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Ovarian histology and fecundity in the evaluation of the reproduction of the invasive species *Serrasalmus marginatus* (Characidae) on a neotropical floodplain

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ABSTRACT. The construction of the Itaipu Hydroelectric Power Plant in 1982 led to the formation of a reservoir, which, in turn, leveled the waters of the Paraná River by flooding the geographic barrier Salto de Sete Quedas. This allowed the piranha *Serrasalmus marginatus* to invade and colonize the upper Paraná River. This study aimed to: i) confirm, through light microscopy, the reproductive phases of *S. marginatus* females; ii) estimate fecundity and iii) evaluate the reproduction areas of the population. A total of 764 females were collected from nine sampling sites on the upper Paraná River floodplain. Microscopic analysis of the ovaries showed the following phases: early developing subphase, developing phase, spawning capable phase, actively spawning subphase, regressing phase and regenerating phase. The frequency distribution of the oocytes shows that spawning is fractional and fecundity indeterminate. Fecundity varied from 410 to 752 oocytes (mean = 584). The continual spawning of oocytes during the long reproductive period, as well as the aggressiveness of the species as regards the defense of its offspring, guarantees more descendants in the Patos, Ventura, Fechada, Guaraná and Garças lagoons and Ivinheima and Baia rivers of the upper Paraná River floodplain.

Keywords: Indeterminate fecundity, type of spawning, oogenesis, oocytes, piranha.

Histologia ovariana e fecundidade na avaliação da reprodução da espécie invasora *Serrasalmus marginatus* (Characidae) em uma planície de inundação neotropical

RESUMO. A construção da Usina Hidroelétrica de Itaipu, em 1982, levou a formação do reservatório que por sua vez nivelou as águas do rio Paraná inundando a barreira geográfica do salto de Sete Quedas. Isto permitiu que a piranha *Serrasalmus marginatus* invadisse e colonizasse o alto rio Paraná. Este estudo teve por objetivos: i) confirmar através da microscopia de luz as fases reprodutivas das fêmeas de *S. marginatus*; ii) estimar a fecundidade e iii) avaliar as áreas de reprodução da população. Um total de 764 fêmeas foram amostrados em nove estações de amostragem na planície de inundação do alto rio Paraná. A análise microscópica dos ovários mostrou fêmeas nas subfases em desenvolvimento inicial, fase desenvolvimento, fase apto à desova, subfase desova ativa, fase regressão e fase regeneração. A distribuição de frequência dos oócitos mostra que a desova é parcelada e a fecundidade é indeterminada. A fecundidade variou de 410 a 752 e em média 584 oócitos. A desova contínua durante o longo período reprodutivo associado à agressividade em relação à defesa da prole garante o sucesso em deixar mais descendentes nas lagoas dos Patos, Ventura, Fechada, do Guaraná, das Garças, nos rios Ivinheima e Baia da planície de inundação do alto rio Paraná.

Palavras-chave: Fecundidade indeterminada, tipo de desova, oogênese, oócitos, piranha.

Introduction

Invasion of species is one of the main causes of biodiversity loss leading to both environmental and economic damage (McNeely, 2001; Molnar, Gamboa, Revenga, & Spalding, 2008; Pejchar & Mooney, 2009; Keller, Geist, Jeschke, & Kühn, 2011; Simberloff et al., 2013). Invasive species have some characteristics that facilitate or allow their establishment in a new environment (e.g. high trophic plasticity and dispersion capacity) (Lodge, 1993; Marchetti, Moyle, & Levine, 2004). As regards freshwater ecosystems, fish have received special attention due to the quantity of invasive species recorded in recent years, especially carp, tilapia and African catfish (Garcia, Loebmann, Vieira, &

Bemvenuti, 2004; Vitule, Umbria, & Aranha, 2006; Russel, Thuesen, & Thomson, 2012).

The upper Paraná River, possessing high species richness, according to Langeani et al. (2007), underwent a massive invasion due to the formation of the Itaipu Reservoir, which eliminated the natural geographic barrier Salto de Sete Quedas (Júlio Jr, Dei Tos, Agostinho, & Pavanelli, 2009), which had separated the faunas of the middle and upper Paraná River. Consequently, about 33 species became invaders, characterizing one of the largest invasions of freshwater species in South America (Skóra, Abilhoa, Padial, & Vitule, 2015).

