Int. J. Aquat. Biol. (2017) 5(3): 218-227
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Reproductive biology of *Pseudotocinclus tietensis* (Siluriformes: Loricariidae: Hypoptopomatinae), a threatened fish species

Jandyr A. Rodrigues-Filho[1, 4], Renato M. Honji[2], Paulo H. Mello[2], Maria I. Borella[3], Alexandre W.S. Hilsdorf[1], Renata G. Moreira*[1, 2]

[1]Universidade de Mogi das Cruzes. Av. Dr. Cândido Xavier de Almeida e Souza, 08701-970, Brasil.
[2]Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, 321, 05508-090, Brasil
[3]Instituto de Ciências Biomédicas, Universidade de São Paulo Av. Prof. Lineu Prestes, 1524, São Paulo 05508-900, Brasil.
[4]Fundação Instituto de Pesca do Estado do Rio de Janeiro, Km 2, 28470-000, Santo Antônio de Pádua, Brasil.

Abstract: *Pseudotocinclus tietensis* is endemic to the Upper Tietê River basin and classified as vulnerable. The reproductive biology of this species is still unknown, therefore, we investigated its reproductive strategy and gonad development during its annual reproductive cycle. The fish were collected throughout one year, and histology of the gonads, fecundity and oocyte diameter was conducted. Three phases of gonad maturation were found in males and females (immature, developing, and spawning capable), and the development stages of the gametes were identified within each stage. In the testes, four stages of gamete development were distinguished: spermatogonia, spermatocytes, spermatids and spermatozoa. During spermiation, the spermatozoa were released into the tubular lumen and then continued through the efferent ducts. In the ovaries, five stages of gamete development were identified: chromatin nuclear, perinucleolar, yolk vesicle formation, vitellogenic and ripe. The minimum diameter of ovulating oocytes was 297 µm, and the absolute fecundity was 64 to 306 oocytes. Males with spermatozoa in the lobular lumen and females with vitellogenic and ripe oocytes were found throughout the year. *Pseudotocinclus tietensis* has asynchronous ovarian development and gametes with fertilization capacity can be eliminated throughout the annual cycle.

Article history:
Received 9 January 2017
Accepted 5 May 2017
Available online 25 June 2017

Keywords:
Reproductive cycle
Gonads
Pseudotocinclus tietensis
Fecundity
Cascudo

Introduction

Reproduction is an important biological process and information on the cyclical development of the gonads, time and place of spawning and length in which individuals enter the reproductive process are critical for understanding the reproductive biology of a species. For teleosts, this information is particularly important for the development of fishery regulations, including the period of capture, the place and size that fish can be caught within a management program, the creation of conservation management measures as well as for the control of undesirable species (Marques et al., 2000).

The upper reaches of rivers such as the Upper Tietê River Basin (UTRB) are characterized by the prevalence of small fish species with high endemism, caused mostly by a low degree of spatial dispersion (Menezes, 1994). The icthyofauna of the UTRB is distinct from those of other upper reaches of drainage in the Upper Paraná Basin (Langeani-Neto, 1989). This is mainly due to the presence of valleys and hills, which provide isolation of the animals, allowing a high rate of speciation and endemism (Castro and Casatti, 1997). This is the case for the *Pseudotocinclus tietensis* (Loricariidae: Hypoptopomatinae), a species that is endemic to UTRB (Langeani-Neto, 1989; Wolf et al., 2005; Marceniuk et al., 2011), listed as "Endangered" in the National List of Threatened Species (Brasil, 2016) and classified as "Vulnerable" in the red list of threatened species (IUCN, 2016).

The subfamily Hypoptopomatinae is a monophyletic group of Loricariids composed of 19 genera

* Corresponding author: Renata G. Moreira
E-mail address: renatagm@ib.usp.br
DOI: https://doi.org/10.22034/ijab.v5i3.260

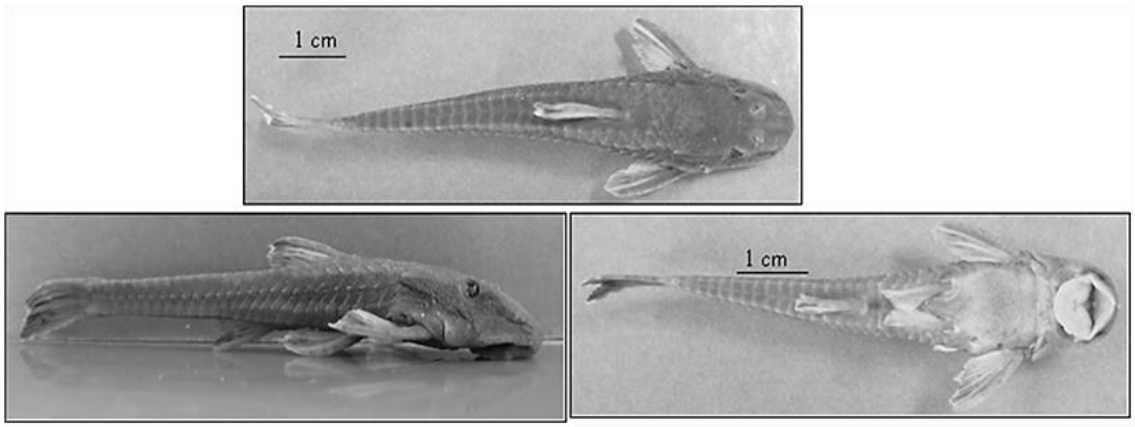

Figure 1. The collected *Pseudotocinclus tietensis* from the river Paraitinga located in the Upper Tietê Basin region, State of São Paulo, Brazil. Dorsal, lateral and ventral view displayed here.

