Grassiotto et al. (2013), which combines the macro and microscopic aspects of gonads throughout the reproductive cycle of teleosts. Voucher specimens were deposited in the Fish Collection of the Laboratory of Fish Ecology, Universidade Federal Rural do Rio de Janeiro, under number LEP-UFRRJ #1873.

Size structure was assessed by length-frequency distributions of the individuals grouped into 20 mm TL size classes. The sex ratio was compared for each size class and for the pooled individuals. A chi-square (χ^2) test was used to assess the significance of differences at confidence level of 95% ($p < 0.05$).

The gonads were assigned to developmental stages, based on form, size, mass, colour and vascularization. However, gonads were ultimately classified as either immature (juveniles and inactive stages) or mature (developing, spawning capable, regressing and regenerating phases) to reduce the chances of identification mistakes of gonadal stages. The gonad classification was adapted from Nuñez and Duponchelle (2009) and West (1990). Oocytes were classified according to their morphology, their affinity to the dyes, and the presence of specific inclusions (lipid droplets, yolk granules, cortical alveoli).

The criteria for identification of oocyte stages and postovulatory follicles (POFs) were adapted from Brown-Peterson et al. (2011). The diameters of the first 50 oocytes and their nuclei were measured to 0.0001 mm using an ocular micrometer. Measurements were taken only on oocytes sectioned through the nucleus in fishes randomly chosen from the monthly samples. Histological identification of the various maturity stages were determined according to development of the ovary and testes and by the presence/absence of different types of oocytes (i.e., whether organized by ovarian lamellae or not) and spermatocytes. Histological classification of ovaries was based on oocyte stage and the occurrence of different stages of postovulatory follicles (POFs). The diameters of the vitellogenic oocytes and the spermatogenic cell nuclei were determined using the computerized image analyser Image-J 1.48 (Schneider et al. 2012), in 20 non-overlapping random fields of the histological slide prepared for each specimen.

In males, the histological characterization of the reproductive phases were based on the morphological changes that occur in the epithelium of the seminiferous tubules throughout the reproductive cycle. This characterization considers the presence or absence of spermatocytes and the types of germinative cells contained in the spermatocytes besides eventual alternations of a continuous or discontinuous germinal epithelium (Brown-Peterson et al. 2011).

The spawning period was determined by variations in the gonadosomatic index, $GSI = 100 \times (GW \times TW^{-1})$. The Fulton's condition factor (K) and hepatosomatic index (HSI) were calculated as indirect indices of energy status. The Fulton's condition factor (K) was calculated following the equation $K = 100\times(TW\times TL^{-3})$. The hepatosomatic index (HSI) was calculated as, $HSI = 100 \times (LW \times TW^{-1})$, where LW is the weight of the liver.

RESULTS

Sex ratio

A total of 94 specimens (56 females, 38 males) were examined. The total length (TL) ranged from 70 mm to 170 mm (female) and from 74 mm to 150 mm (male). The total weight (TW) ranged from 10.7 to 64.3 g (female) and from 7.1 to 43.0 g (male). Females significantly outnumbered males in size larger than 130 mm TL ($p < 0.01$). Highly significant differences were found for the pooled fish ($\chi^2_{(4, 90)} = 29.38$; $p < 0.01$) (Table 1).

Table 1. Chi-square (χ^2) test for sex ratio comparisons of *Astyanax* aff. *bimaculatus* in Funil Reservoir. (EF) Expected frequency, (TL) total length (mm).

Size classes (TL)	Female	Male	Total	EF	χ^2	Significance
70–90	1	6	7	3.5	3.57	*
90–110	2	10	12	6.0	5.33	*
110–130	26	19	45	22.5	1.08	ns
130–150	22	3	25	12.5	14.4	**
150–170	5	0	5	2.5	5.0	*
Total	56	38	94	47	29.38	**

(ns) Non-significant, (*) significant at $p < 0.05$, (**) significant at $p < 0.01$.