With the flooding, the piranha *Serrasalmus marginatus* Valenciennes, 1837 successfully colonized the upper Paraná River basin. Its success increased with the construction of the Piracema Channel in the Itaipu Reservoir, where this species has been recorded (Makrakis, Gomes, & Makrakis, 2007). It is distributed in the Paraná-Paraguay river basin, lives in both lentic and lotic environments, carries out short-distance migrations (Graça & Pavanelli, 2007) and has been indicated as the principal cause of the decrease in the population of its native congener (Agostinho & Júlio Jr, 2002). In addition, *S. marginatus* is an iteroparous, gonochoristic, monomorphic, oviparous species with external fecundation. It reaches sexual maturity at 11.5 cm standard length for males and 12.2 cm standard length for females and all individuals are able to reproduce at 13.0 cm standard length (Suzuki, Vazzoler, Marques, Lizama, & Inada, 2004). Moreover, it possesses batch spawning, parental care and a long reproductive period (September to April) (Vazzoler, 1996; Suzuki et al., 2004), coinciding with the season of higher temperatures and flooding.

One way to evaluate the reasons for the success of *S. marginatus* is to study its reproductive activity and capacity, since reproducing and maintaining the population is one of the precepts for obtaining success in a new environment. The reproductive capacity of fish can be quantified using the following measurements: maturation length or age, type of fecundity, fecundity, duration of the reproductive season, spawning behavior, and spawning fraction. Information about reproductive potential is fundamental to spawning stock biomass evaluation (Hunter, Macewicz, Lo, & Kimbrell, 1992; Murua et al., 2003; Ganias, 2013; Ganias, Lowerre-Barbieri, & Cooper, 2015). Thus, this work aims to: i) validate the reproductive phases of the females using light microscopy; ii) estimate the fecundity and iii) evaluate the reproduction areas of the *S. marginatus* population from the upper Paraná River floodplain.

Material and methods

Study area

The upper Paraná River floodplain is located downstream from the Engenheiro Sérgio Mota (Porto Primavera) Hydroelectric Power Plant and upstream from the Itaipu Reservoir. This distance (approximately 250 km) is the last dam-free stretch of the Paraná River in Brazil (Agostinho & Zalewski, 1996). Although the floodplain is located between two large dams, the Paraná River possesses two important tributaries, the Baia and Ivinheima rivers, which contribute to the maintenance of biodiversity and ideal conditions for the entire aquatic fauna, mainly the ichthyofauna.

Sampling

The fish were collected quarterly (March, June, September and November/December 2013, 2014 and 2015) from 9 sampling sites on the upper Paraná River floodplain: 1 - Baia River (Rbai), 2 - Ivinheima River (Rivi), 3 - Paraná River (Rpar), 4 - Guaraná Lagoon (Lgua), 5 - Patos Lagoon (Lpat), 6 - Paraná River in the Garças Lagoon (Lgar), 7 - Ressaco do Pau Veio Lagoon (Lpve), 8 - Baia River in the Fechada Lagoon (Lfec) and 9 - Ivinheima River in the Ventura Lagoon (Lven) (Figure 1).

The samplings were carried out using 11 gill nets (meshes: 2.4; 3; 4; 5; 6; 7; 8; 10; 12; 14; 16 cm, between opposite knots) and two trammel nets (meshes: 6; 8 cm). The nets were exposed for 24 hours at every site and checked between 8:00 and 9:00 (night-morning), 16:00 and 17:30 (daytime) and 22:00 and 23:30 (evening-night). In addition, bottom otter trawls (20 m long; 0.5 cm mesh) were carried out during the day in the coastal areas of every lagoon. The fish were anesthetized and euthanized using 0.1% ethyl aminobenzoate (benzocaine), according to the protocols of Summerfelt and Smith (1990) and approved by CEUA (Committee for the Ethical Use of Animals) (*Universidade Estadual de Maringá*).

The following data were recorded for each individual: catch site, catch date, standard length (cm), total weight to the nearest 0.01 g, gonadal development phases based on the macroscopic characteristics of the ovary, total weight of the gonads (twg) to the nearest 0.01 g and weight of the ovarian fractions (wof) to the nearest 0.01 g.

