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Female germ cell renewal during the annual reproductive cycle in Ostariophysians fish

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ABSTRACT

The objective was to characterize female germ cell renewal during the annual reproductive cycle in two species of ostariophysian fish with distinct reproductive strategies: a siluriform, *Pimelodus maculatus*, in which oocyte development is group synchronous and the annual reproductive period is short; and a characiform, *Serrasalmus maculatus*, with asynchronous oocyte development and a prolonged reproductive period. These reproductive strategies result in fish determinate and indeterminate fecundity, respectively. Annual reproductive phases were determined by biometric and histologic analysis of gonads and interpreted according to new proposals for phase classification and stages of oocyte development (with special attention to germinal epithelium activity). Histologically, there were two types of oogonia in the germinal epithelium: single oogonia and those in mitotic proliferation. Oogonial proliferation and their entry into meiosis resulted in formation of cell nests (clusters of cells in the ovarian lamellae). Morphometric analysis was used to estimate germ cell renewal. Based on numbers of single oogonia in the lamellar epithelium, and nests with proliferating oogonia or early prophase oocytes throughout the annual reproductive cycle, oogonial proliferation and entrance into meiosis were more intense during the regenerating phase and developing phase, but decreased sharply ($P < 0.05$) during the spawning-capable phase. Oogonial proliferation gradually recovered during the regressing phase. We concluded that, independent of species or features of the reproductive cycle, germ cell renewal occurred during the regenerating phase, ensuring availability of eggs for the spawning event.

1. Introduction

In most Teleostei, reproduction is an annual, cyclic event. During each breeding season, thousands to millions of eggs are produced during successive spawning events, depending on species, life history, and body size. In contrast to most mammals that have a lifetime determinate reproduction, in fish, the renewable source of the germ cells underlies nondeterminate lifetime reproduction, during a female fish's reproductive life history. The renewable source of oocytes and eggs that allows female teleosts to produce new follicles during their entire reproductive life is the germinal epithelium that borders the ovarian lamellae [1,2].

The lamellar germinal epithelium houses oogonia [2–6]; stem cells of the female germinal lineage reside within the lamellar epithelium [4]. Within this germinal epithelium oogonia proliferate and form germline cysts. Formation of these cysts is conserved throughout vertebrate evolution [7–12]. By definition: "Cysts are groups of cells that form

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from a single founder cell. The founder cell undergoes synchronous mitotic divisions that are followed by incomplete cytokinesis to form a cluster of 2^n cells that are interconnected by intercellular bridges" (see [13] for review). It is inside cysts that oogonia enter meiosis, giving rise to oocytes [2,3,6]. Proliferation and differentiation of oogonia forms nests (cell clusters in the epithelium) [14]. Meiosis progresses and subsequently arrests in diplotene of the first meiotic division. Diplotene oocytes become progressively surrounded by prefollicle cells during folliculogenesis. At the completion of folliculogenesis, prefollicle cells become follicle cells when they, and an oocyte, are surrounded by a basement membrane, becoming separated from the cell nest to form a discrete ovarian follicle [2,3,6]. Near the end of folliculogenesis, the follicle basement membrane is encompassed by cells from the ovarian stroma that form a theca. Together, the follicle, basement membrane and theca form a follicle complex in which the oocyte develops [2,15–17].

Renewal of gametes, their development, differentiation, maturation, and release, result in alterations of gonadal features which distinguish phases of the annual reproductive cycle in teleostean fishes [18]. Recognition of reproductive phases constitutes fundamental knowledge of reproductive biology in fish and is applied in management of fish stocks. However, in contrast to males, recognition of female reproductive phases usually does not consider germinal epithelium activity. Determination of the reproductive phases of the females is based on various stages of oocytes present in the ovarian lamellae, whether they are in primary or secondary growth, maturation, or undergoing ovulation [18]. Remarkably, dynamics of female germ cell renewal throughout the reproductive cycle remain, for the most part, unknown.

In the present study, activity of the female germinal epithelium was characterized in two species of ostariophysian fish with distinct reproductive strategies: a Siluriformes, *Pimelodus maculatus*, and a Characiformes, *Serrasalmus maculatus*. In *P. maculatus*, oocyte development is group-synchronous and the annual reproductive period is short [19–21], and in *S. maculatus*, oocyte development is asynchronous, and the reproductive period is prolonged [22–24]. These two types of reproductive strategies result in a fish of determinate and indeterminate fecundity, respectively

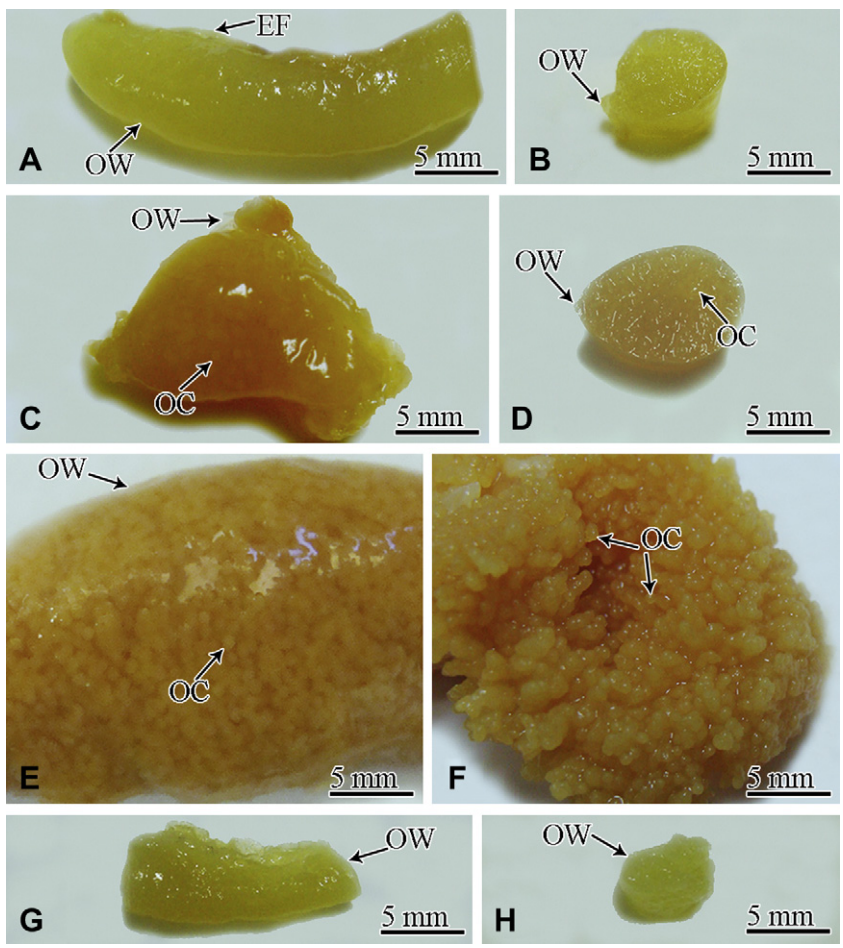

Fig. 1. Macroscopic features of the *Pimelodus maculatus* ovary. (A and B) Medial portion of an ovary in regenerating phase. (C and D) Cranial portion of an ovary in the developing phase. Developing oocytes were visible. (E and F) Medial portion of an ovary in spawning-capable phase. Oocytes of various sizes were present. (G and H) Caudal portion of an ovary in the regressing phase. EF, layer of external fat; OC, oocyte; OW, ovarian wall.

[25–27]. The Ostariophysi constitutes a basal group in the Teleostei, comprised of the Orders Cypriniformes, Characiformes, Siluriformes, and Gymnotiformes. Characiformes and Siluriformes are the predominant fish in the continental fresh waters of Central and South America. Among them are large species with great economic importance in Neotropical regions [28].