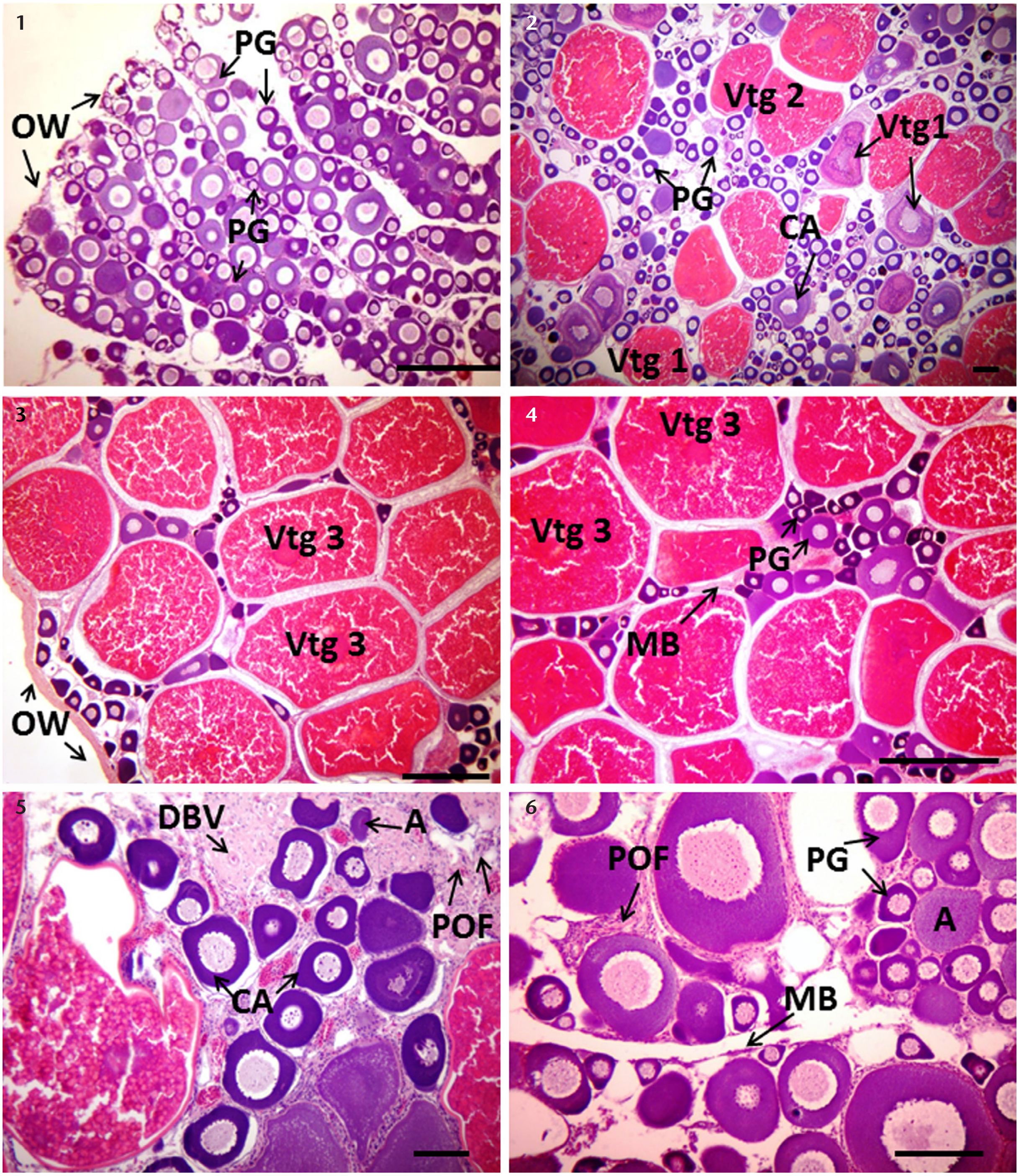
Histological characteristics of cells of the oogenic lineage

Primary growth stage

Oogonia, chromatin nucleolar and perinucleolar stages were present in the ovary throughout the entire annual cycle, and are referred to as primary growth stages (PG). They were clearly observed with the Alcian-blue technique (Table 2).

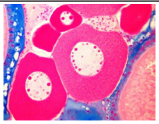
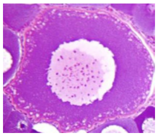
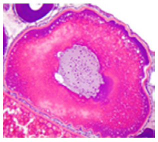
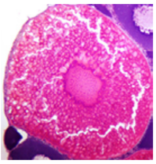
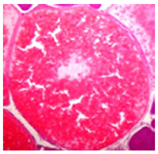
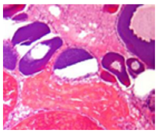
Oogonia: In this stage there is a predominance of the smallest cells of the oogenic lineage. Oogonia stage were characterized by cells with a large (8–10 µm), spherical and basophilic nucleus, and basophilic cytoplasm. The cells can be found isolated or in nests in the ovuligerous lamellae (Fig. 1, Table 2). Chromatin nucleolar (Fig. 2): The cells are similar to oogonia, although somewhat larger (two-fold larger, 20 µm) (Table 2).

Perinucleolar: The cells showed strongly basophilic cytoplasm with irregular contour with large, spherical and well-defined nuclei (Figs 3, 4). There was an eccentric nucleolus. The advanced oocyte had a rounder shape. The cytoplasm was less basophilic and presented irregular contour. The nucleus was



Figures 1–6. Photomicrographs of ovaries of *Astyanax* aff. *bimaculatus* in different phases of gonadal maturation. (1) Immature. Oocytes in primary growth (PG) ovuligerous lamellae (OL). (2) Developing. Presence of oocytes of different sizes (PG, CA, Vitg1 and Vtg2). (3–4) Spawning Capable. Predominance of vitellogenic oocytes (Vtg3). (5) Regressing. Presence of different types of oocytes (PG, CA, Vitg1, POF) and oocytes in atresia (A). (6) Regenerating. Oocytes in primary growth (PG) and POF. (OW) ovarian wall, (CA) cortical alveoli, (Vtg1) primary vitellogenic, (Vtg2) secondary vitellogenic, (Vtg3) tertiary vitellogenic, (MB) muscle bundle, (DBV) dilated blood vessels, (POF) postovulatory follicles, (A) atresia. Staining haematoxylin and eosin (HE). Scale bar: 100 µm.

Table 2. Stages of oocyte development in *Astyanax* aff. *bimaculatus.*

Stage	Characteristics	Image
Primary growth	Oogonias (nests) Chromatin nucleolar Perinucleolar	
Pre-vitellogenic	Cortical alveoli	
Vitellogenic	Primary vitellogenic –Vtg1	
	Secondary vitellogenic – Vtg2 (378.7µm ± 315.3 µm s.d. diameter)	
	Tertiary vitellogenic – Vtg3 (585.4 µm ± 450.1 µm s.d. diameter)	
Postovulatory follicles and atresic	String formation of granulosa cells disorganised or absent. Disintegration of the nucleus	

large (28 µm) and acidophilic and some basophilic nucleoli were seen on the periphery (Table 2). The follicular envelope was composed of only one layer of pavement cells.