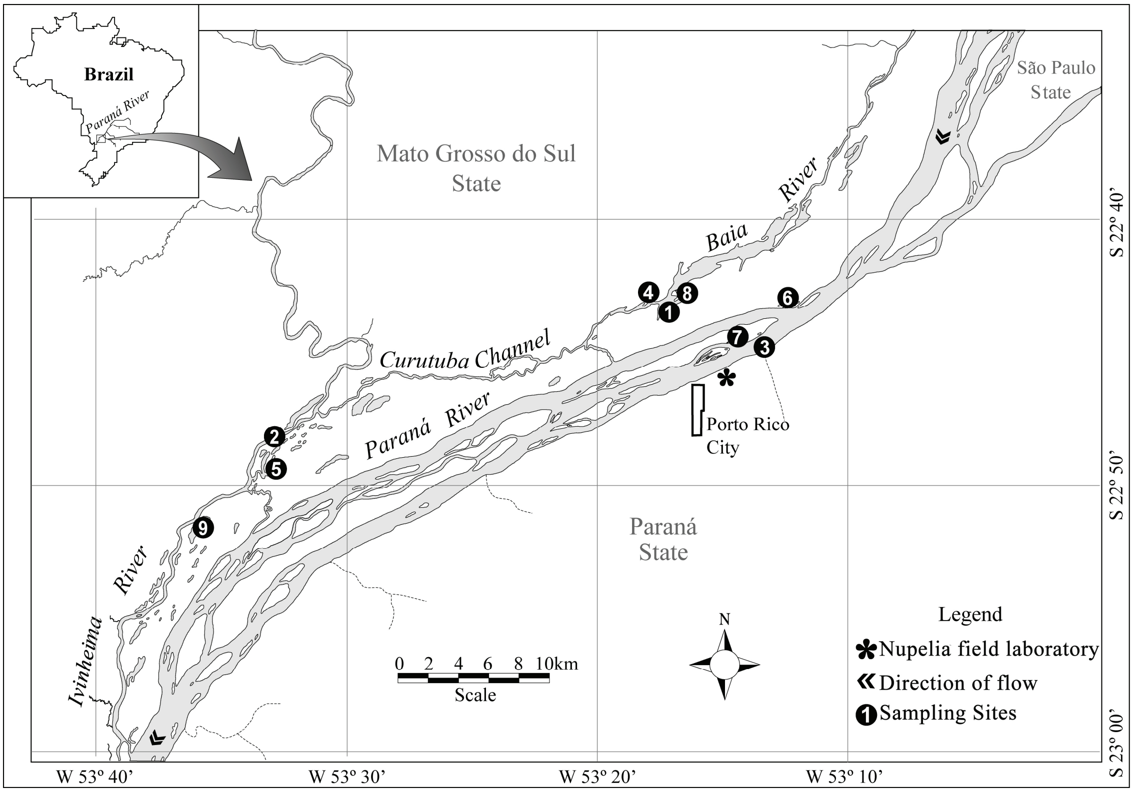

Figure 1. Study area and location of the sampling sites (Baia River – 1; Ivinheima River – 2; Paraná River – 3; Guaraná Lagoon – 4; Patos Lagoon – 5; Garças Lagoon – 6; Ressaco do Pau Veio Lagoon – 7; Fechada Lagoon – 8; Ventura Lagoon – 9) on the upper Paraná River floodplain.

Reproductive characterization

The ovarian development phases were attributed according to the macroscopic characteristics proposed by Brown-Peterson, Wyanski, Saborido-Rey, Macewicz, and Lowerre-Barbieri (2011), Wildner, Grier, and Quagio-Grassiotto (2013) and Quagio-Grassiotto, Wildner, and Ishiba (2013). A fraction of the left lobe of the ovary was fixed in Bouin solution for at least 48 hours, dehydrated in ethanol, infiltrated using Historesin and shaped into blocks, which were cross-sectioned at a thickness of 5 μm and stained using 0.5% Toluidine Blue, Hematoxilin/Eosin and periodic acid Schiff/ hematoxylin/metanil yellow (Quintero-Hunter, Grier, & Muscato, 1991). The germ cells were identified according to Grier, Uribe-Aranzábal, and Patiño (2009) and Wildner et al. (2013) and the reproductive phases attributed macroscopically (early developing subphase, developing phase, spawning capable phase, actively spawning subphase, regressing phase and regenerating phase) were validated by the microscopic characteristics of the development stages of more advanced cell types, according to Brown-Peterson et al. (2011), Quagio-Grassiotto et al. (2013) and Wildner et al. (2013).

In order to determine type of fecundity, ovaries from individuals in the developing phase, spawning capable phase and actively spawning subphase were selected. The right lobe of the ovary was fixed in 10% buffered formalin. Using the gravimetric method, the oocytes were counted and measured from three subsamples of approximately 0.3 g each from the anterior, middle and posterior region of the right lobe of the ovary (Hunter, Lo, & Leong, 1985; Murua et al., 2003).

The diameter of the oocytes from 12 specimens of *S. marginatus* was measured using a stereomicroscope equipped with an ocular micrometer to determine the type of oocyte development and spawning. The type of spawning was determined according to the frequency distribution of the oocytes per diameter class (Murua & Saborido-Rey, 2003).

Thus, absolute fecundity (AF), i.e. the number of oocytes that a female will spawn in the next reproductive period, was calculated according to Vazzoler (1996).