2. Materials and methods

2.1. Fish

Mature female *P. maculatus* and *S. maculatus* were collected monthly, as follows: (1) Jurumirim reservoir, Alto Paranapanema River, São Paulo State, Brazil, from January

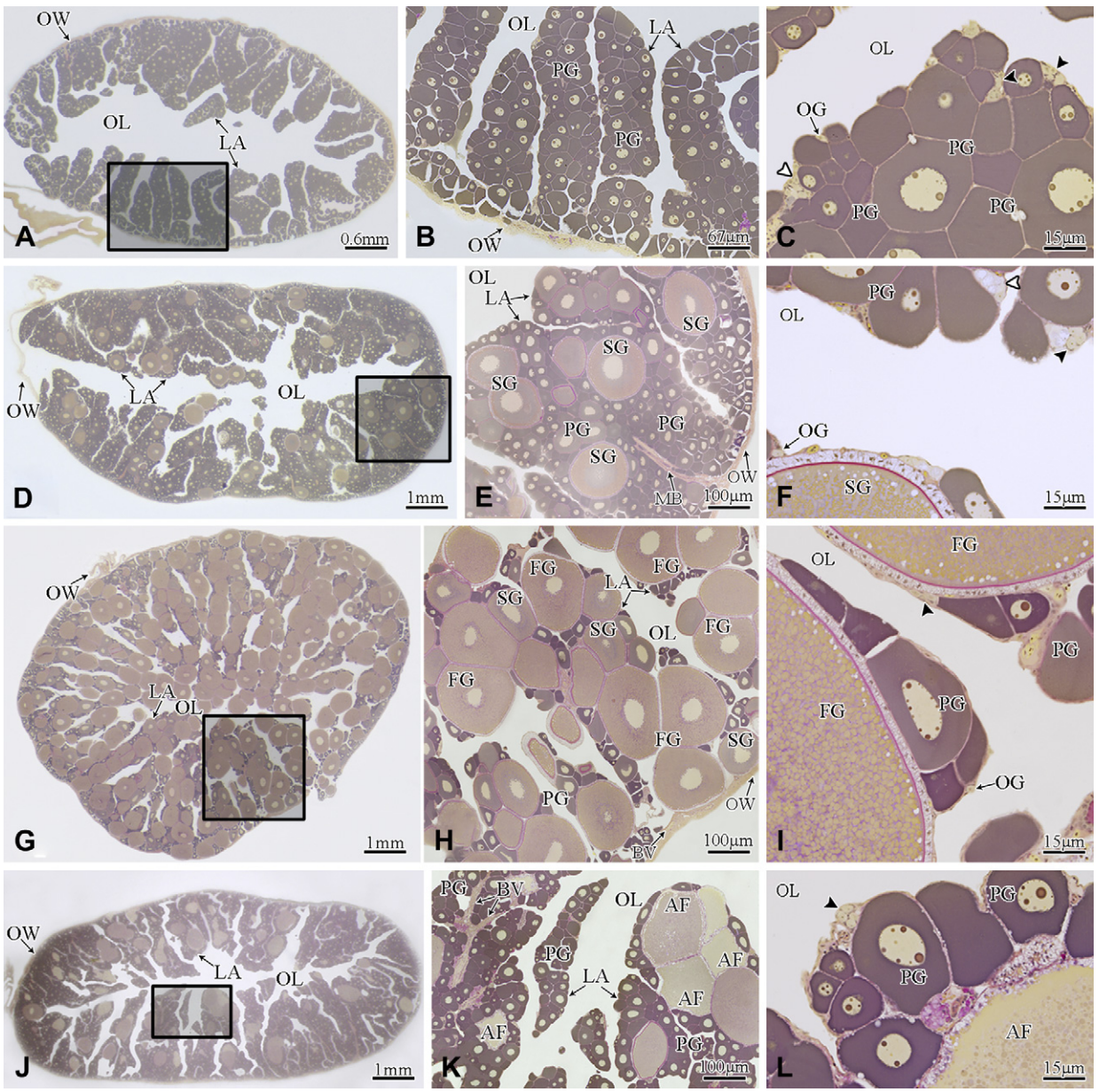

Fig. 2. Phases of the reproductive cycle in *Pimelodus maculatus*. (A) Ovary in the regenerating phase. (B) Detail of the lamellae containing primary growth oocytes. (C) Detail of the epithelium; note the nest with numerous oogonia and a nest with a numerous early oocytes. Single oogonia were also present. (D) Ovary in the developing phase. (E) Detail of the lamellae containing primary and secondary growth oocytes. (F) Detail of the epithelium; note the nest with a few oogonia and a nest with early oocytes. Single oogonia were also present. (G) Ovary in spawning-capable phase. (H) Detail of lamellae containing fully grown oocytes, and secondary and primary growth oocytes. (I) Detail of the epithelium showing a single oogonium. A nest with a few oogonia was also present. (J) Ovary in regressing phase. (K) Detail of the lamellae containing atretic follicles and primary growth oocytes. Early secondary growth oocytes were absent. (L) Detail of the epithelium showing a nest with few oogonia. Nest with oogonia indicated by black arrowheads and white arrowheads indicate nest with early oocytes. AF, atretic follicle; BV, blood vessels; FG, full-grown oocyte; LA, ovarian lamellae; MB, muscle bundle; OG, oogonium; OL, ovarian lumen; OW, ovarian wall; PG, primary growth oocyte; SG, secondary growth oocyte.