and 139 species (Eschmeyer and Fong, 2016) that are widely distributed in continental waters of South America and have been studied in terms of molecular phylogeny (Cramer et al., 2011). This subfamily is divided into two tribes, Hypoptopomatini and Otothyrini, and the genus *Pseudotocinclus* is a member of the latter. Until 2005, this genus included one species. i.e. *P. tietensis* (Langeani-Neto, 1989), but the genus was revised and two new species were described as *P. juquiae* from the Ribeira do Iguape Basin and *P. parahybae* from Paraíba do Sul River Basin (Takako et al., 2005). The presence of a small naked area on the snout tip and transverse dark-brown bands on the dorsal region of the body distinguishes *P. tietensis* from the other species in the genus (Takako et al., 2005).

The main focus of our study, which took place at the upper reaches of the Tietê River and tributaries, was to analyze the gonadal and oocyte development of *P. tietensis* during its annual cycle to gain some expertise on the reproductive biology of this species and to support decisions on conservation management of this threatened species.

Materials and Methods

The specimens of *P. tietensis* (Ihering, 1907) (Fig. 1) were sampled in two tributaries of the URTB, São Paulo State, Brazil (Fig. 2) including the Biritiba Mirim River (23º42'01.81"S, 46º05'27.32"O) (Biritiba Mirim/SP) and the Paraitinga River (23º31'36.09"S, 45º48'52.08"O) (Salesópolis/SP). Fish were captured every two months with sieves and electrofishing device (Honda Generator - EX1000W, CA). Specimens were identified based on Takako et al. (2005).

After sampling, fish were placed in plastic bags and transported to the Laboratory of Metabolism and Reproduction of Aquatic Organisms (LAMEROA), where they were anesthetized with tricaine methanesulfonate (MS-222, INS Sigma Diagnostics, St. Louis, MO) (1 $g.L^{-1}$), neutralized with sodium bicarbonate and killed by decapitation (according to the institutional animal care protocols and approval. The total length and weight (Wt) were recorded, and the ovaries were excised and weighed (Wg) for the calculation of the gonadosomatic index (GSI) according to Vazzoler (1996): GSI = (Wg x100) /Wt.

The middle third of the gonads was fixed in Bouin's solution for 18-20 hours and transferred to ethanol (70^0 GL). The fragments of gonads were dehydrated and embedded in Paraplast, diaphanized (Erv-Plast; Erviegas Surgical Instruments, São Paulo, Brazil), cut with a microtome (5 μm), stained with hematoxylin-eosin and examined under light microscopy (Zeiss Axioskop II light microscope, Zeiss MC80 DX camera and a computer image capture PIXERA professional). The different developmental stages of the gametes were identified according to West (1990) for the ovaries and according to Grier and Aranzábal-Uribe (2009) for the testes. Based on the predominance of gamete development stages, females and males were divided into the different phases of