The floodplain reproduction sites were identified by number of females in different reproductive phases.

Results

A total of 765 females with 3.9 to 24.9 cm standard length were used for macroscopic characterization of the gonads at the different sites of the floodplain. The sites having the greatest representativeness were Patos Lagoon (231 individuals), Ivinheima River (214) and Ventura Lagoon (70). In the Baia River, 145 individuals were collected, while 51 and 18 were collected in the Fechada and Guarana lagoons, respectively. The sites having the fewest number of samples were the Garças Lagoon, Paraná River, and Ressaco do Pau Veio Lagoon, with eight, twenty-three and five individuals, respectively.

Based on the microscopic diagnosis of the germ lineage, cysts with a batch of oogonia surrounded by prefollicle cells, pachytene oocytes, primary growth oocytes, early and late secondary growth oocytes, a full-grown oocyte (Figure 2 and Table 1) and oocyte maturation (Figure 3J) were recognized in *S. marginatus*. The postovulatory follicle complex recorded after ovulation and follicular atresia showed oocytes unable to ovulate (Figure 2H, I).

Histological sections of the ovaries showing the development phases were detailed and described (Figure 3 and Table 2).

The diameter of the vitellogenic oocytes of *S. marginatus* varied from 300 to 1700 μm (Figure 4), and the trends reveal asynchronous development of the oocytes and batch spawning. Only a portion of the oocytes is spawned in each batch after reaching maturation and ovulation.