Secondary growth stage

This stage includes Cortical alveoli (CA) and Vitellogenic (Vtg), which is divided into three substages: primary (Vtg1), secondary (Vtg2), and tertiary (Vtg3).

Cortical alveoli: The cells had nuclei with an irregular contour (Figs 2, 5). The main characteristic was the presence of vesicles and alveoli in the periphery of the cytoplasm and the large diameter (42 µm). The cytoplasm was less basophilic than the previous stage. The nucleus had several nucleoli in its periphery (Table 2). The zona radiata was evident, rather thin and translucent, surrounded by a layer of cubic cells of the granulosa and by pavement cells of the theca.

Vitellogenic stage

Primary vitellogenic (Vtg1): In this stage, the yolk granules, also called yolk spheres or yolk globules, were numerous containing cortical alveoli and occupying the entire cytoplasm (Fig. 2). The nucleus became smaller than the previous stage (18 µm) (Table 2).

Secondary vitellogenic (Vtg2): The nucleus had the irregular contour with many peripheral nucleoli (Fig. 2). The cytoplasm was acidophilic, and the alveoli occupy its cortical portion. At this stage, alveoli appeared, filled with acidophilic material, and yolk granules. The mean diameter of Vtg2 was 378.7µm ± 315.3 µm s.d. (Table 2). The zona radiata was thicker than in the previous phase. The cell layer of the granulosa was well defined and the theca remained composed of pavement cells.

Tertiary vitellogenic (Vtg3): The cytoplasm was markedly acidophilic and completely filled with vitellogenic granules (Figs 3, 4). The lipid inclusions were dispersed in the cytoplasm and in the nucleus. The nucleus had smaller size compared to the previous stage. The mean diameter of Vtg3 was 585.4 ± 450.1 µm s.d. The granulosa cells were long, with a very irregular apical surface and the theca presented a high degree of vascularization.

Atresia

The cells of the granular layer migrated to the interior of the ooplasm, absorbing the yolk. At the end of this stage, the zona radiata disappeared (Figs 5, 6). The postovulatory follicle was recognizable by its disorganized structure, abundant vacuoles and a convoluted follicular wall (Table 2).

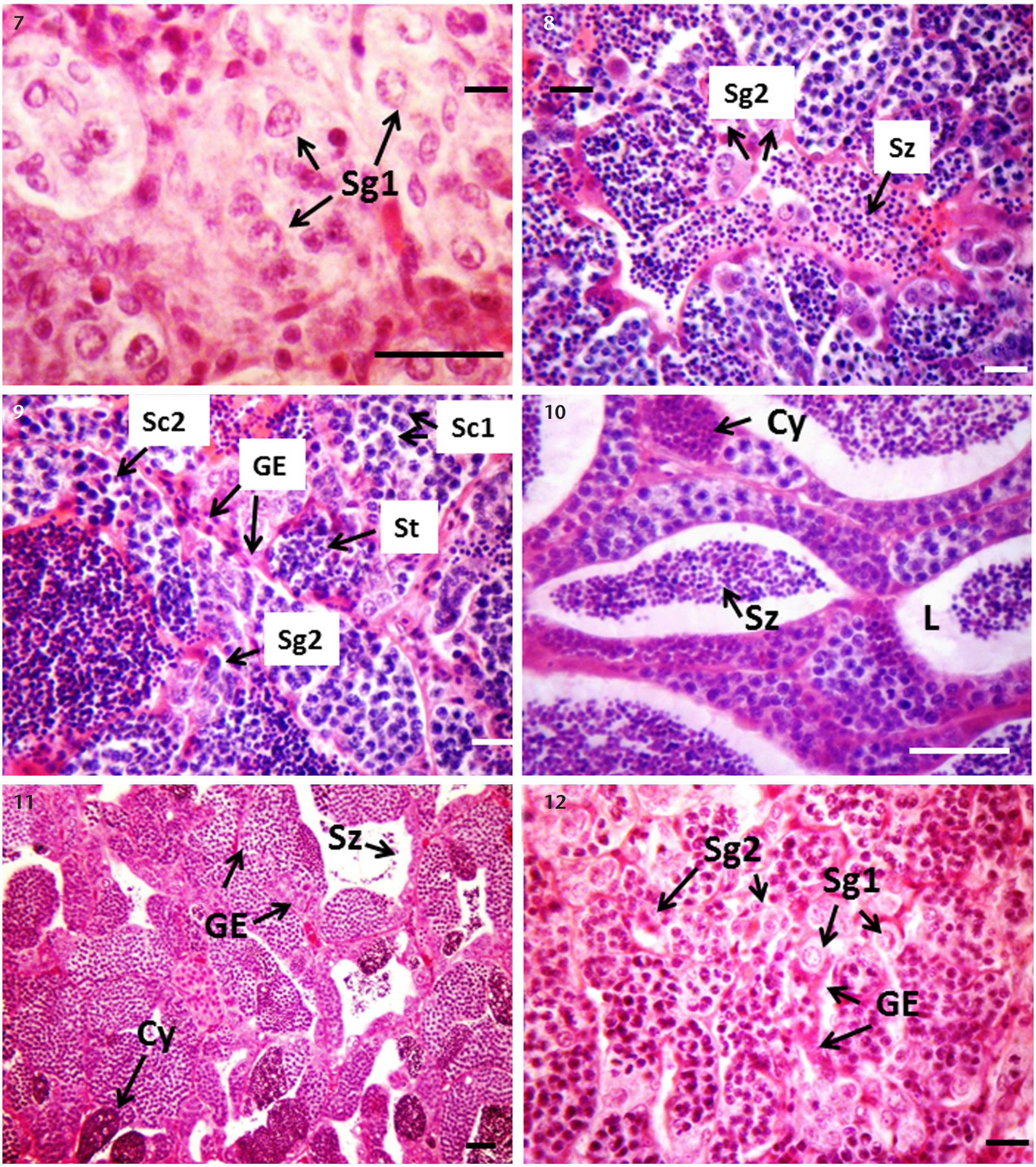
Atresia was frequently observed during the oocytes regressing, undergoing various phases of degeneration and absorption. The post-ovulatory follicles, resulting from the release of the mature oocyte are formed by hypertrophied granulosa cells. On the other hand, the theca cells do not undergo any changes with oocyte release.