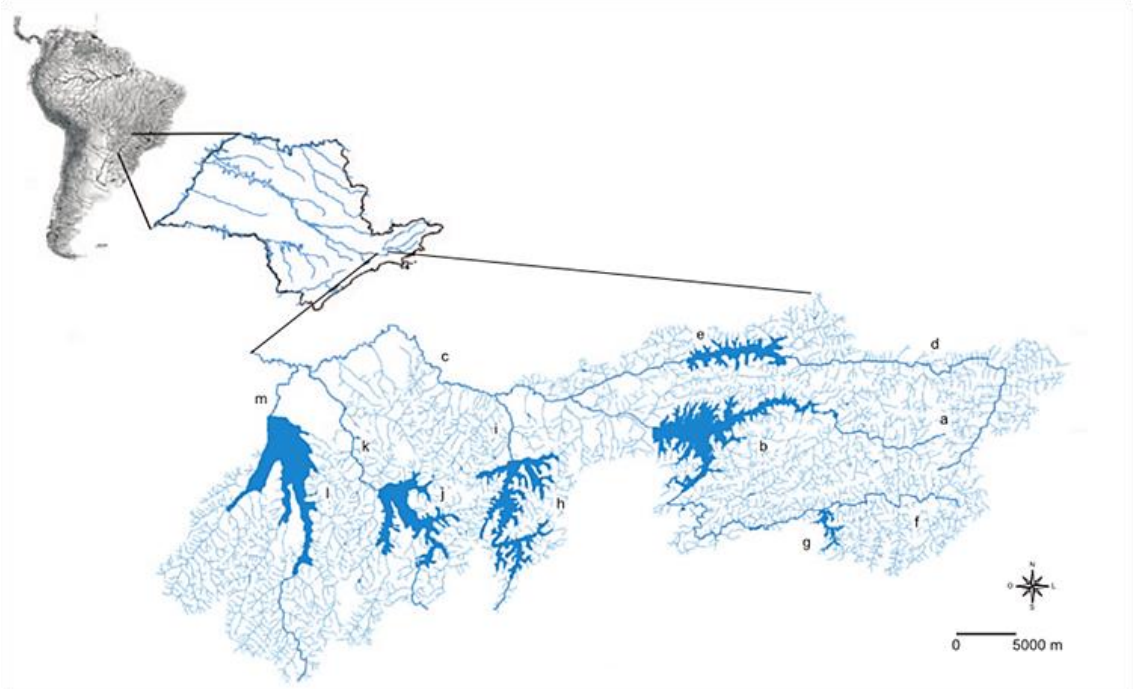

Figure 2. Hydrographic basin from the Upper Tietê River. (a) Head of Tietê River; (b) Ponte Nova Reservoir; (c) Tietê River; d) Paraitinga River; (e) Paraitinga Reservoir; (f) Claro River; (g) Ribeirão do Campo Reservoir; (h) Biritiba Mirim Reservoir; (i) Biritiba Mirim River; (j) Jundiaí Reservoir; (k) Jundiaí River; (l) Taiçupeba Reservoir; (m) Taiaçupeba River (Marceniuk et al., 2011). Sampling points: red arrow (23º31'36.09"S, 45º48'52.08"O; Paraitinga River) and black arrow (23º42'01.81"S, 46º05'27.32"O; Biritiba Mirim River).

gonadal development, as described by Brown-Peterson et al. (2011).

The remaining portion of the ovaries was weighed again for the fecundity analysis, immersed in a Gilson solution (Simpson, 1951) and stored for 30 days for dissociation of the tissue containing the oocytes. After this period, the oocytes were kept in ethanol (70^0 GL), ready to be evaluated for the fecundity and diameter analyses. An acrylic reticulated plate was used for counting and measuring oocyte diameter under a stereomicroscope (Leica MZ125) with micrometric ocular (Vazzoler, 1981; Romagosa et al., 2001; Honji et al., 2009; Gomes et al., 2015). The absolute fecundity was calculated in the developing and spawning capable phases. Relative fecundity was calculated using the mean body mass, total length and gonad weight. The data will show in linear fit of the type $Y = \alpha + bX$, where Y = Fecundity, α = regression constant, b = regression coefficient and X = variable (body weight, ovaries weight and total length).

Analysis of Data: All values were expressed as the mean ± standard deviation (M±SD). Body weight, length, weight of ovaries, GSI, number of oocytes/females and number of oocytes/ovary mass were compared among the different maturation phases using analysis of variance (one-way ANOVA), followed by either the Student–Newmann–Keuls (SNK) test for parametric analyses or Dunn's test for non-parametric analyses. Fecundity correlations were calculated using linear regression applied to total length, ovary and body mass. In all analyses, the differences were considered to be significant when $P<0.05$. These analyses were performed using the statistical software SigmaStat for Windows ver. 3.10 (Systat Software, San Jose, CA).

Results

A total of 41 specimens of *P. tietensis* were collected during the annual cycle, every two months: 20 females (January, n=4; March, n=3; May, n=3; July, n=3; September, n=4; November, n=3) with a total length of 5.9-8.3 cm (7.1±0.6) and body weight of 1.5-3.6 g (2.7±0.4 g), 16 males (January, n=3; March, n=2; May, n=2; July, n=3; September, n=3; November, n=3) with total length of 4.5-7.8 cm (6.2±0.4 cm) and