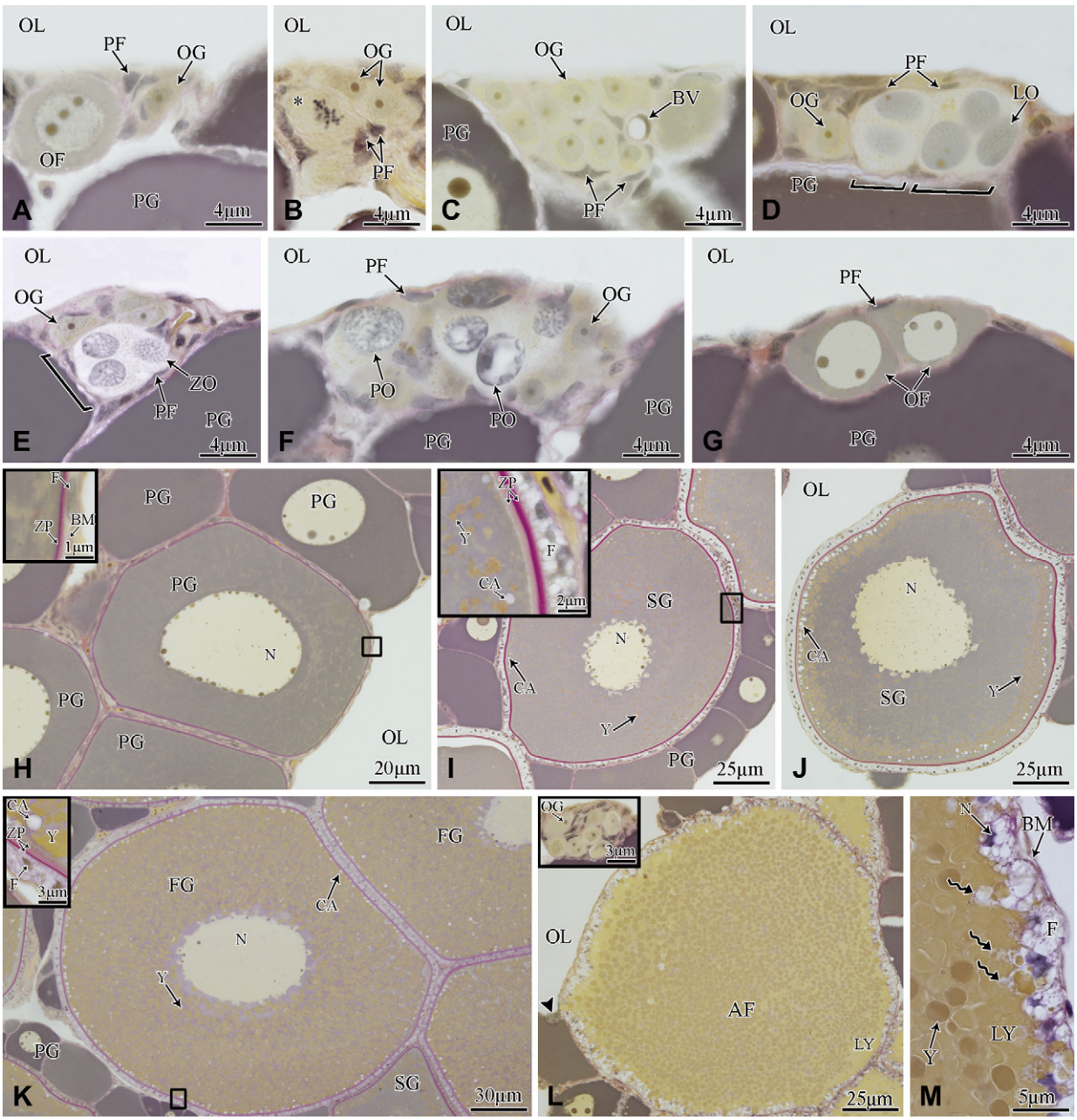

Fig. 3. Oocyte development in *Pimelodus maculatus*. (A) Single oogonium scattered in the germinal epithelium. (B) Oogonia proliferation formed cell nests. In the nest, each oogonium was wrapped by prefollicle cells, giving rise to germline cysts. Oogonium in metaphase of mitosis (black asterisk). (C) Nest with a germline cyst of oogonia. (D) In the nests, oogonia entering meiosis gave rise to germline cysts with leptotene oocytes. The nucleus of the leptotene oocytes was basophilic. Note that germline cysts with oocytes (square bracket) and others with a single oogonium coexisted in the same nest. (E) Nest with zigotene oocytes. (F) Nest with pachytene oocytes. During late pachytene, prefollicle cells extended from the cell layer encompassing the germline cysts, inserted themselves among oocytes, and progressively surrounded each one of them. (G) Ovarian follicles arrested in diplotene. Early diplotene oocytes became completely surrounded by follicle cells (former prefollicle cells), giving rise to ovarian follicles. Inside the newly formed ovarian follicle, the diplotene oocyte began primary growth and left the nest. (H) Developing primary growth oocyte. Inset: zona pellucida composed of a single thin layer. (I) Early secondary growth oocyte in which the first yolk globules and the first vesicles of the cortical alveoli appeared. Inset: zona pellucida composed of two layers. Note the hypertrophic follicular cells. (J) Developing secondary growth oocyte characterized by progressive deposition of yolk. (K) Full-grown oocyte with cytoplasm completely full of yolk and cortical alveoli forming a thin peripheral layer. Inset: zona pellucida composed of two layers. (L) Atretic follicles. Note liquefaction of yolk globules (LY). Inset: high magnification of a nest with oogonia present (black arrowhead). (M) Detail of disintegration and fragmentation of the zona pellucida. Note the phagocytic follicle cells engulfing yolk (undulated black arrows). Mitosis indicated by black asterisk; black arrowhead indicates nest with oogonia; square brackets indicate germline cysts; and undulated black arrows indicate fragmentation of the zona pellucida. AF, atretic follicle; BM, basement membrane; BV, blood vessels; F, follicle cell; FG, full-grown oocyte; LO, leptotene oocytes; LY, liquefaction of the yolk globules; N, nucleus; 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; ZO, zigotene oocytes; ZP, zona pellucida.

1996 to December 1997; and (2) Piracicaba River, São Paulo State, Brazil, from January 2004 to December 2005. During the first sampling period (from January 1996 to December 1997), 50 *P. maculatus* and 55 *S. maculatus* were collected. During the second sampling period (January 2004 to December 2005), 82 *P. maculatus* and 38 *S. maculatus* were collected (total of 255 females). All specimens were anesthetized with 0.1% benzocaine and euthanized, in accordance with institutional animal care protocols and approval. For each fish, the total length was measured to the nearest 0.5 cm and total weight was determined to the nearest 0.1 g. Soon after death, ovaries were removed, weighed (nearest 0.01 g), and then fixed in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 mol/L, pH 7.2) for at least 24 hours.

2.2. Gonadosomatic index

During the first sampling period (from January 1996 to December 1997), the gonadosomatic index (GSI) was calculated for each fish (50 *P. maculatus* and 55 S. *maculatus*) and was used to establish the gonadal maturation curve, to estimate the breeding season. The index is given by GSI = GW/W × 100, where GW = gonad weight, and W = total weight. These data were grouped and analyzed (one-way ANOVA, followed by Tukey test) separately by seasons and phases of the reproductive cycle.

2.3. Histologic analysis

For light microscopy, the right ovaries from all 225 females (132 *P. maculatus* and 93 *S. maculatus*) were dehydrated in ethanol and embedded in Historesin (Technovit 7100), and the left ovaries were maintained in fixative. Sections (3 μm) from all right ovaries were stained with periodic-acid-Schiff/hematoxylin/metanil yellow [29]. The periodic-acid-Schiff method per se or with other dyes [29] is used to detect neutral polysaccharides. Neutral polysaccharides are stained magenta. Histologic sections of 15 ovaries of *S. maculatus* representing all phases of the reproductive cycle (three per phase) were stained with the reticulin method, which enhances basement membranes [6,30,31]. Ovaries were evaluated with a computerized image analyzer (Leica Qwin 2.5, Leica Microsystems, Heerbrugg, Switzerland).

Folliculogenesis and oocyte development were described in accordance with Grier et al. [2] and Quagio-Grassiotto et al. [6]. Fecundity was classified as proposed by Hunter et al. [25], and as used by Murua and Saborido-Rey [26] and Lowerre-Barbieri et al. [27]. Characterization of

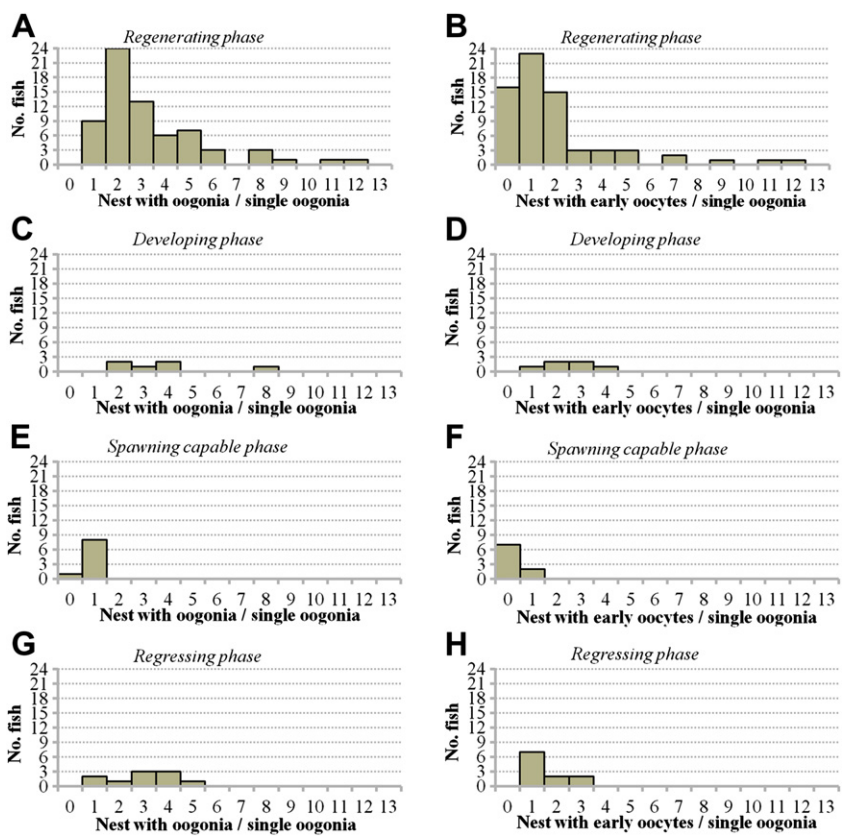

Fig. 4. Frequency of nests containing proliferating oogonia and early prophase oocyte (divided by single oogonia) by each phase of the reproductive cycle in *Pimelodus maculatus*. (A and B) Regenerating phase, N = 68. (C and D) Developing phase, N = 6. (E and F) Spawning-capable phase, N = 9. (G and H) Regressing phase, N =11.

Table 1
Frequency of reproductive phases (1996 plus 1997) and GSI (mean ± SEM) by seasons.

Species	Season	Regenerating	Developing	Spawning-capable	Regressing	GSI	No.
P. maculatus	Summer	40%	0%	40%	20%	2.32 ± 0.59^{A}	15
	Autumn	100%	0%	0%	0%	0.62 ± 0.10^{B}	12
	Winter	100%	0%	0%	0%	0.88 ± 0.07^{B}	13
	Spring	60%	20%	0%	20%	1.26 ± 0.20 (A,B)	10
S. maculatus	Summer	24%	53%	18%	6%	1.13 ± 0.24^{A}	17
	Autumn	36%	55%	0%	9%	0.69 ± 0.07^{A}	11
	Winter	8%	25%	67%	0%	3.19 ± 0.56^{B}	12
	Spring	20%	13%	60%	7%	3.43 ± 0.63^{B}	15

[A and B] Within a species, means without a common superscript differed ($P < 0.05$).

reproductive phases was performed according to Brown-Peterson et al. [18].

2.4. Morphometric analysis

For morphometric analyses, ovaries from 94 *P. maculatus* (50 from the first sampling period and 44 from the second) and 93 *S. maculatus* (55 from the first sampling period and 38 from the second) were used to enumerate sites of proliferation.

For each fish, three random, histologic sections of the right ovary were analyzed. In each of these sections, all single oogonia in the lamellar epithelium were counted. Also proliferation sites (cell nests) were counted. Numbers of nests containing proliferating oogonia, and those containing early prophase oocytes, were recorded separately. The number of nests with oogonia and those containing early oocytes were each, by their turn, divided by the number of single oogonia present in the germinal epithelium and the mean for each animal was recorded. The frequency of these nests (divided by single oogonia) was graphically represented at each phase of the reproductive cycle. Analysis of the results was performed by Kruskal–Wallis nonparametric one-way ANOVA ($P < 0.05$ was considered significant).