During oocyte development, the follicle formation occurs, with the zona radiate (ZR) separating the oocyte from the follicular wall with a basal membrane between the follicular cell layer (granulosa cells) and the theca layer of connective tissue. Only in the vitellogenic oocytes, the presence of acid glycoproteins in the theca and granulosa cells was detected with positive reaction to AB. In addition, the neutral glycoproteins were found in the ZR, in the cortical alveoli and between the yolk globules because of the positive reaction to the PAS.

Histological characteristics of cells of the spermatogenic lineage

The testes were covered by a capsule of dense connective tissue, the tunica albuginea, which protrudes into the organ delimiting and supporting the seminiferous tubules. The determination of the types of male germ cells was performed according to the histological characteristics of the cytoplasm, nucleus and size of the cells. Based on these observations, the following spermatogenic cells were identified: primary (sg1) and secondary (sg2) spermatogonia; primary (sc1) and secondary (s2) spermatocytes; spermatids, (sd) and spermatozoa.

Primary spermatogonia (G1): They were the largest (10 µm) cells of the germ lineage with abundant eosinophilic cytoplasm (Figs 7–12), and large spherical nucleus with isolated nucleolus. The nucleus had a mean diameter of 5.76 ± 0.79 µm

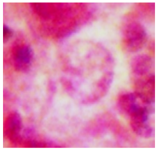
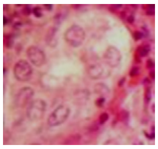
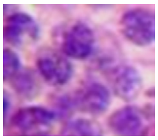
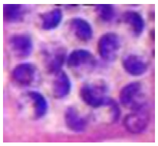
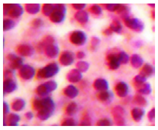
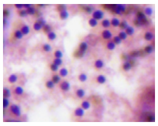


Figures 7–12. Photomicrographs of testes of *Astyanax* aff. *bimaculatus* in different phases of gonadal maturation. (7) Immature. Only primary spermatogonia (Sg1) without lumen. (8–9) Developing. Various types of spermatocytes evident along lobules (Sg2, Sc1, Sc2, St, Sz) and germinal epithelium (GE) continuous throughout. (10) Spawning Capable. Predominance of Sz in lumen seminiferous tubules. (11) Regressing. Presence of cysts (Cy), residual spermatozoa (Sz) and germinal epithelium (GE) in regeneration. (11) Regenerating. Proliferation of spermatogonia (Sg1, Sg2) and GE continuous throughout. Staining haematoxylin and eosin (HE). Scale bar: 20 µm.

(Table 3). They were observed isolated from the wall of the seminiferous tubules at all maturation stages.

Secondary spermatogonia (G2): They originated from the division of primary spermatogonia. These cells were observed grouped in cysts (Figs 8, 9, 12). The cytoplasm was clear and reduced. The nucleus was more basophilic; nucleolus were clearer. Nucleus had smaller size than in the previous stage with mean diameter of 4.50 ± 0.64 µm (Table 3).

Table 3. Stages of nuclear diameter (µm) of the spermatogenic cells development in *Astyanax* aff. *bimaculatus*.

Spermatogenic cells	Nuclear diameter ± s.d.	Image
G1 – primary spermatogonia	5.76 ± 0.79	
G2 – secondary spermatogonia	4.50 ± 0.64	
C1 – primary spermatocyte	3.70 ± 0.56	
C2 – secondary spermatocyte	3.53 ± 0.48	
T – spermatid	1.80 ± 0.54	
Z – spermatozoa	1.67 ± 0.42	

Spermatocytes (C1, C2): They were smaller than the secondary spermatogonia (Fig. 9). The nucleus was more basophilic than in the previous phase and the cytoplasm was hardly visible. The primary spermatocytes (C1) had a nucleus with a mean diameter of 3.70 ± 0.56 µm, whereas the secondary spermatocytes (C2) had a nucleus with a mean diameter of 3.53 ± 0.48 µm (Table 3).

Spermatids (T): They had indistinguishable limits, spherical and basophilic nuclei (Fig. 9). The nucleus mean diameter was 1.80 ± 0.54 µm (Table 3) and the cells were observed in cysts.