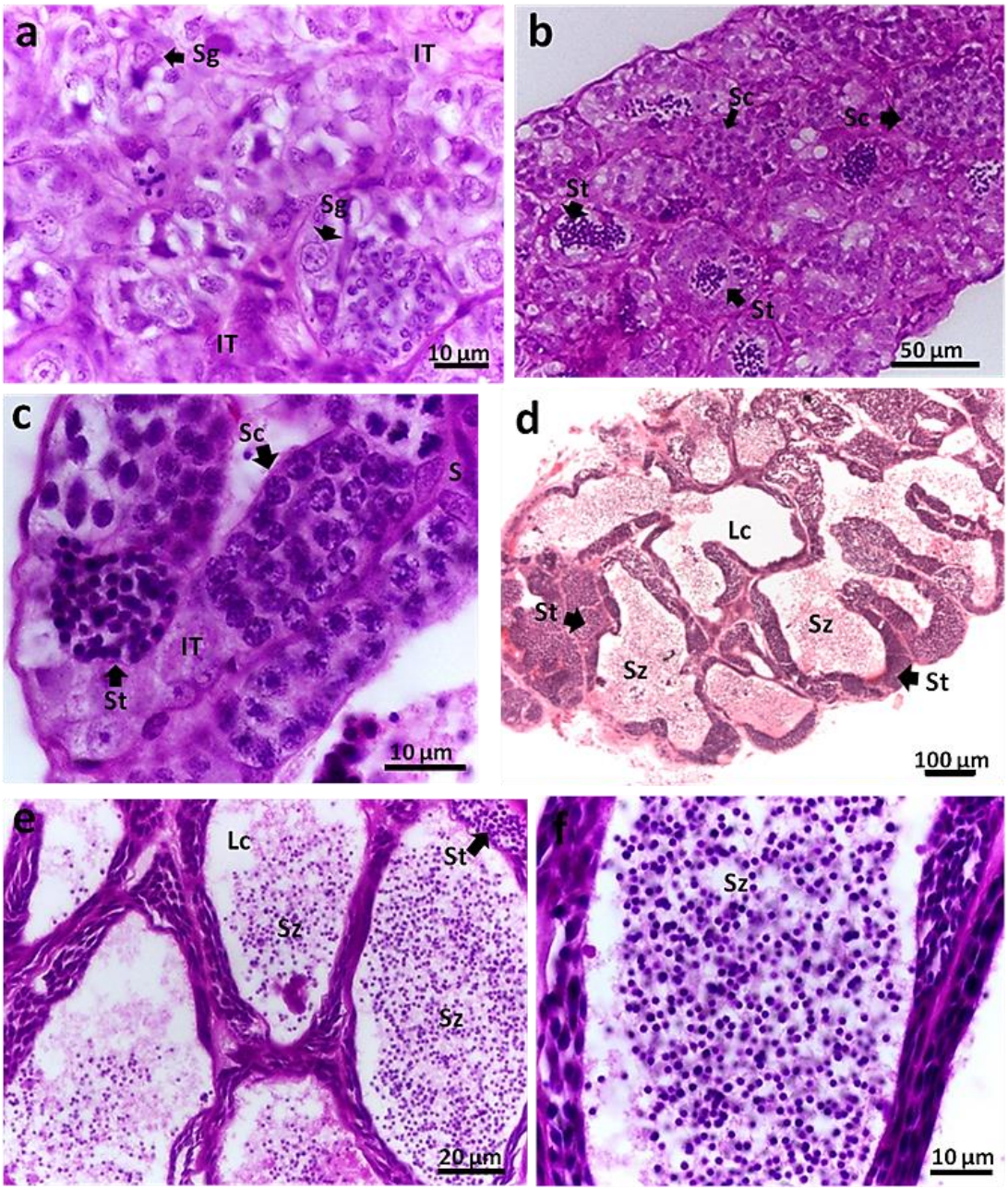

Figure 3. Histological sections showing the maturation phases and the stages of cellular development in the testes of *Pseudotocinclus tietensis*. (a) Immature, (b-c) Developing, (d) Spawning capable, and (e-f) Spawning capable (Sg=spermatogonial, IT=interstitial tissue, S=Sertoli cells; Sc=spermatocytes; St.=spermatids; Sz=sperm, Lc=luminal cavity).

body weight of 1.2-2.9 g (2.0±0.4 g). Five specimens with total length below 4 cm (3.5±0.3 cm) and body weight of 1 g (1±0.2) that we were not able to remove their gonads, discarded.

The testes were paired structures located along the bladder and connected to the dorsal wall of the coelom. The posterior end of the organs continued through a common duct that opened to the outside via the urogenital papilla. The testes were surrounded by the tunica albuginea, from which septa of connective tissue were directed towards the internal part of the organs.

Microscopically, the testes were classified as unrestricted spermatogonial testes, anastomosing tubular type, composed of numerous tubules containing the germinal compartment, separated by interstitial tissue (Fig. 3a, c). The germinal compartment was surrounded by Sertoli cells (Fig. 3c) and contained seminiferous tubules and cysts containing germ cells (Fig. 3d, e). It was possible to