3. Results

3.1. Reproductive phases in P. maculatus

3.1.1. Regenerating phase

The lowest value of the GSI occurred during the regenerating phase (0.81 ± 0.05, N = 36). Anatomically, ovaries in this phase were small and oocytes were not

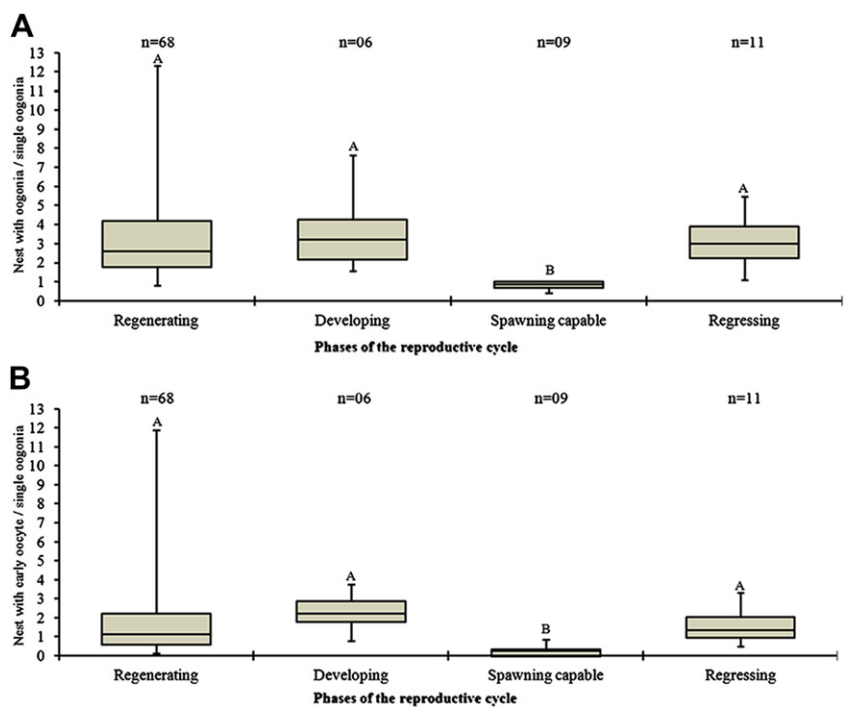

Fig. 5. Relationship between ratio of the number of nests containing proliferating oogonia (A) and early prophase oocytes (B) by the number of single oogonia in *Pimelodus maculatus* throughout the reproductive cycle. The central line of box marks the median. The bottom of the box marks the first quartile and the tip of the third quartile, demonstrating the distribution of 50% of the total sample. The upper and lower rods extend, respectively, from the first quartile value to the minimum and from the third quartile to the maximum value. Different letters indicate differences ($P < 0.05$).

grossly visible (Fig. 1A and B). In histologic sections (Fig. 2A and B), besides several forming follicles (Fig. 3G), the ovarian lamellae contained only primary growth oocytes (Fig. 3H). Scattered among them, there were a few atretic oocytes. In the regenerating phase, the number of nests with proliferating oogonia (Figs. 2C and 3B and C), and nests containing early prophase oocytes (Figs. 2C and 3D–F), reached peak values (Fig. 4A and B) in relation to each single oogonium (Fig. 3A) present in the germinal epithelium.

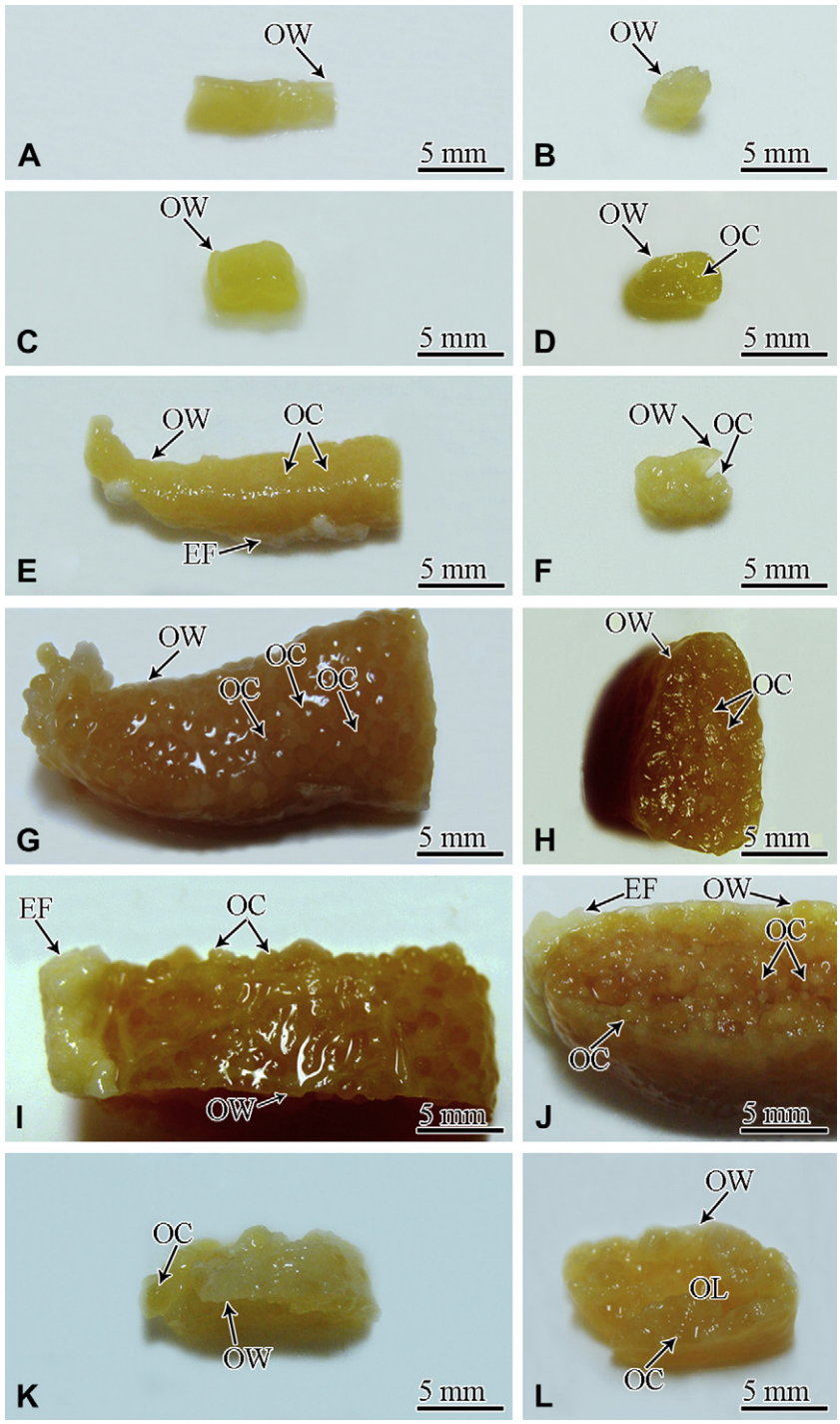

Fig. 6. Macroscopic features of the ovary in *Serrasalmus maculatus*. (A and B) Medial portion of an ovary in the regenerating phase. (C and D) Medial portion of an ovary in the early developing subphase. Small oocytes were present. (E and F) Cranial portion of an ovary in the developing phase. Secondary growth oocytes were present. (G and H) Cranial portion of an ovary in the spawning-capable phase. Oocytes (various sizes) were present. (I and J) Medial portion of an ovary in the actively-spawning subphase. (K and L) Medial portion of an ovary in regressing phase. EF, external layer of fat; OC, oocyte; OL, ovarian lumen; OW, ovarian wall.