Spermatozoa (Z): They were the smallest cells of the germ line with spherical, very basophilic nucleus. They occupied the central region of the seminiferous tubules (Figs 8, 10, 11). During the spermatogenesis, there was a reduction of 80–90% in the diameter of the nucleus. The nucleus mean diameter was 1.67 ± 0.42 µm (Table 3).

Phases of the reproductive cycle

Females

The ovaries were enveloped by simple pavement epithelial tissue in the early stages of development changing to simple cubic in the final stages of maturation. Underneath the epithelium, we found the albuginea composed by dense connective tissue and regions of muscle fibers. The tunica albuginea emits septa into the ovarian lumen, delimiting the ovigerous lamellae where oogonia and oocytes are found at different stages of development.

The microscopic characteristics of the ovaries (Table 4) and testes (Table 5) were composed of five phases with slight differences in microscopic characteristics.

Immature: It was the primary stage of young ovaries that have not yet begun reproductive activity. Macroscopically, the ovaries were thin and translucent, not distinguished from the males testes. Histologically, a thin layer of ovarian wall (OW) and ovuligerous lamellae (OL) were observed, which were occupied by PG (Fig. 1, Table 4).

Table 4. Macroscopic and histological description of the phases of the reproductive cycle of female of *Astyanax* aff. *bimaculatus*. Adapted Brown-Peterson et al (2011) and Quagio-Grassiotto et al. (2013).

Phases	Features of ovaries	
	Macroscopic	Histological
Immature	Laminar form and small occupying less than one – third of the celomatic cavity, translucent, measuring 15–25 mm and weighing between 0.01 and 0.03 g; oocytes not visible to the naked eye	Primary growth (PG) present and the ovarian wall is thin
Developing	Wider ovaries, occupying less than one-third of the coelomatic cavity measuring 19–36 mm and weighing 0.7–2.3 g. Pale cream colour to whitish-yellow, visible blood vessels	PG, pre-vitellogenic (CA) and some in early vitellogenesis (Vtg1, Vtg2)
Spawning Capable	Oval and large shape occupying entire celomatic cavity, (24–43 mm; 2.5–4.8 g), yellowish-green colour, visible oocytes, blood vessels more evident	Prevalence of large vitellogenic oocytes (Vtg3), but oocytes in other stages of development is observed
Regressing	Flaccid, occupying less than one-third of the coelomic cavity (20–35 mm; 0.1–1.2 g) slightly brown and orange with haemorrhagic appearance; small oocytes visible to the naked eye	Disorganization of ovarian tissue with reabsorption of empty follicles (POF) and atresic oocytes (A); dilated blood vessels. Many different oocytes are still found
Regenerating	Small and broad ovaries, slightly brown colour (28–50 mm; 0.9–1.1 g), blood vessels less prominent	Presence of empty follicles (POF), oocytes in atresia and muscle bundle. Ovarian lamella partially occupied by oocytes at oocytes in development

Table 5. Macroscopic description of the phases of the reproductive cycle of male of *Astyanax* aff. *bimaculatus*. Adapted Brown-Peterson, Wyanski, Saborido-Rey, Macewicz and Lowerre-Barbieri et al. (2011) and Quagio-Grassiotto et al. (2013).

Phase	Features of the testes	
	Macroscopic	Histological
Immature	Filiform, occupying less than one-third of the coelomic cavity, measuring 14–30 mm and weighing between <0.01 and 0.01 g, translucent	Prevalence of primary spermatogonia (Sg1). Lumen of the tubules imperceptible
Developing	Flat shape, occupying less than one-third of the coelomic cavity, measuring 15–35 mm and weighing between 0.03 and 0.17 g, whitish colour.	Secondary spermatogonia (Sg2) and spermatocytes primary (Sc1) identified in spermatocytes along the germinal epithelium. Presence of primary, secondary spermatocytes (Sc1, Sc2), spermatids (St) and spermatozoa (Sz) in the lumen of the seminiferous tubules
Spawning Capable	Sinuous and flat shape, occupying one-third and nearly two-third of the coelomic cavity, measuring 24–45 mm and weighing between 0.12 and 0.47 g. Opaque white colour, unclear irrigation	Spermatozoa (Sz) present in the lumen of seminiferous tubules. Spermatogonia (Sg1, Sg2), spermatocytes (Sc1, Sc2) and spermatids (St) may be present in the spermatocytes
Regressing	Flaccid, haemorrhagic and occupy an average of 2/3 of the coelomic cavity, measuring 24–30 mm and weighing between 0.17 and 0.25 g. Reddish white colour	Spermatozoa present in the lumen of disorganized seminiferous tubules; germinal epithelium may be continuous or discontinuous. Spermatocytes containing non-released spermatids (St) dispersed by the seminiferous tubules
Regenerating	Small and bulky measuring 17–20 mm and weighing between 0.08 and 0.12 g	Lumen of the seminiferous tubules discrete or undetectable. Spermatogonia Sg1 and Sg2 in proliferation. Germinal epithelium continuous

Developing: The ovaries have began to mature; in this stage they presented whitish-yellow color, and increase in weight and length. Histologically, the alveolar cortical oocytes were also observed (Fig. 2, Table 4).

Spawning capable: The ovaries were distinctly large (24–43 mm) and occupy a large part of the coelomatic cavity. They were greenish yellow and vitellogenic oocytes, and a thick zona radiata was observed. (Figs 3, 4, Table 4).

Regressing: At this stage, there was a change in color and reduction in size (less than half of the anterior phase) and weight of the ovaries. The ovaries were flaccid and wrinkled. Some unreleased oocytes begun the process of atresia and postovulatory follicles were visible (Fig. 5, Table 4).