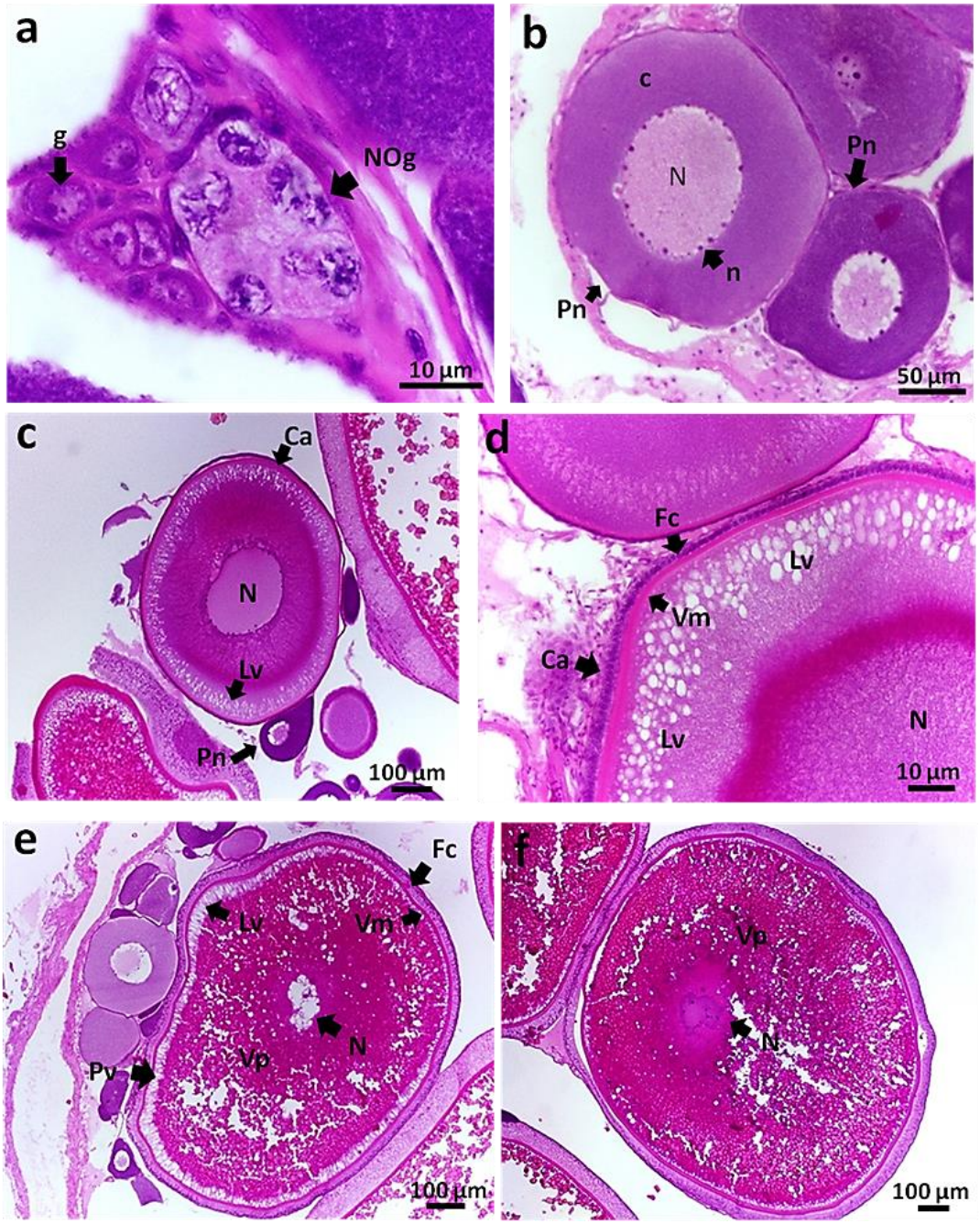

Figure 4. Histological sections showing the maturation phases and stages of development of gametes in the ovary of *Psudotocinclus tietensis*. Immature: oocytes in stage I (Chromatin nucleolar, a) and II (perinucleolar, b-c); Developing: oocytes in phase III (cortical alveoli, c-d) and IV (Vitellogenic, e); Spawning Capable: phase V oocytes (Ripe, f) (Cn=Chromatin nucleolar, Nog="nests" of oogonia, Og=oogonia, Pn=perinucleolar, c=cytoplasm, N=nucleus, n=nucleolus, Ca=Cortical alveolar, Lv=lipid vacuoles, Zp=Zona pellucida, Fc=follicular cells, Pv=Protein vitellogenesis).

establish three stages of testicular development during the annual cycle: Immature, Developing and Spawning capable. These stages of testicular development were distinguished due to the following characteristics:

Immature: Testes with a great amount of interstitial tissue and spermatogonia predominated within the germ cells (Fig. 3a). Spermatogonia were larger cells among the germline, with a well-developed central nucleus, prominent nucleoli and a clear but abundant cytoplasm (Fig. 3a).

Developing: The interstitial tissue diminished and the number of spermatogonia was reduced (Fig. 3b). Spermatocytes and spermatids (Fig. 3b, c) were

Table 1. Ovarian parameters of *Pseudotocinclus tietensis* during late maturation phases.

Maturation Phase	Gonads weight (g)	GSI	Absolute Fecundity	Relative Fecundity
Developing	0.04±0.07[a]	1.8±0.24[a]	586.1±67.36[a]	861.5±71.75[a]
Spawning Capable	0.24±0.06[b]	7.4±1.80[b]	1795.3±210,57[b]	2190.1±233.27[b]

Values (mean ± standard deviation) followed by different letters are statistically different among maturation phases ($P \leq 0.05$).
GSI: gonadosomatic index

located within the intratubular cysts. Spermatocytes were smaller than spermatogonial cells, contained a clear cytoplasm and a large central nucleus and were organized as groups of rounded cells (Fig. 3b, c). The spermatids were smaller arising from the division of spermatocytes that were present in large numbers within the cysts (Fig. 3b). These spermatids are completely spherical with little cytoplasm and a rounded nucleus with a very compact chromatin (Fig. 3c).

Spawning Capable: At this stage, the interstitial tissue was scarce and spermatid cysts were still present (Fig. 3d, e), but in small numbers. The spermatocysts were opened to release the spermatozoa (Fig. 3d, e) in the luminal cavity, the main locale of spermatozoa (Fig. 3e, f).

The microscopic classification of the ovaries was based on cytologic features of the developmental stages of the follicles and structures. The development resulted in changes of the germ cells that characterized the different phases of gonadal maturation. The ovaries were paired organs located laterally to the gas bladder and connected to the dorsal wall of the coelom. Each ovary showed free ends, and the rear extremity continued through a common duct that was opened to the outside through the urogenital papilla. Histological analysis allowed for identification of three phases of gonadal development in females throughout the annual cycle: Immature, Developing and Spawning capable. These stages were observed in all seasons. The ovaries presented lamellae filled with oocytes and surrounded by follicular cells. It was possible to identify five stages of oocyte development, described below, together with the description of gonadal development phases:

Immature: This is the initial phase of gonadal development that includes the following stages of oocyte development: (1) Chromatin nucleolar: These cells were grouped into nests (Fig. 4a), embedded into the lamellae, and were either oogonia or oocytes in the initial phases of development. The cytoplasm of the cells was slightly stained, and each cell had a large nucleus that was spherical and intensely basophilic, with one nucleolus in the central position (Fig. 4a). (2) Perinucleolar. The oocytes detached themselves from their nests and began a growth phase; the basophilic cytoplasm was developed, and the nucleus contained numerous rounded nucleoli of different sizes, located on the periphery of the nucleus (Fig. 4b).