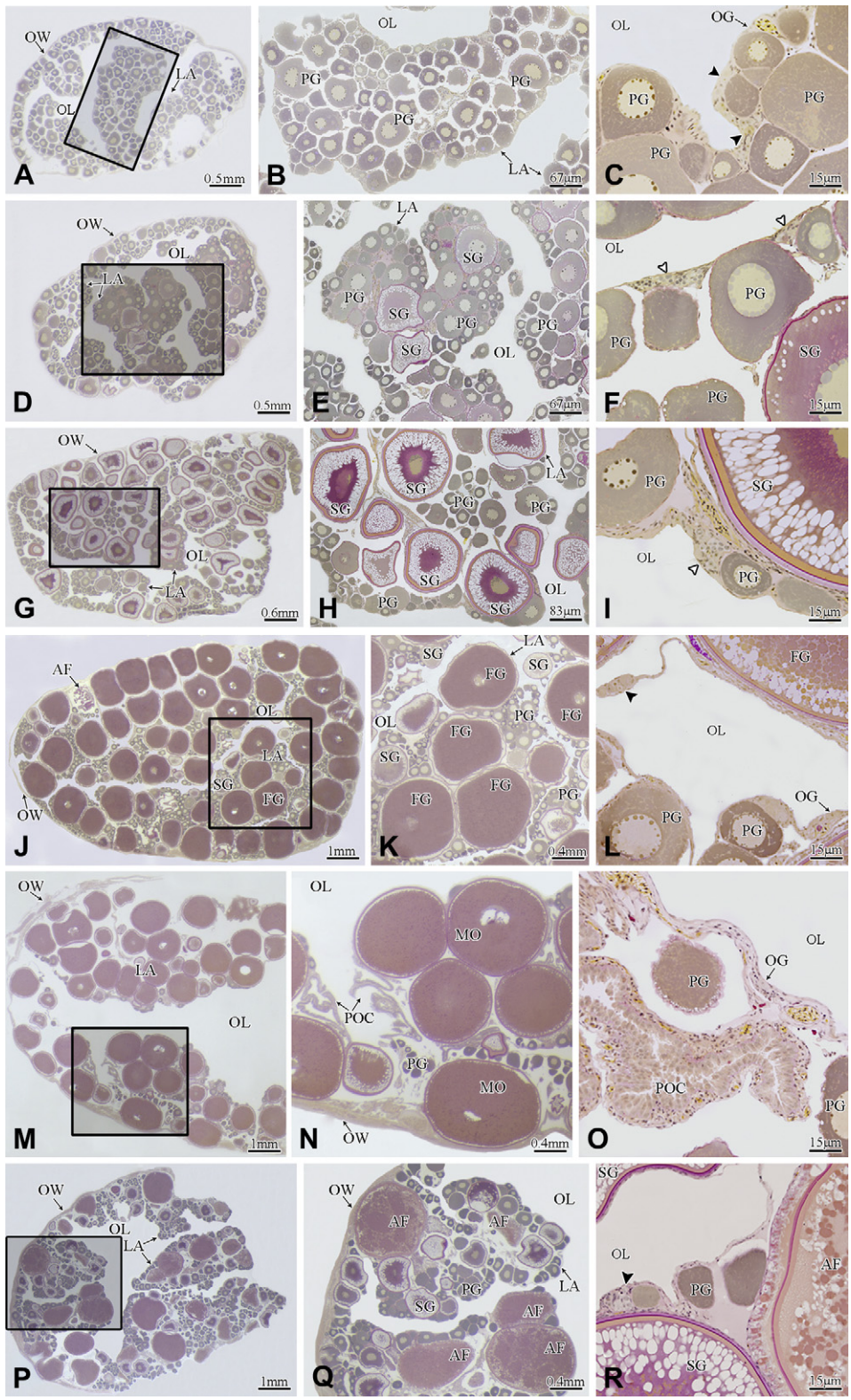

Fig. 7. Phases of reproductive cycle in the *Serrasalmus maculatus*. (A) Ovary in the regenerating phase. (B) Detail of lamellae containing primary growth oocytes. (C) Detail of epithelium containing a nest with numerous oogonia. (D) Ovary in early developing subphase. (E) Detail of the lamellae containing primary and early secondary growth oocytes. (F) Detail of the epithelium, including nest with numerous oocytes. (G) Ovary in developing phase. (H) Detail of the lamellae containing primary and early to late secondary growth oocytes. (I) Detail of the epithelium showing a nest with numerous early oocytes. (J) Ovary in the spawning-

3.1.2. Developing phase

In the developing phase, the GSI value increased (1.08 ± 0.03, N = 2). Ovaries were enlarged, and small oocytes were grossly visible (Fig. 1C and D). With the progression of the reproductive cycle, some oocytes initiated and others advanced in vitellogenesis (Fig. 2D and E). During the developing phase, together with deposition of yolk, cortical alveoli were progressively appearing and forming a discrete layer close to the oolemma (Fig. 3I and J). During the developing phase, additional ovarian follicles were formed from cell nests. In the germinal epithelium (Fig. 2F), there were more cell nests that contained proliferating oogonia than nests with early prophase oocytes or single oogonia in the epithelium (Fig. 4C and D).

3.1.3. Spawning-capable phase

The maximum value of the GSI was reached in the spawning-capable phase (4.03 ± 1.11, N = 6). In this phase, ovaries were large (Fig. 1E and F), and oocytes were readily visible. Full-grown oocytes filled most of the lamellae (Fig. 2G and H). In the ooplasm of these oocytes, yolk globules fused to each other, and the cortical alveoli formed only a single, discrete, and discontinuous layer close to the oolema (Fig. 3K). Late vitellogenic oocytes and other previtellogenic oocytes were also present (Fig. 2H). In the lamellar epithelium (Fig. 2I), the number of nests with proliferating oogonia was very close to the number of the single oogonia (Fig. 4E). Nests with early prophase oocytes were scarce (Fig. 4F).

3.1.4. Regressing phase

Postspawning, during the regressing phase, the value of GSI decreased (1.42 ± 0.33, N = 6). Regressing ovaries were flaccid, blood vessels were prominent, but oocytes were not grossly visible (Fig. 1G and H). In the lamellae, atresia of unovulated oocytes (Fig. 3L and M) coexisted with previtellogenic oocytes (Fig. 2J and K). In the lamellar epithelium (Fig. 2L), the number of nests containing oogonia increased and nests with early prophase oocytes were also observed (Fig. 4G and H).

3.2. Gonadosomatic index versus reproductive phases in P. maculatus

The GSI of *P. maculatus* (Table 1) increased progressively from winter to spring, peaked in summer, and decreased abruptly in autumn. In addition to reflecting seasonal changes in oocyte development and production, the strong decline in GSI from summer to autumn was clear evidence that the spawning season of *P. maculatus* was short and occurred mostly in the summer.

Fish in the spawning-capable phase were only identified during summer (Table 1). In addition to reproducing individuals, there were also postspawning individuals with ovaries in the regression phase and in regenerating phase. During the autumn and winter, all individuals were in the regenerating phase, whereas in the spring, individuals in the developing phase appeared. These data, in combination with GSI and gonadal histology information, confirmed that *P. maculatus* spawned during summer.

3.3. Activity of the germinal epithelium versus reproductive phases in P. maculatus

It was in the regenerating phase that the highest values of nests containing proliferating oogonia and nests containing the early prophase oocytes were present (Fig. 5). Conversely, in fish in the spawning-capable phase, the number of nests containing proliferating oogonia was nearest to the number of the single oogonia in the epithelium (Fig. 5A). Also, the number of nests with oocytes was very small in relation to all the other phases of the annual reproductive cycle (Fig. 5B). Postspawning, in the regressing phase, oogonial proliferation and entrance into meiosis were higher compared with in the spawning-capable phase, but with less intensity compared with the regenerating phase.

3.4. Reproductive phases and oocyte development in S. maculatus

3.4.1. Regenerating phase

The lowest value of the GSI occurred during the regenerating phase (0.51 ± 0.06, N = 12). Anatomically, ovaries in this phase were small, and oocytes were not grossly visible (Fig. 6A and B). In histologic sections, they had a thick wall, numerous blood vessels, and the ovarian lamellae contained only primary growth oocytes (Fig. 7A and B). Scattered among these, there were a few atretic oocytes. Follicle formation was intense and occurred in the epithelium above the basement membrane (Fig. 8G and H). As oocytes developed, the first vesicles of the cortical alveoli appeared close to the oolema (Fig. 8I). In the regenerating phase, oogonial proliferation became evident (Fig. 7C) by increasing numbers of nests containing oogonia on the basement membrane in comparison with the number of single oogonia in the epithelium (Figs. 8A–C, and 9A). Nests containing the initial prophase oocytes were also present (Figs. 8D–F and 9B).

3.4.2. Developing phase

During the developing phase, GSI increased (0.95 ± 0.10, N = 20). Progressively, ovaries enlarged and small oocytes were grossly visible (Fig. 6C–F). With the progress of the reproductive cycle, some oocytes initiated vitellogenesis (Fig. 7D–I).

capable phase. (K) Detail of lamellae containing full-grown oocytes, and secondary and primary growth oocytes. (L) Detail of epithelium (single oogonium). Nests with only a few oogonia were also present. (M) Ovary in the actively-spawning subphase. (N) Detail of the lamellae containing maturing oocytes, and secondary and primary growth oocytes. Note the postovulatory follicle complex. (O) Detail of the epithelium (single oogonium near a postovulatory follicle complex). (P) Ovary in regressing phase. (Q) Detail of the lamellae containing atretic follicles, and primary and secondary growth oocytes. (R) Detail of the epithelium (nest with only a few oogonia). Black arrowhead indicates nest with oogonia and white arrowhead, nest with early oocytes. AF, atretic follicle; FG, full-grown oocyte; LA, ovarian lamellae; MO, maturing oocyte; OG, oogonium; OL, ovarian lumen; OW, ovarian wall; PG, primary growth oocyte; POC, postovulatory follicle complex; SG, secondary growth oocyte.

