

Induced reproduction in a migratory teleost species by water level drawdown

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Captive reproduction is one of the problems faced in aquaculture requiring the manipulation of environmental factors and/or hormonal treatment. Thus, we seek to verify experimentally which gonadal changes were present in mature individuals of *Astyanax altiparanae* arising from decreased water level. Collections were made every four hours, initiated four hours before and finished 28 hours after stimulation, at the Fish Farming Station of Companhia Energética de São Paulo, São Paulo, Brazil. The gonads were analyzed by light microscopy. The females had ovaries in the spawning capable phase until 12h; in 16h, in a more advanced stage of spawning capable phase; and, from 20h, in the regressing phase. Males had testes in the spawning capable phase until 8h; in 12h, in a more advanced stage of spawning capable phase; and, from 16h, the return to the spawning capable phase. The morphological description was corroborated by the proportion of cell classes. Females presented variation on the gonadosomatic index, but it was not found an emptying of the gonad for neither sex. The process of inducing reproduction with water level drawdown was considered satisfactory, since both sexes presented a reduction in the number of mature gametes at the end of the sample period.

Reprodução em cativeiro é um dos problemas enfrentados na aquicultura exigindo a manipulação de fatores ambientais e/ou tratamento hormonal. Assim, verificou-se experimentalmente as alterações gonadais presentes em indivíduos adultos de *Astyanax altiparanae* decorrentes da diminuição do nível da água. As coletas foram realizadas a cada quatro horas, iniciadas quatro horas antes e encerradas 28 horas após a estimulação, na Estação de Piscicultura da Companhia Energética de São Paulo, São Paulo, Brasil. As gônadas foram analisadas por microscopia de luz. As fêmeas apresentaram ovários na fase de apto à reprodução até 12h; em 16h, um estágio mais avançado desta fase; e, a partir das 20h, na fase de regressão. Os machos tiveram os testículos em fase de apto à reprodução até 8h; em 12h, um estágio mais avançado desta fase; e, a partir das 16h, o retorno à fase de apto à reprodução. A descrição morfológica foi corroborada pela proporção das classes celulares. As fêmeas apresentaram variação no índice gonadosomático, mas não foi estabelecido o esvaziamento das gônadas para nenhum dos sexos. O processo utilizado para a indução à reprodução foi considerado satisfatório, uma vez que ambos os sexos apresentaram redução no número de gametas maduros no final do período de amostragem.

Keywords: Asynchronous reproduction, Characidae, Gametogenesis, Gonadosomatic index, Histology.

Introduction

The seasonal reproduction in fish occurs due to the interaction of physiological functions with environmental cycles (Bollough, 1939; Sundararaj & Vasal, 1976; Sundararaj *et al.*, 1980). Environmental factors act such as flags of a favorable station to reproduction, which are perceived by external receptors, and therefore affect the central nervous system, pituitary, and gonads (Rocha & Rocha, 2006). The specific environmental factors that stimulate fish reproductive period as in elimination of gametes, gonadal maturation, or even in migratory movements, are still not

very clear. A combination of factors such as photoperiod, temperature, increasing of water level and food availability associated with physiological variations of the individual may possibly be involved in the regulation of different stages of the reproductive cycle (Bailly *et al.*, 2008).

Increases in temperature and photoperiod have been the main factors associated with gonadal maturation (Suzuki *et al.*, 2004). However, in latitudes where the seasonal temperature fluctuations are small, flooding seems to be the main trigger for the reproduction of many fish species (Agostinho *et al.*, 2004). The beginning of the flooding cycle seems to start a synchronization process for events of

spawning and the peak of flood level would signal its end (Suzuki *et al.*, 2004). Thus, the floods have an essential role in spawning stage (Godoy, 1975), while the first rains after the dry season are considered stimuli for the formation and movement of migratory fish shoal (Agostinho *et al.*, 2003).

Migratory species need to move in order to find an ideal environment to spawn, so significant environmental changes to successful reproduction of the species occur during migration. However, when they are contained in tanks, they are deprived of these changes, which results in failure in reproduction (Mylonas & Zohar, 2007). Thus, the reproduction of migratory species in captivity in most cases does not occur naturally, and to obtain reproductive success in these conditions the manipulation of environmental parameters or some hormonal induction is necessary (Mylonas & Zohar, 2007). The main problem encountered in aquaculture is the dysfunction in the maturation of oocytes (Mylonas & Zohar, 2007) that usually happens due to the lack of an appropriate environmental stimulus or by the presence of a stressor in captivity (Schreck *et al.*, 2001). Another failure is the release of oocytes to the external environment (spawning) (Bromage & Cumaranatunga, 1988; Zohar *et al.*, 1989), which probably occurs by the deficiency in the synchronization of gonadal changes in females.