Developing: During this phase, two stages of oocyte development are found: (3) Yolk vesicle formation (Cortical alveolar), in which oocyte size increased and the oocytes entered the phase in which endogenous vitellogenesis occurs, characterized by intense lipid deposition, which was represented by vacuolization of cytoplasm (empty spherical structures located close to the cell membrane resulting from the processing of histological analyses) (Figs. 4c, d). The cytoplasm became acidophilic. Zona pellucida became evident. (4) Vitellogenic (yolk) characterized by the deposition of highly acidophilus protein granules (Fig. 4 e). This deposition occurred in the form of platelets from the periphery of the oocyte cytoplasm, causing a further increase in size.

Spawning Capable: In this phase, germ cells were present in the ovaries at most stages of development, as described above. Additionally, at stage (5), Rip (mature) oocytes were present, with larger and more numerous protein granules (Fig. 4f). Lipid vesicles on the periphery were still present but reduced. The end of this phase is characterized by the migration of the nucleus from the core to the periphery of the cytoplasm (Fig. 4f), a process known as germinative vesicle breakdown (VGBD).

Table 2. Linear correlation between absolute fecundity (F_{abs}) with body mass (BM), total length and gonad weight of *Pseudotocinclus tietensis*.

	Relationship	α	b	r^2
Body weight	F_{abs}=33.99BM–18.96	18.96	33.99	0.361
Total Length	F_{abs}=46.43TL–253.27	253.27	46.43	0.429
Gonad Mass	F_{abs}=269.36GM+40.98	40.98	269.36	0.581

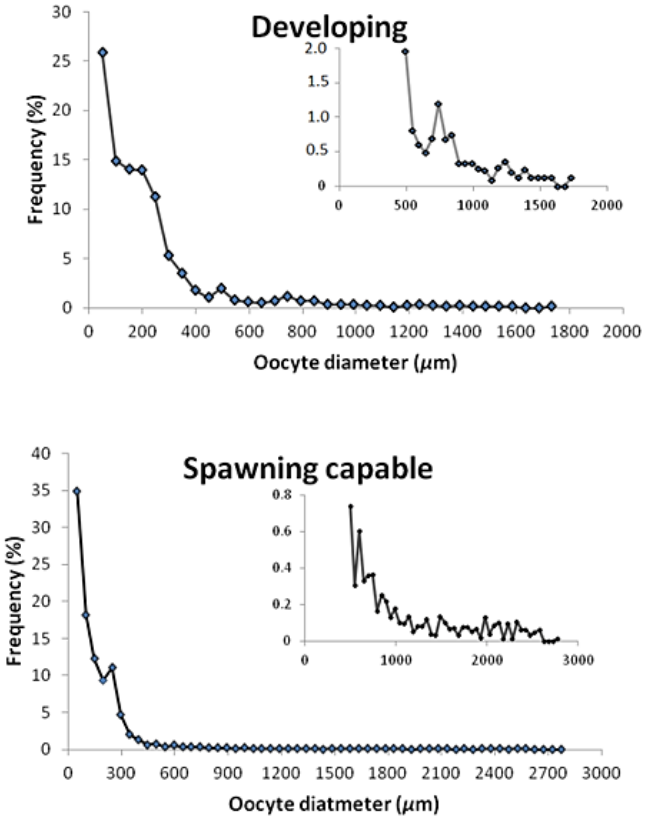

Figure 5. Frequency distribution of oocyte diameters in *Pseudotocinclus tietensis*. (A) Developing and (B) spawning capable.

There was no relationship between body weight and stage of gonadal maturation; the animals at all phases of gonad development presented no difference in body weight. However, there was a relationship between the total length and the phase of gonadal development (P=0.024), showing that the animals in the developing phase were shorter. The data in Table 1 show that females at the spawning capable phase presented with larger ovaries and GSI calculations than those in developing phase (P=0.006 and P=0.012, respectively). Furthermore, the absolute number of oocytes per female (absolute fecundity) and the ovarian mass were also higher in the spawning capable females than the Developing females (P<0.001 for both parameters).

The frequency distribution of oocyte diameters, calculated based on the average value of females in each maturation stage is presented in Figure 5. This pattern of oocyte diameter distribution defines *P. tietensis* ovaries as asynchronous. Vitellogenic oocytes were defined as those with a diameter greater than or equal to 297 μm. Below this diameter, all oocytes are still at the cortical alveolar or perinucleolar stages. Based on these analyses, the absolute fecundity of *P. tietensis* was 64-306 oocytes, all which were eliminated every spawning.

The relationships between fecundity and body weight, gonad weight and total length are presented in Figure 6a, b, and c, respectively. Table 2 presents the correlation between fecundity and body weight, total length and gonad weight. The linear correlation showed that fecundity in *P. tietensis* was more related to gonad weight than to body weight or total length. These data were corroborated the fact that body mass did not vary with the maturation phase.