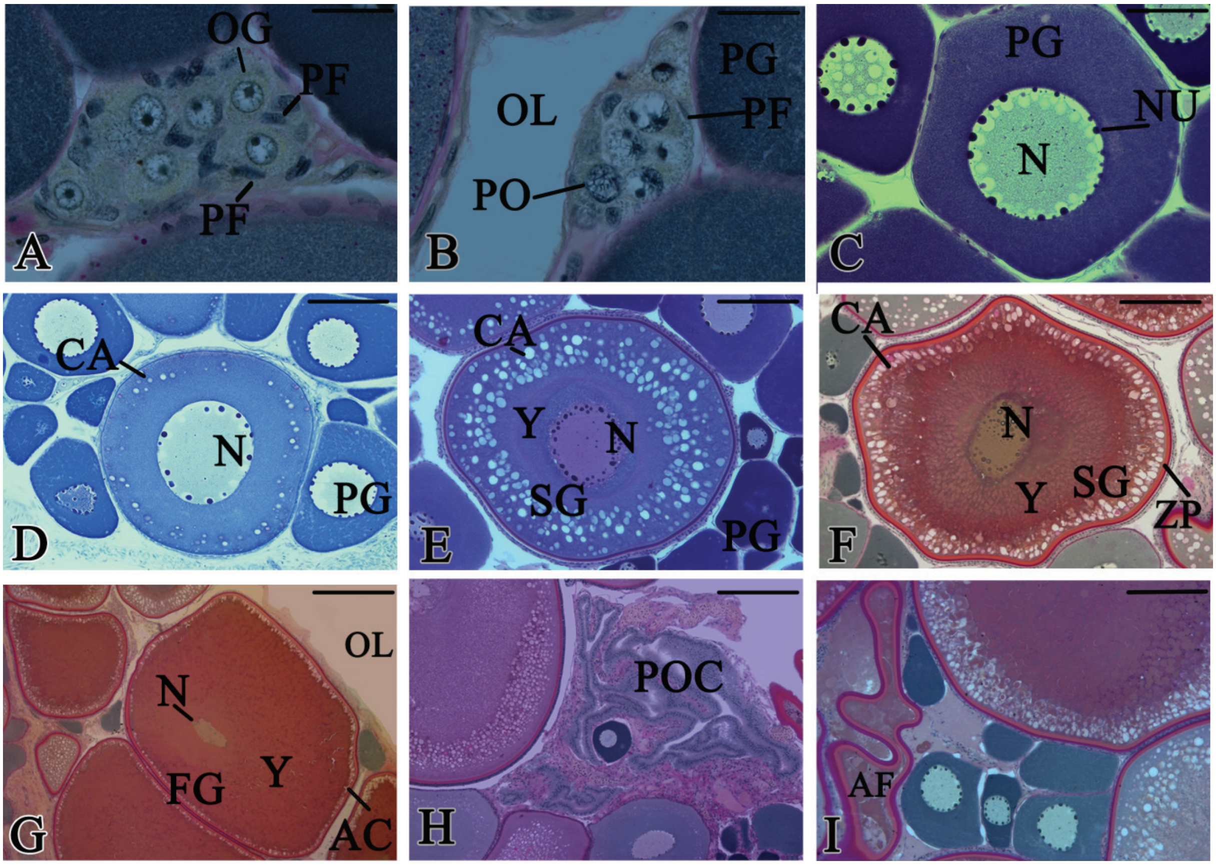

Figure 2. Oocyte development in *Serrasalmus marginatus*. Light microscopy, PAS/Hematoxylin/ Metanil Yellow (A, B, F, G, H, I), Hematoxilin/Eosin (C, E) and Toluidine Blue (D). (A) Nest of oogonia is surrounded and among pre-follicle cells, forming germline cysts. Inside this nest the oogonia are spherical and voluminous and their nucleus widens with an evident nucleolus, bar = 22 μm. (B) Cell nest with pachytene oocytes, bar = 29 μm. (C) Ovarian follicle with primary growth oocyte, bar = 70 μm. (D) Late primary growth oocyte shows the beginning of the formation of the cortical alveoli, bar = 145 μm. (E) Early secondary growth oocyte begins the deposition of yolk, formation of the cortical alveolus, bar = 295 μm. (F), Late secondary growth oocyte, bar = 295 μm. (G) Full-grown oocyte, bar = 590 μm. (H) The postovulatory follicle complex, bar = 295 μm. (I) Follicular atresia, bar = 295 μm. CA, cortical alveoli; AF, atretic follicle; BM, basement membrane; F, follicle cell; FG, full-grown oocyte; LO, leptotene oocytes; N, nucleus; NU, perinuclear nucleoli; OF, ovarian follicle; OG, oogonium; OL, ovarian lumen; PF, prefollicle cells; PG, primary growth oocyte; PO, pachytene oocyte; SG, secondary growth oocyte; Y, yolk globule; ZP, zona pellucida.