Regenerating: The ovaries begun the cycle of gonadal development by increasing weight and becoming more turgid (Fig. 6, Table 4).

Males

Immature: The testes were like two silvery or translucent threads, thinner and longer than immature ovaries (Fig. 7, Table 5).

Developing: The testes were translucent and thin. testes are longer, wider, often of triangular or circular section and whitish to pinkish (Figs 8, 9, Table 5).

Spawning capable: The testes were larger and whitish. Histologically, the tubules filled with spermatozoa were observed (Fig. 10, Table 5).

Regressing: The testes were shorter and lighter, but flaccid, empty-like (Fig. 11, Table 5).

Regenerating: In this phase, the testes begun the cycle of gonadal development. A gonad of a regenerating male is difficult to distinguish from a regressing phase, except at the end of the regeneration period, when the testes are relatively turgid. (Fig. 12, Table 5).

Spawning season

The highest percent of ovaries in the ripe stage (spawning capable) occurred in January-February (more than 80%), followed by September-October (30–75%), and the lowest between March and August (<10%) (Fig. 13). Similarly, the highest occurrence of testes in the ripe stage occurred in January-February (45%), followed by September to December (40–50%) and the lowest in March-April (18%).

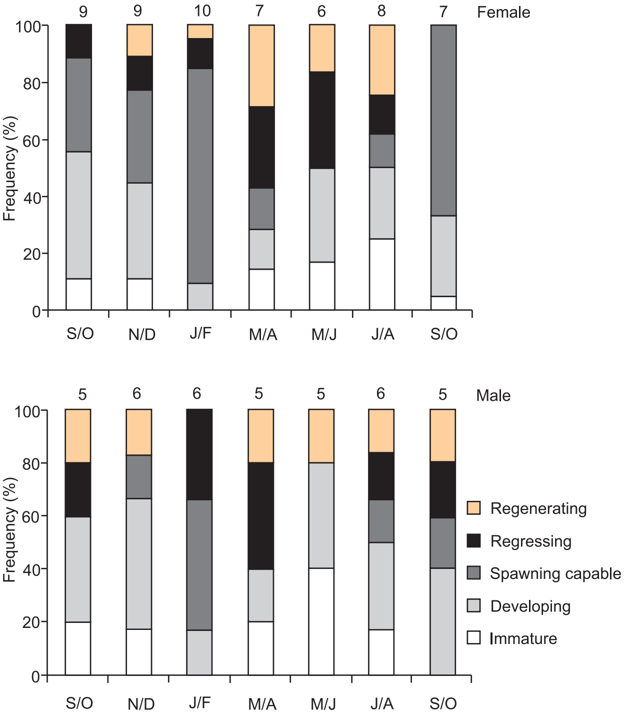


Figure 13. Bi-monthly changes in frequency distribution of the reproductive phases of *Astyanax* aff. *bimaculatus*. Number of individuals indicated above each bar. S/O, September-October; N/D, November-December; J/F, January-February; M/A, March-April; M/J, May-June; J/A, July-August.

The highest GSI values were recorded in January-February for both sexes and the lowest in May-August for females, and in March-June for males (Fig. 14). Values comparatively high of GSI (> 5) were also recorded for both sexes between September and December, and for females in March-April. The GSI values was directly related to the water temperature and reached the peak in January-February, when the rainfall was at the highest levels.