Discussion

The results showed that *P. tietensis* presents an asynchronous oocyte development, with females in the capable spawn phase found throughout the year. Even considering the conservation status of the species and the difficulties in capturing the fish, we realize that the number of specimens captured in this study is reduced, but enough to understand the gonad development.

According to Grier et al. (1980), the testicular structure of teleosts can be divided into unrestricted spermatogonial (spermatogonia are distributed over the entire length of the seminiferous lobules) and restricted spermatogonial (spermatogonia are confined to the distal tubule terminal). In *P. tietensis,* testicular structure was identified as unrestricted spermatogonial type, with spermatogonia present

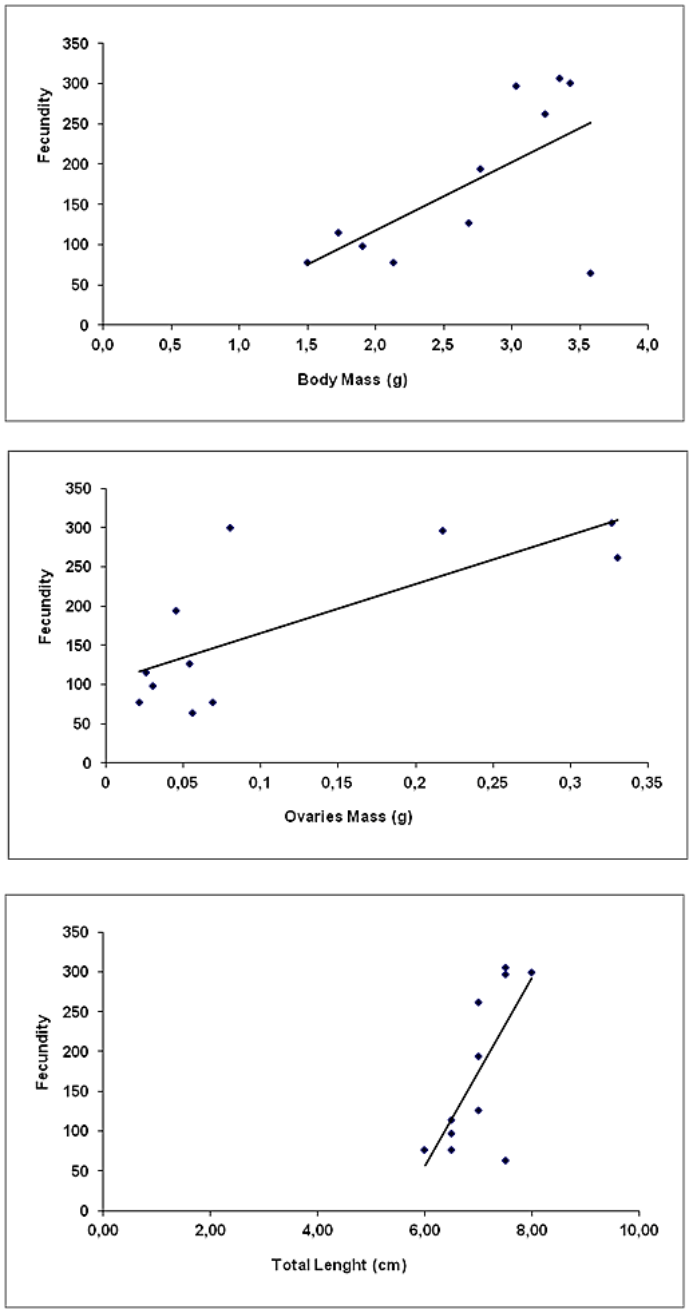

Figure 6. The relationship between fecundity and body weight (A), fecundity and total length (C), and fecundity and gonad mass (B) in *Pseudotocinclus tietensis* by considering all animals sampled.

across the length of the seminiferous tubules, forming germinal cysts all over the walls of the tubules, according to Grier (1996).

The traditional method for classification of the reproductive stages establishes four basic stages: resting, maturing, mature and spent. The criteria for defining these stages and appropriate terminology vary widely between authors. Menezes et al. (2000) classify *Pseudotothyris obtuse*, a species from the same subfamily (Hypoptopomatinae) of *P. tietensis*, as having seven microscopic stages of development of the testes. In the present study, the testes were classified into only three stages, according to the recent criteria established by Brown-Peterson et al. (2011) that standardizes the classification of the reproductive phases. Most of the traditional studies that classify testicular maturation phases consider only four phases: resting, maturing, mature and regression/exhausted. This four-phase classification scheme is based on the principle that a smaller number of stages should decrease the chances of errors in classification. However, it is important to highlight that the regressing phase was not found in *P. tietensis,* suggesting that either the gametes can be successfully released in the regions that fish were sampled or that the regressing and regenerating phases were so fast that the number of samplings did not allow the capture of animals in these phases.