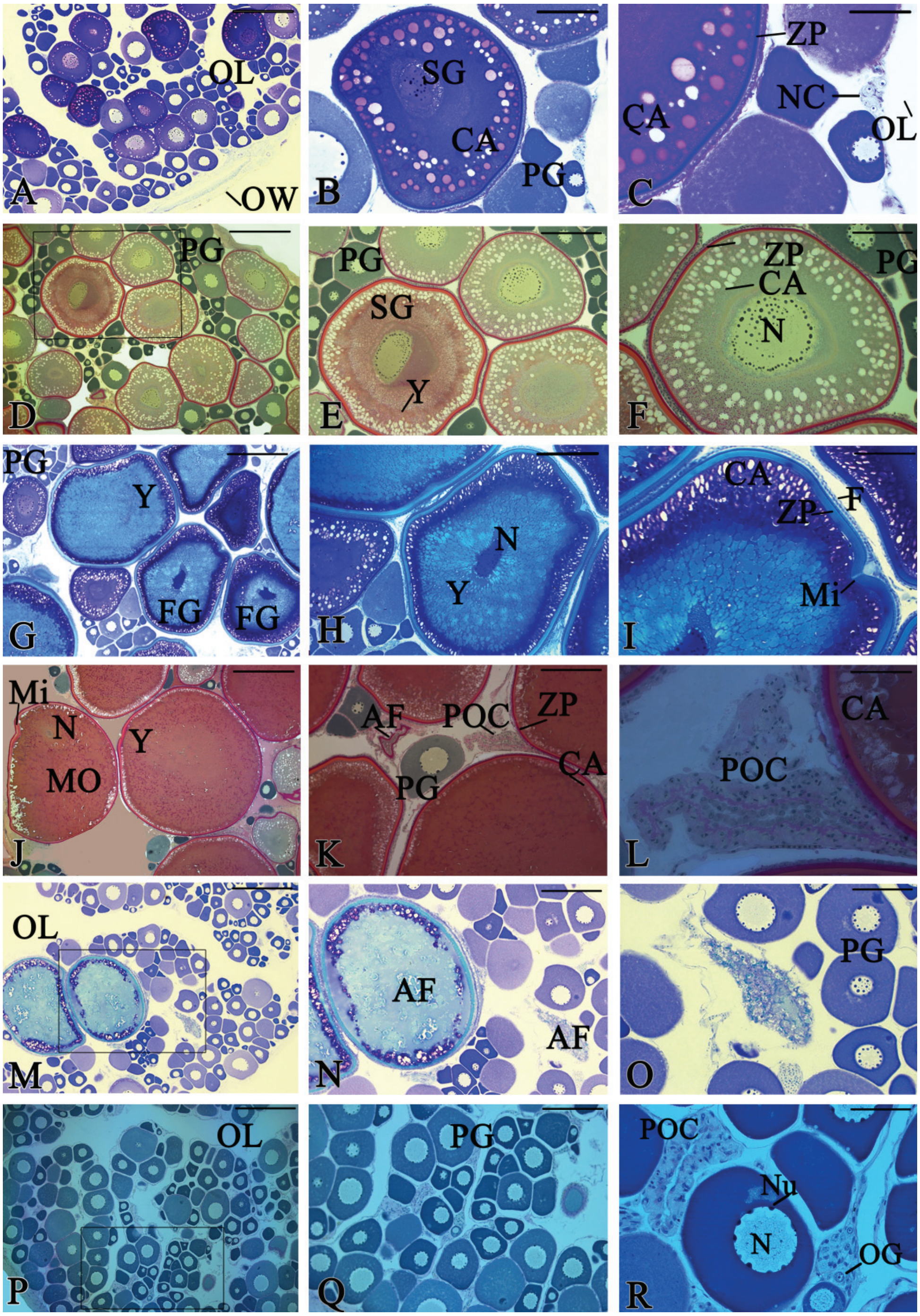

Figure 3. Reproductive phases of the ovarian cycle of *Serrasalmus marginatus* according to oocyte differentiation stages. Light Microscopy, Toluidine Blue (A, B, C, G, H, I, M, N, O) and PAS/Hematoxylin/Metanil Yellow (D, E, F, J, K, L, P, Q, R). (A) Early development subphase, stroma contains primary and secondary oocytes, bar = 550 μm. (B) Lamella contains early secondary growth oocytes, bar = 140 μm. (C) Oogonia nest in the germinal epithelium, bar = 70 μm. (D) Developing phase, primary and secondary growth oocytes, bar = 600 μm. (E) Secondary growth oocytes present cortical alveoli and yolk globules, bar = 300 μm. (F) Oocytes with numerous cortical alveoli, bar = 150 μm. (G) Spawning capable phase, shows primary growth and full-grown oocytes, bar = 550 μm. (H) Full-grown oocyte with central nucleus, bar = 280 μm. (I) Full-grown oocyte showing amicropyle, bar = 140 μm. (J) Actively spawning subphase, showing a mature oocyte, bar = 600 μm. (K) Postovulatory follicle complex and primary growth oocytes, bar = 300 μm. (L) Postovulatory follicle complex, bar = 80 μm. (M) Regressing phase, bar = 550 μm. (N) Atretic follicle, bar = 280 μm. (O) Atretic follicle and primary growth oocyte, bar = 140 μm. (P) Regenerating phase, bar = 600 μm. (Q) Primary growth oocyte, bar = 270 μm. (R) Postovulatory follicle complex, primary growth oocyte and oogonia nest, bar = 6 μm. AF = atretic follicle; CA = cortical alveolus; F = follicle cells; FG = full-grown oocytes; Mi = micropyle; MO = maturing oocyte; N= nucleus/germinal vesicle; NC = nest of oogonia; Nu = nucleolus; OG = oogonium; OL = ovarian lumen; OW = ovarian wall; PG = primary growth oocytes; POC = postovulatory follicle complex; SG = secondary growth oocytes; Y = yolk globules; ZP = zona pellucida.

Table 1. Diagnosis of germinal cells in different stages of oocyte development, postovulatory follicle complex (POC) and atretic ovarian follicles of *Serrasalmus marginatus* on the Paraná River floodplain.

Stages/POC/Atresia	Microscopic Characteristics
Primary growth	Ovarian follicle with primary growth oocyte. It shows intense basophilic ooplasm and the nucleus or germinal vesicle with several perinuclear nucleoli (Figure 2C). A gradual increase of ooplasm and the appearance of cortical alveoli record the end of primary growth oocytes (Figure 2D).
Early secondary growth	This oocyte (Figure 2E) is showing the gradual increase of yolk in the ooplasm and cortical alveoli are arranged on the periphery of the ooplasm during development.
Late secondary growth	Late secondary growth oocyte has nuclear outline more irregular, cortical alveoli are seen on periphery of ooplasm and zona pellucida more developed (Figure 2F).
Full-grown oocyte	This oocyte (Figure 2G) contains a slightly eccentric nucleus, surrounded by ooplasm completely full of yolk globules and the cortical alveoli develop as a thin peripheral layer in the ooplasm.
Postovulatory follicle complex	After oocyte maturation the evidence of ovulation in the ovarian stroma was the formation of the postovulatory follicle complex observed in the lamella (Figure 2H).
Follicular atresia	Unovulated oocyte becomes atretic and its degeneration and removal from the ovarian follicle occurs (Figures 2I).

Table 2. Phases and subphases of reproduction based on the microscopic characteristic of germinal cells of *Serrasalmus marginatus* females on the Paraná River floodplain.