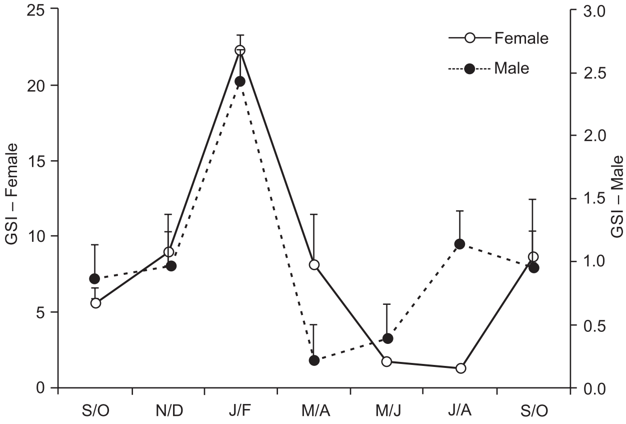


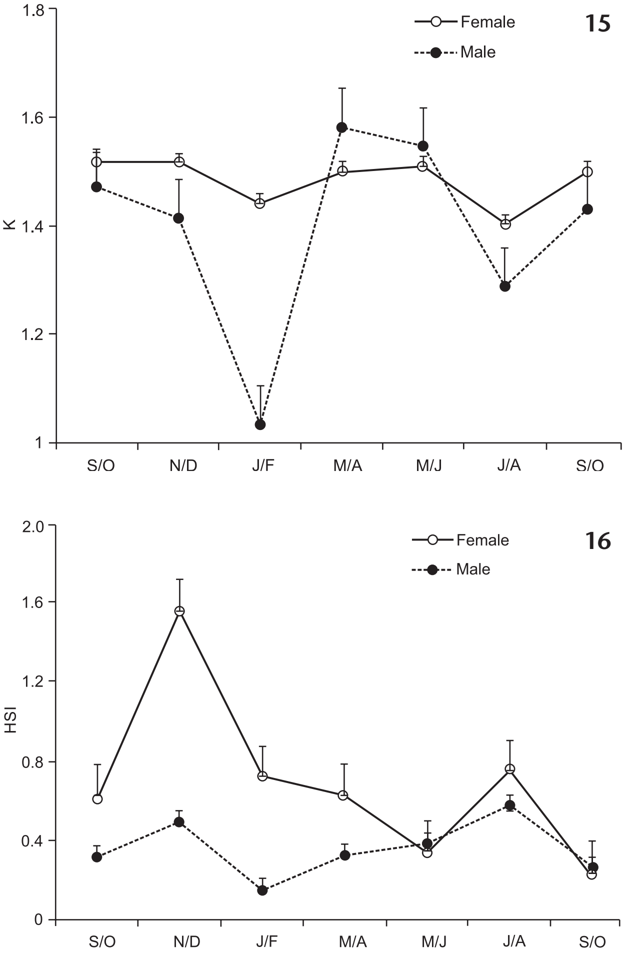
Figure 14. Bi-monthly changes in gonadosomatic index (means + standard error) for female and male of *Astyanax* aff. *bimaculatus* S/O, September-October; N/D, November-December; J/F, January-February; M/A, March-April; M/J, May-June; J/A, July-August.

The condition factor (K) had the highest values in September-December and in March-June and the lowest in January-February and July-August for females (Fig. 15). For males, the highest K was recorded in March-June and the lowest in January-February. The HSI had two peaks for both sexes, one in November-December and another less conspicuous in July-August. The highest values in November-December were followed by a decreasing in the following months with a slight increase in July-August for both sexes (Fig. 16).

DISCUSSION

We found compelling evidence from macro and microscopy observation that *Astyanax* aff. *bimaculatus* is a batch spawner. Its small size, with small oocytes and long spawning season, are evidence that this species fits to an opportunistic strategy (sensu Winemiller and Rose 1992). Vazzoler and Menezes (1992) reported that large oocytes offer better conditions for larval development and survival, but small oocytes can be produced in greater numbers. Opportunist reproductive strategy is common in small-sized species, with early maturation, high mortality rates, multiple spawning, small eggs, low fecundity, rapid population turnover and capacity for rapid colonization (Lamouroux et al. 2002, Blanck et al. 2007).

The opportunist strategy is not universal among species of *Astyanax*. Winemiller (1989) classified *A. bimaculatus* and *Astyanax henseli* de Melo & Buckup, 2006 as having a seasonal reproductive strategy. Among the life history strategies proposed by Winemiller (1989), the reproductive characteristics presented by *A. henseli* are more similar to the seasonal strategy, i.e., late maturation, seasonal reproduction, intermediate or high fecundity and low investment in the offspring, with absence of parental care and reduced juvenile survival. In addition, synchronous oocyte development in two groups presented by *A. henseli* (Dala-Corte and Azevedo 2010) suggests that the species presents total spawning, that is, a single batch of oocytes is spawned within a reproductive period. On the other hand, Abelha and Goulart (2008) found a large reproductive period for *Astyanax paranae* Eigenmann, 1914 (October to April) in a small reservoir from state of Paraná, which is an indicative of opportunist strategy. Similarly, the reproductive period for *A.*



Figures 15–16. Bi-monthly changes in (15) condition factor (means + standard error) and (16) hepatosomatic index (means + standard error) for female and male of *Astyanax* aff. *bimaculatus*. S/O, September-October; N/D, November-December; J/F, January-February; M/A, March-April; M/J, May-June; J/A, July-August.

paranae in the stream population was characterized as long, with spawning extending from July to March (Veregue and Orsi 2003), which also did not fit to a seasonal reproductive strategy.

We observed ovaries in the regression phase in different months and this in an indication that *A.* aff. *bimaculatus* spawns in parcels (batch spawn). This type of spawning favours reduction of predation on offspring and competition among individuals for food and shelter (Suzuki et al. 2000, Hojo et al. 2004, Bailly et al. 2008). Spawning in parcel (batch spawning) has been described for *A. bimaculatus* (Agostinho et al. 1984), *A. scabripinnis* (Jenyns, 1842) (Barbieri 1992, Veloso-Júnior et al. 2009) and *A. fasciatus* (Cuvier, 1819) (Carvalho et al. 2009), whereas total spawning has been reported for *Astyanax schubarti* Britski, 1964 (Nomura 1975), A. fasciatus (Gurgel 2004) and *Astyanax lacustris* (Lütken, 1875) (Súarez et al. 2017). Batch spawning allows several spawning events during the same reproductive cycle. Consequently, different niches in space and time are occupied with different size classes in a variety of habitats. This leads to lower competition among adults for spawning sites and among larvae for available food sources (Ratton et al. 2003). Garutti (1989), studying populations of *A. lacustris* from the Paraná River Basin, suggested that the species has batch spawning and prolonged reproductive period in habitats such as streams and headwaters, and total spawning and short reproductive period in rivers with greater water volume, where individuals would be less exposed to abrupt changes. Fish can transition from total spawn to fractional spawn and vice-versa because of physiological or environmental alterations.

We observed that the spermatozoa of *A.* aff. *bimaculatus* had rounded heads, a characteristic of fish with external fertilisation (Grier 1981). The reduction in size of spermatogenic lineage cells throughout the development is a widely accepted general rule (Sprando and Russel 1988), and it was found in *A.* aff. *bimaculatus*. During the spermatogenesis, a reduction of 80–90% of the nuclear diameter of the spermatogenic cells was observed.