The histological examination of the ovaries provides information about transitions and structural and morphological changes that occur during oocyte development, considering specific stages through which the oocytes pass during their maturation (Dias et al., 1998). Researchers studying the classification of maturation phases of *P. obtusa* females (Menezes et al., 2000), as well as in males, identified seven phases of ovarian development. Following the classification of maturation phases proposed by Brown-Peterson et al. (2011) for *P. tietensis*, we found three phases of maturation. However, because parental care is common in loricariids and it is believed that females could be burrowed by keeping the eggs, there is the potential that females are inaccessible for sampling at the regressing/spent phase.

The fecundity, which was found to be between 64 and 306, was calculated using the oocytes that were ready to be released in the next spawning. These oocytes included those that were in the end of maturation phase and those that were already mature. A wide range in fecundity is common in asynchronous species, as observed in *Oreochromis niloticus* (Duponchelle et al., 2000), which was found to have a total fecundity ranging from 149 to 2797. The number

of oocytes varied mainly in relation to the gonadal mass; for the length and body mass, there was no correlation with the number of oocytes to be ovulated. At all stages of maturation, there are a large number of residual oocytes (oogonia each with a small diameter that would be recruited in the next reproductive cycle.

The fact that *P. tietensis* lacks one unique batch of oocytes with a large diameter but contains many small batches of oocytes above 297 μm that would be ovulated in the next ovulation cycle characterizes the species as a multiple spawner. These data were corroborated by the presence of gametes in various stages of development in the gonads throughout the annual cycle, with no predominance of one cell type. However, the calculation of fecundity in asynchronous species presents some problems regarding the choice of the batch of the smaller oocytes to be ovulated. According to Narahara et al. (1989), the choice of this batch, assumes that an oocyte at this diameter will be successfully ovulated. However, many processes are known to trigger the reabsorption of oocytes, which can impair ovulation.

In conclusion, even considering the threatened status of *P. tietensis*, the ability to follow the gonadal development of a representative number of males and females throughout the annual cycle allows for classification of the species as a multiple spawner, with a variable number of oocytes to be ovulated at each spawn. These data will help fill current gaps regarding the biology and reproductive strategy of this species, which as of yet is still not known (www.fishbase.org). We suggest that conservation attempts for this species could be successful using proper management practices, such as the establishment of pairs of fish in fish farms and ongoing supervision of reproductive behavior throughout the year.

Acknowledgements

The authors would like to thank the employees of the Ponte Nova Fish Farm for providing the facilities for sampling in the Upper Tietê River Basin and S. Batlouni for his help with the testes analysis. This work was supported by an undergrad student fellowship (CNPq/PIBIC).

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Int. J. Aquat. Biol. (2017) 5(3): 218-227
E-ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

چکیده فارسی

زیست شناسی تولید مثل گونه در معرض تهدید *Pseudotocinclus tietensis* (**Siluriformes: Loricariidae: Hypoptopomatinae**)

جاندیر آ. رودریگز-فیلهو[۱،۴]، رناتو م. هونجی[۲]، پائولو ح. ملو[۲]، ماریو آی. بورلا[۳]، الکساندر و.س. هیلسدروف[۱]، رناتا ج. موریرا [۱،۲]*

[۱] دانشگاه د موگی داس کروزس، آو. دکتر کاندیدو د آلمیدا ای سوزا، ۹۷۰-۰۸۷۰۱، برزیل.
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چکیده:

ماهی بومزاد *Pseudotocinclus tietensis* ساکن ناحیه بالادست حوضه رودخانه تیته بوده و به‌عنوان یک گونه آسیب‌پذیر طبقه‌بندی می‌شود. زیست‌شناسی تولیدمثل این گونه تاکنون ناشناخته است، بنابراین ما استراتژی تولیدمثل و توسعه گنادی آن را در طی چرخه تولیدمثل سالانه مورد بررسی قرار دادیم. نمونه‌ها در طی یک‌سال جمع‌آوری شدند، بافت‌شناسی گناد، هم‌آوری و قطر اووسیت آن‌ها مطالعه شد. سه مرحله بلوغ گنادی در نرها و ماده‌ها (نابالغ، در حال توسعه و قادر به تخمریزی) یافت شد و مراحل توسعه گامت‌ها در هر مرحله مشخص شد. در بیضه‌ها، چهار مرحله توسعه گامتی شامل اسپرماتوگونیا، اسپرماتوسیت، اسپرماتید و اسپرماتوزوآ تشخیص داده شد. در طی مرحله اسپرم‌ریزی، اسپرماتوزوآ به درون لوله لومن آزاد شده و سپس از طریق مجاری وابران له خارج هدایت می‌شوند. در تخمدان‌ها ، پنج مرحله توسعه گامتی شامل هسته کروماتینی، پیش هستک، تشکیل وزیکول زرده، زرده‌زایی و رسیدگی تشخیص داده شد. حداقل قطر تخمک ۲۹۷ میکرومتر و هم‌آوری مطلق ۶۴ تا ۳۰۶ بود. نرهای دارای اسپرماتوزوآ در لوله لومن و ماده‌ها با تخمک‌های زرده‌سازی شده و رسیده در طول سال یافت شدند. ماهی *P. tietensis* دارای تخمدان با توسعه غیر همزمان بوده و گامت‌های با قابلیت لقاح در طول چرخه سالانه می‌توانند خارج شوند.

کلمات کلیدی: چرخه تولیدمثل، گنادها، *Pseudotocinclus tietensis*، هم‌آوری، کاسکودو.