Phase/subphase	Microscopic Characteristics
Initial Development subphase	Ovarian stroma contains more primary growth oocytes (PG) and some secondary growth oocytes (SG) (Figure 3A). In the SG the cortical alveoli and formation of yolk globules begins to appear (Figure 3B). Nests containing oogonia in proliferation are observed at the edge of ovarian lumen (Figure 3C).
Developing	Primary and secondary growth oocytes (Figure 3D). In the early and late vitellogenic oocytes, the formation of yolk globules progressed in their oolema (Figure 3E, F).
Spawning Capable	Primary growth and secondary growth (early, late vitellogenic and full-grown oocytes) (Figure 3G). Full-grown oocytes with nucleus situated at the center of the ooplasm. Its yolk globules abundant except on periphery (Figure 3H). Micropyle recorded in the full-grown oocytes (Figure3I).
Actively Spawning subphase	Maturing oocyte with germinal vesicle takes an eccentric position on the periphery of the ooplasm at the animal pole near the micropyle (Figure 3J). In this subphase atretic follicle and postovulatory follicle complex also occur (Figure 3K, L).
Regression	Predominance of primary growth oocytes and atretic follicles (Figure 3M, N, O), and an absence of secondary growth oocytes.
Regeneration	Presence of primary growth oocytes, proliferating oogonia forming cell nests and degenerating postovulatory follicles were recorded (Figure 3P, Q, R).

The diameter of the vitellogenic oocytes of *S. marginatus* varied from 300 to 1700 μm (Figure 4), and the trends reveal asynchronous development of the oocytes and batch spawning. Only a portion of the oocytes is spawned in each batch after reaching maturation and ovulation.

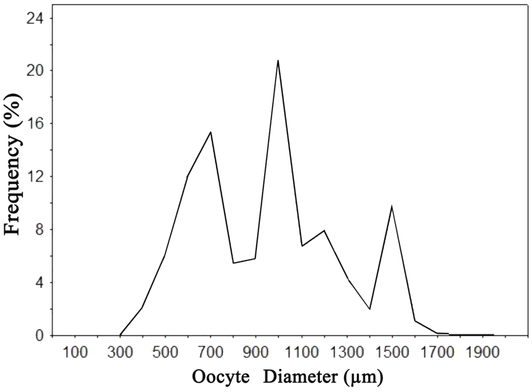

Figure 4. Frequency of the vitellogenic oocyte diameter (μm) of the ovaries of the piranha *Serrasalmus marginatus* sampled on the upper Paraná River floodplain.

The absolute fecundity estimated for six individuals whose total length varied from 16.4 to 20.2 cm, varied from 410 to 752 oocytes. As regards the different rivers of the upper Paraná River floodplain, the piranha *S. marginatus* shows reproductive activity in every studied environment; however, it is more frequent in Patos Lagoon and the Ivinheima and Baia rivers, successfully occupying the lotic waters of the rivers and the lentic waters of the lagoon (Figure 5). Among the principal environments (Ivinheima, Baia and Paraná), *S. marginatus* is least reproductively active in the Paraná River. The main channel of the Paraná River did not have any individuals in advanced stages of gonadal development (Figure 5).

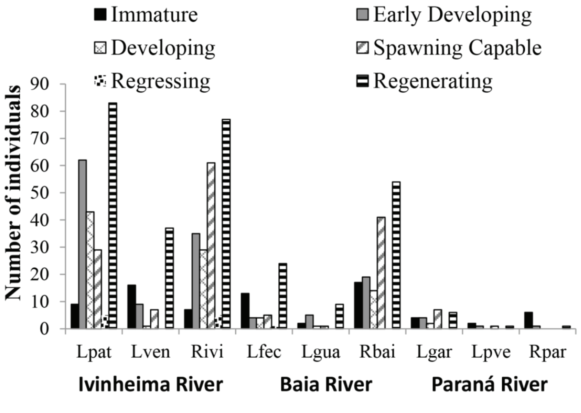

Figure 5. Spatial distribution of the gonadal development phases per river and sampling site of the piranha *Serrasalmus marginatus* sampled on the upper Paraná River floodplain. Lpat = Patos Lagoon; Lven = Ventura Lagoon; Rivi = Ivinheima River; Lfec = Fechada Lagoon; Lgua = Guaraná Lagoon; Rbai = Baia River; Lgar = Garças Lagoon; Lpve = Pau Veio Lagoon; Rpar = Paraná River.