A large group of migratory species belongs to the Characidae family, which represents the largest number of fish species in Brazil, with worldwide distribution (Britski, 1972). In this group lies the genus *Astyanax*, considered the most representative of the Tetragonopterinae subfamily, being one of the dominant genera in South America (Eigenmann, 1921). This genus shows wide distribution in the Neotropics and has approximately a hundred species (Garutti & Britski, 2000).

Astyanax includes species which, in general, are reofilics, small, and are characterized by having small oocytes, high relative fecundity, rapid development, external fertilization and no parental care (Agostinho *et al.*, 1999; Suzuki *et al.*, 2005). Among the species of this genus, *Astyanax altiparanae* is characterized by being small, fast growing, reaching sexual maturity at about four months of age in farming conditions, and displays sexual dimorphism (Porto-Foresti *et al.*, 2001). According to Dias *et al.* (2005), the duration of the reproductive period seems to gradually become longer after the flood, however Garutti (1989) and Peres *et al.* (2005) found that *Astyanax altiparanae* shows a period of prolonged mating in shallow waters, whereas in deep water environments it tends to have a short reproductive period.

The construction of dams, overfishing, pollution, introduction of exotic species, and other anthropogenic factors are leading to a population decline of some native species, after that the captive reproduction becomes an important tool for the conservation of fish species (Machado, 1975). However, it is important to have knowledge of reproduction techniques, so the containment of the species would not be a hindrance to reproduction. Thus, the *Astyanax altiparanae* was used as a model due to the characteristics already

mentioned and the fact that this species is heavily livestock potential and high plasticity. Moreover, as no standard for characterizing reproduction has been described so far, and considering what has been described in the literature for *Astyanax altiparanae*, we seek to find experimentally which gonadal alterations are present in mature individuals under induced reproduction by water level drawdown.

Material and Methods

The experiment and the collections were conducted in February and March 2012, in Fish Farming Station of Companhia Energética de São Paulo (CESP) in Paraibuna, São Paulo, Brazil (23°24'47"S 45°36'7"W). Voucher specimens are deposited at the Museu de Zoologia da Universidade de São Paulo (MZUSP 113746). Before the experiment the specimens of both sexes were kept together in an outdoor tank with natural photoperiod regime and hand fed once a day. For the induction process the following steps were carried out:

- 1) Mature individuals of both sexes were separated and acclimated in indoor tanks under natural photoperiod and running water (23.3±0.7°C, pH 6.5±0.3, 336.7±16.4 mV, 7.6±1.2 mg/L DO) in ratio of 4 females per male;
- 2) On the next day, the specimens were put together around 8 AM in a tank with water level reduced by approximately 50%, but keeping the running water to oxygenation;
- 3) For this study, samples were collected every four hours interval, beginning four hours before reducing the water level and maintained until 28 hours after.

At each sampling time, the gonads of 16 females and four males were collected, keeping the initial sex ratio. The animals were anesthetized by an alcoholic solution of benzocaine (100 mg/L), there after they were euthanized by spinal cord section, in accordance with the procedure approved by the Ethics Committee on Animal Experiments of the Institute of Biomedical Sciences (USP Proc. 2011/017). The classification of male and female individuals was based on their sexual dimorphism, where males presented spines on the anal fin (Garutti & Britski, 2000). Individuals had the total weight (Wt) and gonad weight (Wg) measured on an analytical balance (Marte AL500) with an accuracy of 0.001 g.

The gonads were isolated and fixed in Bouin's solution for 24 hours. After fixing procedure, they were washed in water, dehydrated in increasing alcohol concentrations, cleared in xylene solution and embedded in Paraplast. The blocks were cut in 5 µm thick, obtaining semi-serial sections with approximately 200 µm of distance between each other. Each slide contained three sections, subsequently stained with hematoxylin/eosin. The sections were used for morphological characterization of gonads throughout the sampling times previously determined.

To check the gonadal variation over sampling times, the gonadosomatic index (GSI) of both sexes was estimated. The comparison between the GSI through times, after

verification of data normality by Lilliefors test, was made using parametric statistical test ANOVA for males and non-parametric statistical test of Kruskal-Wallis for females.

All the histological sections were used to quantitatively evaluate the distribution of germ cell types present in the gonads during the sampling times. For females, $n=10$ individuals for each sampling time were randomly selected. The cell types were counted in the total area of each section. The germinal epithelium was discriminated in: oogonia, initial oocyte, pre-vitellogenic oocyte, vitellogenic oocyte, mature oocyte, post-ovulatory complexes, and atresia (adapted from Quagio-Grassiotto *et al.*, 2011). All males were measured according to the area occupied by each cell type in 10 tubules by section. The germ cell types were discriminated in: spermatogonia, primary spermatocyte, secondary spermatocyte, spermatid and spermatozoid (adapted from Schulz *et al.*, 2010).