Astyanax aff. *bimaculatus* has an unbalanced sex ratio, with females outnumbering and reaching larger sizes than males. These results are in accordance with the majority of freshwater fish in the tropics (e.g., Duarte and Alcaraz 1989, Duarte et al. 2007, Gomes et al. 2011, 2015) with predominance of females in the largest size classes. Sex ratio is an important trait to estimate the reproductive biomass and total population fecundity, being also one of the most important drivers of the reproductive potential (Marshall et al. 2006). An imbalance in the sex ratio, particularly in adults, is relatively common in fish and is related to sex differences in growth, mortality and/or the energy costs of reproduction (Potts and Wootton 1984). Predominance of females in larger sizes favours higher fecundity (Nikolsky 1963, Shine 1990, Gross 2005) since large females have a larger peritoneal cavity and, thus, can lay a larger number of eggs. Although fecundity and the number of fertilized eggs increase with female body size, a general pattern in teleosts (Gross and Sargent 1985, Duarte and Alcaraz 1989), the ecological importance of the sex ratio is still not fully explained (Gross 2005). The smaller-sized male may be a consequence of selection for early male maturation and reproductive effort, which reduce male growth compared to that of females (Parker 1982, Endler 1983, Andersson 1994). Smaller-sized males were also observed for the congeneric *Astyanax fasciatus* in another reservoir in southeastern Brazil (Carvalho et al. 2009), which are in accordance with our findings.

The peak of reproductive activity indicated by GSI was January-February, followed by a less conspicuous period of reproductive activity in September-October. Studies on the reproductive biology of other species of *Astyanax* have shown that there are variations in the reproductive period, ranging from only two months (e.g., Godinho et al. 2010) to nine months (e.g., Veregue and Orsi 2003, Mazzoni et al. 2005). Overall, species of *Astyanax* have seasonal reproductive strategy, peaking in the rainy season between spring and summer (Dala-Corte and Azevedo 2010). Few studies with species of *Astyanax* reported individuals sexually active during the autumn or winter (Garutti 1989, Veregue and Orsi 2003). Gurgel (2004) found that the congeneric *A. fasciatus* has a long reproductive period, with the highest GSI in mid-summer, coinciding with the peaks in rainfall. These results are in accordance with our findings for *A.* aff. *bimaculatus*.

During the peak of GSI, we also observed decreases in the condition factor (K) and in the hepatosomatic index (HSI). The inverse relationship between both the HSI and K with the GSI suggests the mobilization of hepatic energy and body reserves to gonadal development during the spawning season, which is likely to be associated concomitantly with a decrease of feeding activity. Moreover, the highest K values between March and June for both sexes, after the peak of GSI, indicate a recover in body mass after the reproductive effort. The K factor has been used as a proxy of the spawning period, because in this period, the food intake may cease and K should reach the lowest values (Barbieri et al. 1996).

The HSI has been reported as a more accurate condition index to measure the energy reserves of fish compared with other indices (Dominguez-Petit and Saborido-Rey 2010, Alonso-Fernandez and Saborido-Rey 2012). In the present study, HSI had two peaks for both sexes, one in November-December and another less conspicuous in July-August. During the reproductive period individuals allocate less effort to food search, and consume reserves stored in the liver (capital breeders *sensu* McBride et al. 2015), resulting in a reduction in HSI values, particularly in females (Nikolsky 1963). These highest HSI values before the peaks of GSI are indications of the use of stored energy reserves in liver for gonadal maturation. These findings corroborate the hypothesis that changes in HSI are associated with the role of the liver in the reproductive activity (Alonso-Fernandez and Saborido-Rey 2012) and ovarian maturation. Other species of *Astyanax* had similar patterns of inverse relationship between GSI and HSI. In *Astyanax aeneus* (Günther, 1860), the highest HSI values were found during periods of reproductive inactivity and this was interpreted as an increase in the reserve materials stored in the liver for subsequent use in gamete production (Trujillo-Jiménez et al. 2013). *Astyanax henseli* had higher HSI before the breeding period and lower at the reproductive peak, suggesting greater use of liver reserves for vitellogenesis and gonadal maturation (Dala-Corte and Azevedo 2010, Trujillo-Jiménez et al. 2013), thus confirming the pattern of transference of energy from the liver to the gonads during the reproductive process

Opportunistic species have a higher resistance to environmental and anthropogenic alterations (Winemiller 1989). However, *A. bimaculatus* showed disruptions in their reproductive activity when present in environments with adverse physical-chemical conditions (Bailly et al. 2008, Vasconcelos et al. 2014). Disturbances such are dam construction, chemical pollution and habitat degradation that are frequent in rivers and reservoirs in developing countries can impair the reproductive success of opportunist species. Considering that environmental pressures vary according to the characteristics of each system, variations in the reproductive tactics of closely related species are expected (Dala-Corte and Azevedo 2010). The influence of damming tended to be with lower intensity on fish with opportunist reproductive strategy (Vasconcelos et al. 2014). Conversely, long-distance migratory species respond more markedly to spatiotemporal variations, indicating that the ecosystem dynamics exert greater effects on populations of these species (Bailly et al. 2008). Short-lived species with fast growth or reproductive compensation are expected to have survival advantages, and this may explain, at least in part the great success of *A.* aff. *bimaculatus* and other congeneric species to colonize successfully a large number of lotic and lentic aquatic environments in the Neotropical region.

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