Discussion

A fish must allocate time and resources for reproduction to be represented genetically in the next generation (Wootton, 1998). The reproductive success of fish thus depends on their reproductive rate, the survival rate of their descendants until the age of reproduction, spawning type and number of reproductive opportunities (Wootton, 1998; Murua & Saborido-Rey, 2003; Lowerre-Barbieri, 2009). The reproductive strategy of *S. marginatus* may be considered opportunist, which is characteristic of species that have a short gestation period, are small in size and invest little in their offspring (Winemiller & Rose 1992; Winemiller, 2005), enabling them to rapidly populate different habitats and invade new ones. The capacity to spawn during the entire reproductive season is a species life-history trait that allows the maintenance of population levels even in situations of environmental disturbance (e.g. suppression of Sete Quedas barrier that permitted the passage of species from the middle to upper Paraná River (Júlio Jr et al., 2009)).

Over each reproductive cycle, the renewal of germ cells, their differentiation, development, maturation and release result in gonadal alterations that characterize different reproductive phases. The reproductive phases attributed macroscopically to *S. marginatus* were confirmed through light microscopy. They are developing, spawning capable, regressing and regenerating, according to Brown-Peterson et al. (2011). These phases are used because they are simpler and in Brazil have been adopted by Wildner et al. (2013) for *Serrasalmus maculatus*, Quagio-Grassiotto et al. (2013) for *Hoplias malabaricus* and *Sorubim lima* and Agostinho et al. (2015) for *Hemiodus orthonops*.

Fish species with asynchronous ovarian development exhibit strategies of determinate or indeterminate fecundity (Hunter et al., 1985; Murua et al., 2003; Murua & Saborido-Rey, 2003). These strategies relate to the pattern and time lag in which the pre-vitellogenic oocytes (primary growth) are recruited to compose the stock of vitellogenic oocytes (secondary growth). In indeterminate fecundity, vitellogenesis continues after the start of spawning (Hunter et al., 1985; Murua & Saborido-Rey, 2003; Murua et al., 2003; Ganias et al., 2015).

The fecundity of the batches, spawning frequency and duration of the reproductive season must be known to estimate indeterminate fecundity (Hunter et al., 1992; Murua & Saborido-Rey, 2003; Murua et al., 2003). The patterns of total spawning and partial spawning and the type of fecundity (determinate or indeterminate) have been recorded for marine and freshwater species (Brown-Peterson et al., 2011). Among the 41 species from the upper Paraná River that were studied as regards spawning type, 73% possess batch spawning and, therefore, indeterminate fecundity (Vazzoler, 1996).

The frequency of the diameter of the oocytes and the microscopic record of the different types of oocytes that develop in the reproductive cycle show that their development is asynchronous in *S. marginatus*; therefore, it exhibits a reproductive strategy of batch spawning and indeterminate fecundity. Fecundity (estimated) varying from 410 to 752 has guaranteed the reproductive success of this species. This strategy of batch spawning and indeterminate fecundity has been recorded for *Loricariichthys castaneus* (Gomes, Araújo, Uehara, & Sales, 2011) and *Serrasalmus maculatus* (Wildner et al., 2013).

Adequate environmental conditions and sites for larval development and juvenile growth are fundamental to the population success of any species. Our results demonstrate that most of the reproductive stages predominate in the Baia and Ivinheima rivers, mainly spawning capable individuals, which are in great number in the lagoons associated with these two rivers. These rivers belong to the upper Paraná River floodplain and are two of the main tributaries responsible for the maintenance of ichthyofaunistic diversity and serve as nursery and growth areas, mainly for migratory species (Agostinho, Thomaz, Minte-Vera, & Winemiller, 2000; Reynalte-Tataje, Agostinho, & Bialetzki, 2013). Therefore, these sites are characterized by possessing adequate conditions for the establishment of *S. marginatus* and, consequently, the population increase of this species results in negative impacts for the native species *S. maculatus*, as the two are congeners that have similar environmental and feeding requirements (Agostinho & Júlio Jr, 2002; Agostinho, 2003; Alexandre, Luiz, Piana, Gomes, & Agostinho, 2004).

Conclusion

The reproductive success of *S. marginatus* on the upper Paraná River floodplain is attributed to parental care and greater aggressiveness in defending its feeding and reproduction territories (Agostinho, 2003; Alexandre et al., 2004). However, it is also associated with fecundity, because the long reproductive period with the continual spawning of oocytes guarantees more descendants. *Serrasalmus marginatus* reproduction is intense in lotic (river) environments and moderate in channels and lagoons of the upper Paraná River floodplain (Vazzoler, 1996; Agostinho, 2003; Suzuki et al., 2004). This study reveals that its reproductive success continues to be recorded in the Ivinheima River and in the lagoons of the upper Paraná River floodplain.

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