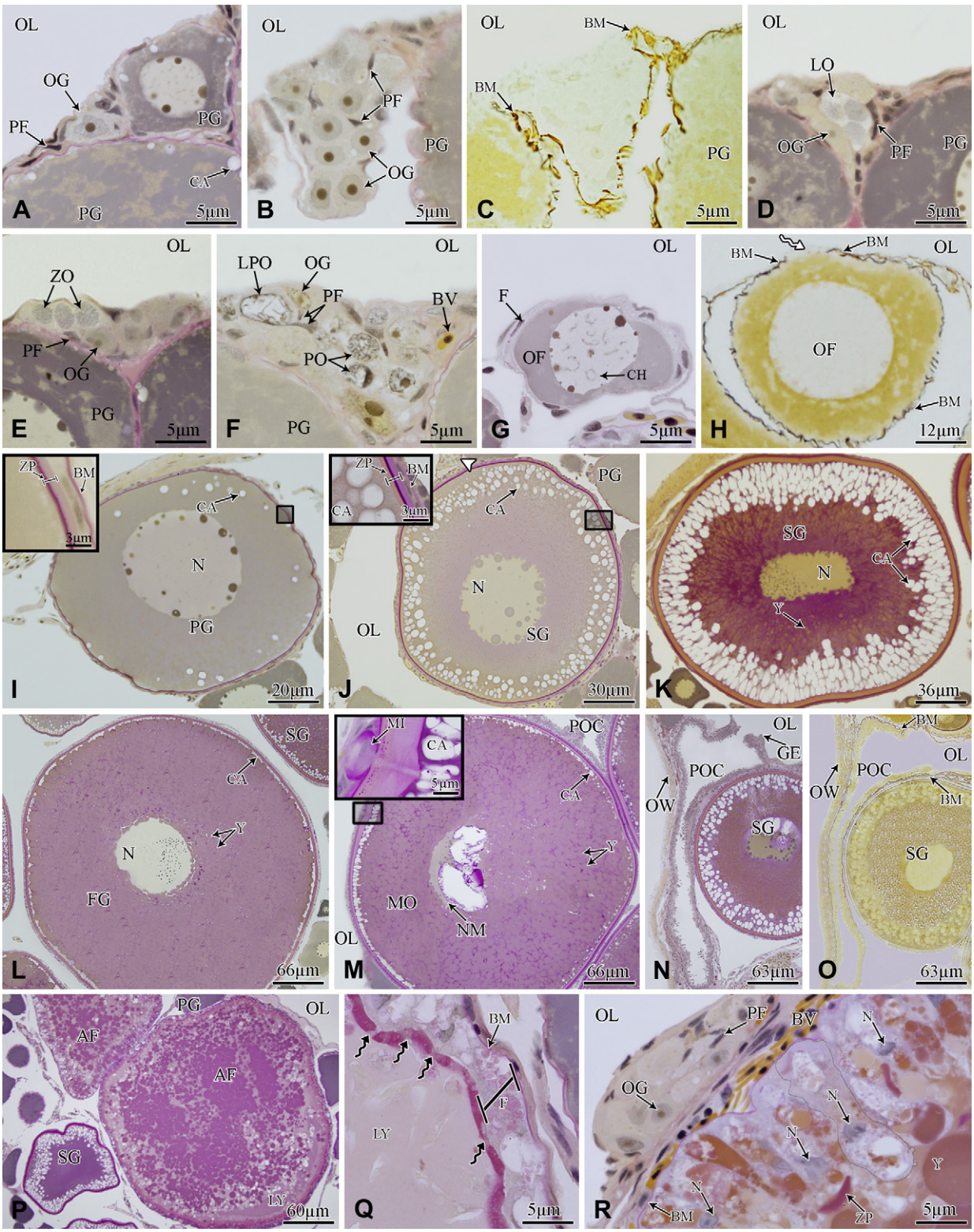

Fig. 8. Oocyte development in *Serrasalmus maculatus*. (A) Single oogonium scattered in the germinal epithelium. (B) Oogonia proliferation formed cell nests that extended into the stroma. Inside the nest, clusters of oogonia were intermingled and individually surrounded by prefollicle cells, forming germline cysts. (C) Reticulin method showing continuity of the basement membrane under the lamellar epithelium and under the cell nest. (D) In the nests, entry of oogonia into meiosis gave rise to germline cysts with leptotene oocytes. The nucleus of the leptotene oocytes was basophilic. Note that germline cysts with oocytes and others with only a single oogonium coexisted in the same nest. (E) Nest with germline cysts containing zigotene oocytes. Cysts with oogonium were also present.

In the early developing subphase (Figs. 6C and D, and 7D and E), formation of yolk globules began in oocytes, and vesicles of the cortical alveoli increased in number and size (Fig. 8J). In the lamellar epithelium (Fig. 7F), in addition to large nests containing numerous prophase oocytes (Figs. 8D–F and 9D), there were nests with proliferating oogonia (Fig. 9C) and also with single oogonia.

During the developing phase (Fig. 6E and F), in oocytes, vesicles of cortical alveoli kept increasing in number and size, formation of yolk globules progressed (Fig. 8K), and the layers of the zona pellucida became thicker. Because of asynchronous vitellogenesis, oocytes in distinct stages and steps of development were concurrently present in the lamellae, including previtellogenic ones (Fig. 7G and H). In the epithelium (Fig. 7I), nests with a few proliferating oogonia (Fig. 9E) and others with the early prophase oocytes were present (Figs. 8D and F and 9F). Also, there were single oogonia, albeit in lower numbers in relation to proliferating sites.

3.4.3. Spawning-capable phase

The maximum value of GSI occurred during the spawning-capable phase (4.46 ± 0.33, N = 20). In this phase, ovaries were large (Fig. 6G–J) and oocytes were readily visible. Full-grown oocytes filled most of the lamellae (Fig. 7J and K). In the full-grown oocyte, the ooplasm was completely full of yolk; yolk globules fused to each other, and the cortical alveoli formed only a single layer close to the oolema. The nucleus (or germinal vesicle) was situated at the center of the oocyte (Fig. 8L). Oocytes in distinct steps of vitellogenesis and others that were previtellogenic were present (Fig. 7K). In the lamellar epithelium (Fig. 7L), single oogonia (Fig. 8A) predominated and some nests with a few proliferating oogonia were present (Fig. 9G and H).

The actively spawning subphase (Fig. 6I and J) was marked by oocyte maturation and spawning (Fig. 7M and N). Nuclear (or germinal vesicle) migration in the direction of the animal pole signaled the onset of oocyte maturation (Fig. 8M). In ovarian lamellae, postovulatory follicle complexes (Fig. 8N and O) appeared concurrent with maturing and full-grown oocytes (Fig. 7M and N). Postspawning, the postovulatory follicle complex remained attached to the lamellar epithelium. It was formed by follicle cells surrounded by the theca. Also previtellogenic and vitellogenic oocytes were present (Fig. 7N). In the actively spawning subphase, the number of single oogonia in the lamellar epithelium (Figs. 7O and 8A) was high compared with the amount of nests with proliferating oogonia and nests with early prophase oocytes (Fig. 9I and J).

3.4.4. Regressing phase

Postspawning, during the regressing phase, the value of GSI decreased (0.78 ± 0.06, N = 3). Regressing ovaries were flaccid. In them, blood vessels were prominent and the ovary lumen and some oocytes were grossly apparent (Fig. 6K and L). In atretic follicles (Fig. 8P), follicle cells become hypertrophic and phagocytic (Fig. 8Q and R), and zona pellucida fragments (Fig. 8Q) and follicle cells invaded the oocyte (Fig. 8R). In the ooplasm, yolk globules disintegrated, and the yolk became liquefied (Fig. 8P and Q). In the ovarian lamellae, atretic unovulated oocytes coexisted with previtellogenic and vitellogenic oocytes (Figs. 7P–R and 8P). In the lamellar epithelium (Figs. 7R and 8R), the number of nests containing oogonia increased compared with the number of single oogonia (Fig. 9K). Nests with the early prophase oocytes were also present, with a frequency similar to single oogonia (Fig. 9L).

3.5. Gonadosomatic index versus the reproductive phases in *S. maculatus*

Values of GSI of *S. maculatus* were lowest during summer and autumn (Table 1). Furthermore, there was a major incidence of individuals in the developing phase during summer. In autumn, individuals in the developing phase were predominant, and there were no spawning-capable individuals during autumn, consistent with lower values of the GSI during summer and autumn.

In the winter, spawning-capable fish dominated (Table 1), followed by those in the developing or regenerating phases. Similarly, in the spring, spawning-capable fish dominated. These observations were in accordance with the highest values of the GSI (Table 1), which occurred in winter and spring, the breeding season of *S. maculatus*.

(F) Nest with pachytene oocytes. During late pachytene, prefollicle cells extended from the cell layer encompassing germline cysts, inserted themselves among oocytes, and progressively surrounded each one. (G) Ovarian follicles arrested in diplotene. Note the presence of chiasmata. Early diplotene oocytes became completely surrounded by follicle cells (former prefollicle cells), giving risen to ovarian follicles. Inside the newly formed ovarian follicle, diplotene oocytes began primary growth and left the nest. (H) Reticulin method showing that the ovarian follicle remained attached to the germinal epithelium throughout the basement membrane (undulated white arrow). (I) Developing primary growth oocyte in which the cortical alveolus began to be formed. Inset: zona pellucida composed of two thicker layers. (J) Early secondary growth oocyte with the first yolk globules. In it, vesicles of cortical alveoli increased. Inset: detail of the zona pellucida (three layers). (K) Developing secondary growth oocyte, with deposition of yolk and formation of cortical alveoli. (L) Full-grown oocyte with cytoplasm completely full of yolk and cortical alveoli forming a thin peripheral layer. Note the nucleus (or germinal vesicle) situated at the center of the oocyte. (M) Maturing oocyte. Note migration of the germinal vesicle or nucleus (NM) toward the animal pole. Inset: detail of the micropyle. (N) Postspawning, the postovulatory follicle complex remained attached to the lamellar epithelium. (O) Reticulin method showing the basement membrane of the postovulatory follicle complex in continuity with the basement membrane of the germinal epithelium. (P) Unovulated oocyte became atretic. Note the hypertrophic follicle cell and liquefaction of the yolk globules (LY). (Q) Detail of the disintegration and fragmentation of the zona pellucida of an atretic follicle. (R) Detail of phagocytic follicle cells engulfing the yolk (undulated black arrow). Enlarged blood vessels and a nest with oogonia were also present. Undulated black arrow indicates fragmentation of the zona pellucida; undulated white arrow indicates connection with the basement membrane of the germinal epithelium; and white arrowhead, nest with early oocytes. AF, atretic follicle; BM, basement membrane; BV, blood vessels; CA, cortical alveoli; CH, chiasmata; F, follicle cell; FG, full-grown oocyte; GE, germinal epithelium; LO, leptotene oocytes; LPO, late pachytene oocyte; LY, liquefaction of the yolk globules; MI, micropyle; MO, maturing oocyte; N, nucleus; NM, nuclear migration; OF, ovarian follicle; OG, oogonium; OL, ovarian lumen; OW, ovarian wall; PF, prefollicle cells; PG, primary growth oocyte; PO, pachytene oocyte; POC, postovulatory follicle complex; SG, secondary growth oocyte; Y, yolk globule; ZO, zigotene oocytes; ZP, zona pellucida.

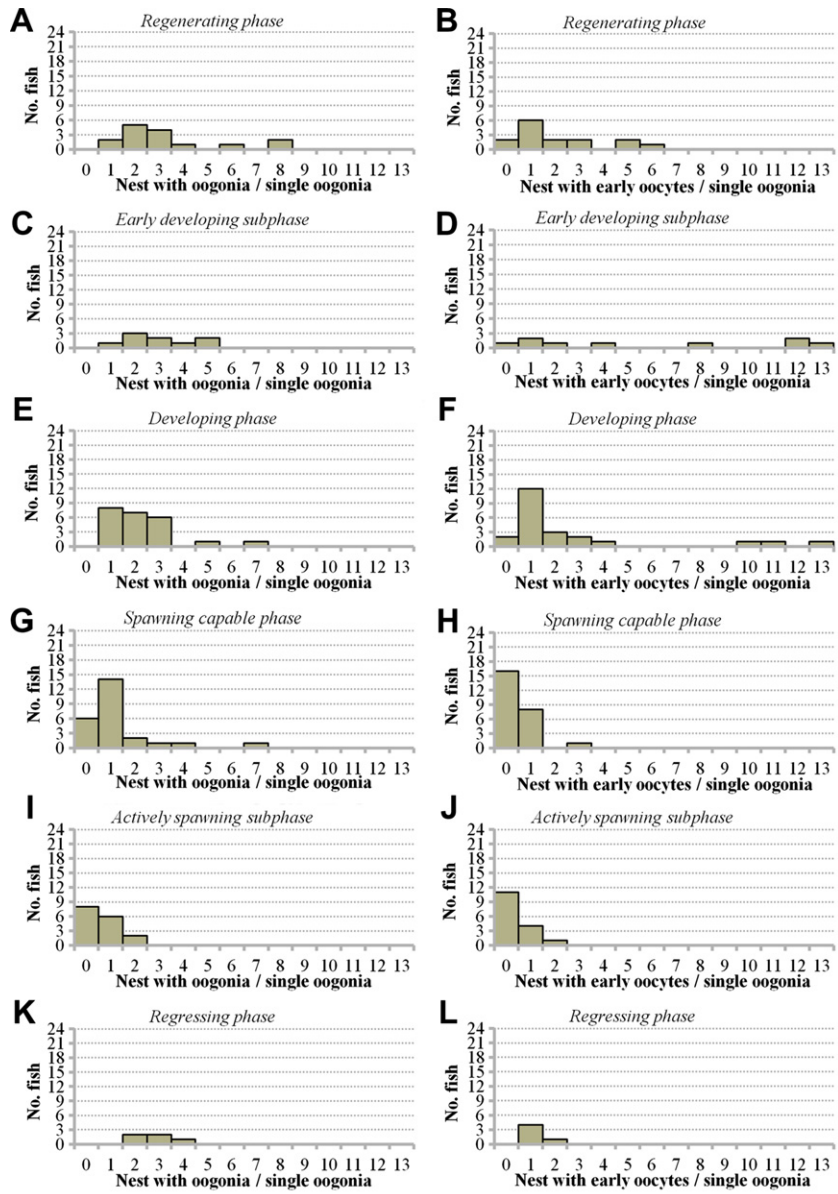

Fig. 9. Frequency of nests containing proliferating oogonia and early prophase oocyte (divided by single oogonia) during each phase of the reproductive cycle in *Serrasalmus maculatus*. (A and B) Regenerating phase, N = 15. (C and D) Early developing subphase, N = 9. (E and F) Developing phase, N = 23. (G and H) Spawning-capable phase, N = 25. (I and J) Actively-spawning subphase, N = 16. (K and L) Regressing phase, N = 5.

3.6. Activity of the germinal epithelium versus reproductive phases in S. maculatus

In *S. maculatus*, the ratio between the number of nests with proliferating oogonia and single oocytes was particularly high in the regenerating phase, signaling that oogonial proliferation was intense (Fig. 10A). It progressively decreased in the developing phase. In the spawning-capable phase and actively-spawning subphase, there were lower values of this ratio (nests with proliferating oogonia/single oogonia), indicating decreased oogonial mitotic activity in this phase, because most of the oogonia remained quiescent. Postspawning, in the regression phase, there was an increased number of nests with oogonia in the epithelium, indicating recovery of oogonial proliferation.

Nests with early prophase oocytes were always detected during the regenerating phase, the early-developing subphase, the developing phase, and the regressing phase (Fig. 10B). However, these nests were most common during the developing phase and the early developing subphase, indicating entrance into meiosis and an intense production of oocytes. In the spawning-capable phase and actively-spawning subphase, there were lower values of this ratio (nests with early prophase oocyte/single oogonia).

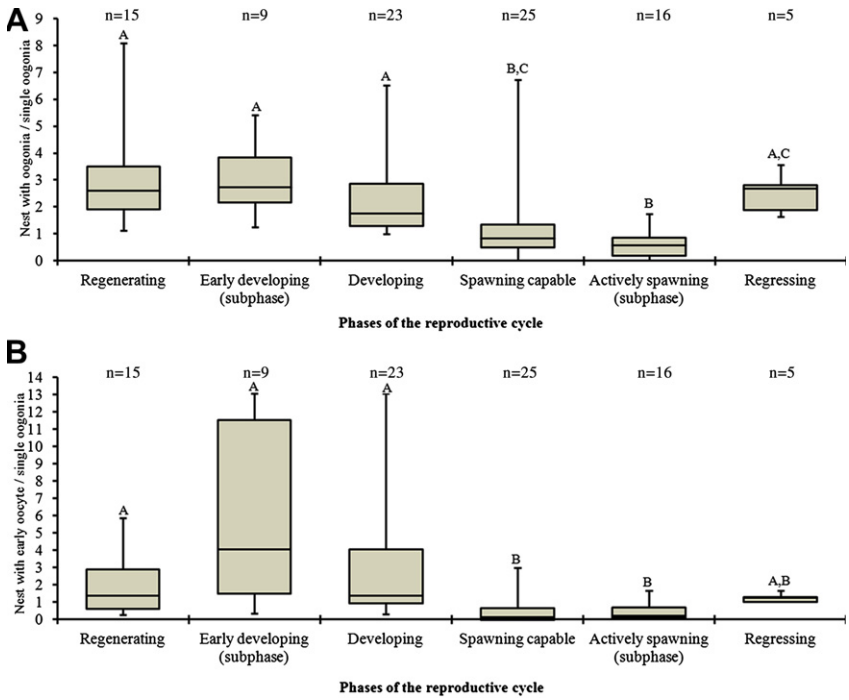

Fig. 10. Relationship between the ratio of the number of nests containing proliferating oogonia (A) and early prophase oocytes (B) by the number of single oogonia in *Serrasalmus maculatus* throughout the reproductive cycle. The central line of box marks the median. The bottom of the box marks the first quartile and the tip of the third quartile, demonstrating the distribution of 50% of the total sample. The upper and lower rods extended, respectively, from the first quartile value to the minimum and from the third quartile to the maximum value. Different letters indicate differences ($P < 0.05$).

3.7. Classification of reproductive phases in the Ostariophysi, taking into account activity of the germinal epithelium

Classification of reproductive phases [18], characterization of the stages and steps of oocyte development [2], descriptions of folliculogenesis [6], and renewal of female germ cells during the reproductive cycle (current study) are summarized (Table 2). This table completes previous studies performed primarily with species of marine Perciformes and extends their application to freshwater Ostariophysi.

4. Discussion

4.1. Germ cell renewal during the reproductive phases

Based on our analysis of female germ cell renewal, in both *P. maculatus* and *S. maculatus*, two types of oogonia were present in the ovarian lamellar epithelium. The first was a single oogonium that probably remained quiescent throughout the reproductive cycle, and the second had proliferating activity and gave rise to cell nests. According to Nakamura et al. [4], single oogonia have a prolonged cell cycle and are stem cell candidates. They also have distinct morphologic features [6]. In that regard, single oogonia are dark (ultrastructural studies) and basophilic (histologic studies), whereas proliferating oogonia are clear and understained, respectively. Dark/basophilic single oogonia are stem germ cell candidates. This is similar to the situation observed for spermatogonia in the males [33]. Proliferation of dark/basophilic oogonia gave rise to clear oogonia that can be committed to oogenesis. In females, probably during the regenerating phase, dark, single oogonia divide by mitosis. They produce only clear oogonia that proliferate and differentiate into oocytes for the next spawning season.

4.2. Origin of ovarian follicles in fish

Oogenesis in teleost fish has been a continuing focus of attention. There are considerable new data on morphologic, physiologic, and molecular aspects of the origin of oocytes and their subsequent development. Although new knowledge has greatly increased comprehension of the reproductive life history of female teleosts [2,17], identification of the locus in the fish ovary where female germ cell renewal occurs is very recent. The first information, reported in *Centropomus undecimalis*, Perciformes [1], was that the ovarian lamellar epithelium housed oogonia in adult females and was a germinal epithelium. Further studies confirmed the origin of follicles from a germinal epithelium in fishes [2,6]. Therefore, it is to be expected that the lamellar epithelium is the source of stem cell candidates of the germinal lineage in female fish [6]. Elegant studies by Nakamura et al. [4,5], using transgenic methods and clonal analysis, clearly demonstrated oogonia stem cells in the ovarian lamellar epithelium of the Beloniformes, *Oryziaslatipes*. According to these authors, oogonia stem cell proliferation formed clusters of germ cells interwoven by somatic prefollicles cells (*sox9b*-expressing cells). These

Table 2
Description of reproductive phases in female fish.

Phase	Previous terminology	Macroscopic and histologic features	Activity of the germinal epithelium
Regenerating: sexually mature, reproductively inactive	Resting, regressed, recovering, inactive	MF: Small ovaries; blood vessels might be reduced HF: PG oocytes present advanced to perinuclear step, with or without cortical alveoli; thick ovarian wall, gamma and/or delta atresia, degenerating POCs might be present	Proliferation of oogonia is more intense than in the previous phases; more nests in the germinal epithelium and fewer single oogonia Indeterminate fecundity: more nests with oogonia Determined fecundity: more nests with oogonia and nests with early oocytes
Developing: ovaries beginning to develop, but not ready to spawn	Maturing, early developing, early maturation, midmaturation, ripening, previtellogenic	MF: Enlarging ovaries, blood vessels becoming more distinct HF: PG, PGca, SGe, and SGl oocytes present. No evidence of POCs or SGfg oocytes. Some atresia can be present Subphase: early developing: PG and PGca oocytes only	More nest with oogonia larger than single oogonia, however fewer than in regenerating phase Indeterminate fecundity: more nests with early prophase oocytes than nests with oogonia Determined fecundity: fewer nests with early prophase oocytes than nests with oogonia
Spawning-capable: fish are developmentally and physiologically able to spawn	Mature, ripe, late developing, late maturation, late ripening, total maturation, fully developed, partially spent, running ripe, prespawning, gravid, final OM, vitellogenic, spawning, ovulated	MF: large ovaries, blood vessels prominent; individual oocytes visible macroscopically; hydration HF: SGfg oocytes present or POCs present in batch spawners; atresia of vitellogenic and/or hydrated oocytes might be present; initial steps of OM can be present; oocytes with cortical alveoli, SGe, and SGl can be present in species with indeterminate fecundity Subphase: actively-spawning: oocytes undergoing OMgvm, OMgvb, hydration (not many ostariophysian fish), or ovulation	Number of nests with oogonia is equivalent to number of single oogonia; nests with early prophase oocytes are rare Indeterminate fecundity: might have more nests with oogonia, however, fewer than in other phases In actively-spawning subphase, more single oogonia present
Regressing: cessation of spawning	Spent, regression, postspawning, recovering	MF: flaccid ovaries, blood vessels prominent HF: atresia (any stage) and POCs might be present; some PGca and/or vitellogenic (SGe, SGl) oocytes might be present	Gradual recovery of oogonia proliferation and entrance into meiosis; the number of nests with oogonia and also with oocytes increases

Abbreviations: GSI, Gonadosomatic Index; HF, histologic; MF, macroscopic; OM, oocyte maturation; OMgvb, germinal vesicle breakdown; OMgvm, germinal vesicle migration; PG, primary growth including multiple nucleoli, perinucleolar, and cortical alveolar oocytes; PGca, cortical alveolar; POC, postovulatory follicle complex; SGl, late secondary growth; SGe, early secondary growth; SGfg, full-grown oocyte.

clusters were designated as a germinal cradle, which were present between epithelial cells of the germinal epithelium and the basement membrane bordering the stromal compartment [4]. In reality, the germinal cradles defined by Nakamura et al. [4,5] topologically corresponded to cell nests described by Selman and Wallace [32]. Therefore, germinal cradles or nests are niches of stem cells of the female germinal lineage [4], and these constitute epithelial niches [34]. By definition, a niche "consists of a local tissue microenvironment capable of housing and maintaining one or more stem cells" [34]. In these niches, stem cells lie dormant; they can be activated at particular stages during the reproductive life cycle [34]. This is relevant for oogonial stem cells throughout the reproductive cycle of teleostean fish.

To generate new knowledge in this area, we have used, as a model, two species of Ostariophysi with distinctly different reproductive strategies to describe the early female germ cell behavior throughout the annual reproductive cycle. In seasonal breeders, *P. maculatus* and *S. maculatus*, external environmental factors trigger internal physiologic mechanisms activating oogonial proliferation and differentiation that give rise to ovarian follicles. Production and development of these follicles result in a series of gonadal transformations (reproductive phases).

4.3. Reproductive phases

As proposed by Brown-Peterson et al. [18], reproductive phases in adult female fish include: regenerating phase, developing phase, spawning-capable phase, and regression phase. The description of annual reproductive phases was described primarily for marine, perciform fishes that produce pelagic eggs. However, the classification by Brown-Peterson et al. [18] was flexible enough to contemplate different reproductive strategies and also some subphases, e.g., early developing subphase and actively-spawning subphase. However this classification lacked adaptation

to freshwater ostariophysian fish that produce demersal eggs. Our proposition, based on analyses reproductive cycles in the Siluriformes, *P. maculatus*, and the Characiformes, *S. maculatus*, was intended to eliminate this gap by helping to promote the universality of the characterization of the reproductive phases of female fish, as proposed by Brown-Peterson et al. [18].

Until now, apparently no classification of the phases of the female reproductive cycle in fish, including Brown-Peterson et al. [18], had considered the activity of the ovarian lamellar germinal epithelium. In that regard, our data characterizing female germ cell renewal, in *P. maculatus* and *S. maculatus*, are original and promote a new understanding of the female reproductive cycle.

4.4. Conclusions

In the Siluriformes, namely *P. maculatus* (determinate annual fecundity and a short spawning season), the regenerating phase comprised the longest period during the annual reproductive cycle. During this phase, oogonial proliferation was more intense, as was the entrance of oogonia into meiosis. Most oocytes for the spawning season were produced during the regenerating phase. In the developing phase, as oocytes were recruited into vitellogenesis, oogonial proliferation and entrance of oocytes into meiosis decreased relative to other phases. Oogonial activity was sharply decreased during the spawning-capable phase, but their proliferation was recovered during the regressing phase. Conversely, in the Characiformes, as studied in *S. maculatus* (prolonged spawning season and indeterminate annual fecundity), oogonial proliferation was more intense during the regenerating phase, whereas entrance into meiosis was more frequent in the developing phase. In this species, given the prolonged spawning season, the spawning-capable females, whose oocyte stock were not depleted, continued to have discreet oogonial proliferating activity, but their entrance into meiosis became rare. At the time of oocyte release (actively-spawning subphase), most oogonia remained quiescent. Similarly, in *P. maculatus* and *S. maculatus*, mitotic activity of the oogonia decreased sharply during the spawning-capable phase. Gradually, oogonial proliferation was recovered during the regressing phase. In summary, independent of the species or features of the reproductive cycle, in *P. maculatus* and *S. maculatus* (and perhaps generally in fish), germ cell renewal occurred during the regenerating phase, ensuring availability of eggs for the next spawning event.

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