Subsequently, the quantitative data of both sexes were used for analysis of the cellular proportion of classes, by sampling time, which allowed the classification of gonadal cycle phases in accordance with Brown-Peterson *et al.* (2011). Finally, to determine if there was emptying of the gonads, it was compared the total number of post-ovulatory complex of each female and the total area occupied by spermatozoid in each male per sample time, using Kruskal-Wallis and ANOVA statistical tests, respectively.

Results

The females had ovaries filled with vitellogenic oocytes, some pre-vitellogenic oocytes and few initial oocytes between the sampling periods -4h and 4h (Fig. 1A). Added to the above characteristics, in the periods of 8h and 12h, some post-ovulatory complexes were observed (Fig. 1B), featuring the gonads in spawning capable phase. At 16h, was verified the presence of many oocytes with germinal vesicle breakdown, also known as mature oocytes, few post-ovulatory complexes and initial oocytes (Fig. 1C), indicating the spawning capable phase, but in the advanced actively spawning subphase. Then, gradually the females had ovaries with post-spawning characteristics: with loads of post-ovulatory complexes, initial oocytes and atresia, and some pre-vitellogenic oocytes, setting the regressing phase (Fig. 1D).

Males showed a great amount of all germ cell classes during the sampling periods from -4h to 8h, with emphasis on the proliferation of spermatogonia and primary spermatocytes (Fig. 1E), showing spermatozoa in the lumen of the tubule, characterizing the spawning capable phase. At 12h, the gonads were full of sperm and secretion, with almost complete differentiation of other cell classes (Fig. 1F), in the spawning capable phase, but in the advanced actively spawning subphase. Then, the gonads showed a partial elimination of sperm and retrieval of other germ cell types (Fig. 1G), returning of the primary stage of spawning capable phase.

For quantitative analysis, it was observed that the proportion of cell classes corroborated the descriptive results for both females and males (Fig. 2A and 2C). The gonadosomatic index of females showed variation over the sampling periods ($H=16.388$, $p=0.037$), indicating that they exhibit significant changes in gonad weight according to reproductive stimulation (Fig. 2B). Further, regarding the maximum emptying of ovary over time, it was found that the same does not occur during the sampled period ($H=7.708$, $p=0.463$). For males, the gonadosomatic index did not vary ($F=1.806$, $p=0.120$) and they did not exhibit an emptying of the testis during the sampling periods ($F=1.612$, $p=0.168$) (Fig. 2D), so spermatozoa were always present in the gonads.

Discussion

The water level drawdown applied to specimens in spawning capable phase of *Astyanax altiparanae* to induce reproduction was effective. The reducing of the water level can be considered a stressor and it is known that stress, most often, triggers a negative response in the reproductive success for both males and females (Consten *et al.*, 2001; Mosconi *et al.*, 2006; Cleary *et al.*, 2007). The main factor involved in negative response to stress is cortisol, that in females appears to inhibit the synthesis of vitellogenin, which is modulated by estrogen (Pankhurst & Van Der Kraak, 2000; Berg *et al.*, 2004), and in males cortisol inhibits androgen synthesis, then slowing spermatogenesis (Consten *et al.*, 2002; Ozaki *et al.*, 2006).

However, these deleterious effects are associated with prolonged stimuli of aggressive agents resulting in high levels of plasma cortisol (Bayunova *et al.*, 2002; Schreck, 2010). In animals at the final stages of the reproductive cycle, stress appears to have a positive effect on reproduction (Small, 2004; Westring *et al.*, 2008; Milla *et al.*, 2009). Previous studies have found that this effect may be observed especially in females, where treatment with corticosteroids (cortisol, 11-deoxycortisol, 11-deoxycorticosterona) induces ovulation in various fish species (Goetz & Theofan, 1979; Haider & Rao, 1994; Milla *et al.*, 2009). Also, in males, high levels of cortisol in specimens of *Morone saxatilis* in captivity caused a precocious spermiation and prolonged the spermiation period (Castranova *et al.*, 2005). This may explain what was observed in males of *Astyanax altiparanae*, which responded to the stimulus of water level stress before the females, suggesting that males show a greater sensitivity to a stress situation.

Furthermore, it is important to note that, in general, the natural process of reproduction demands a high energetic rate for the behavior of cohort and maturation of gametes, especially for migratory fish species (Schreck, 2010), as *Astyanax altiparanae*. This suggests that water stress may have been essential to achieve a favorable energy demand for this process, mimicking a physiological response as expected in natural reproduction.

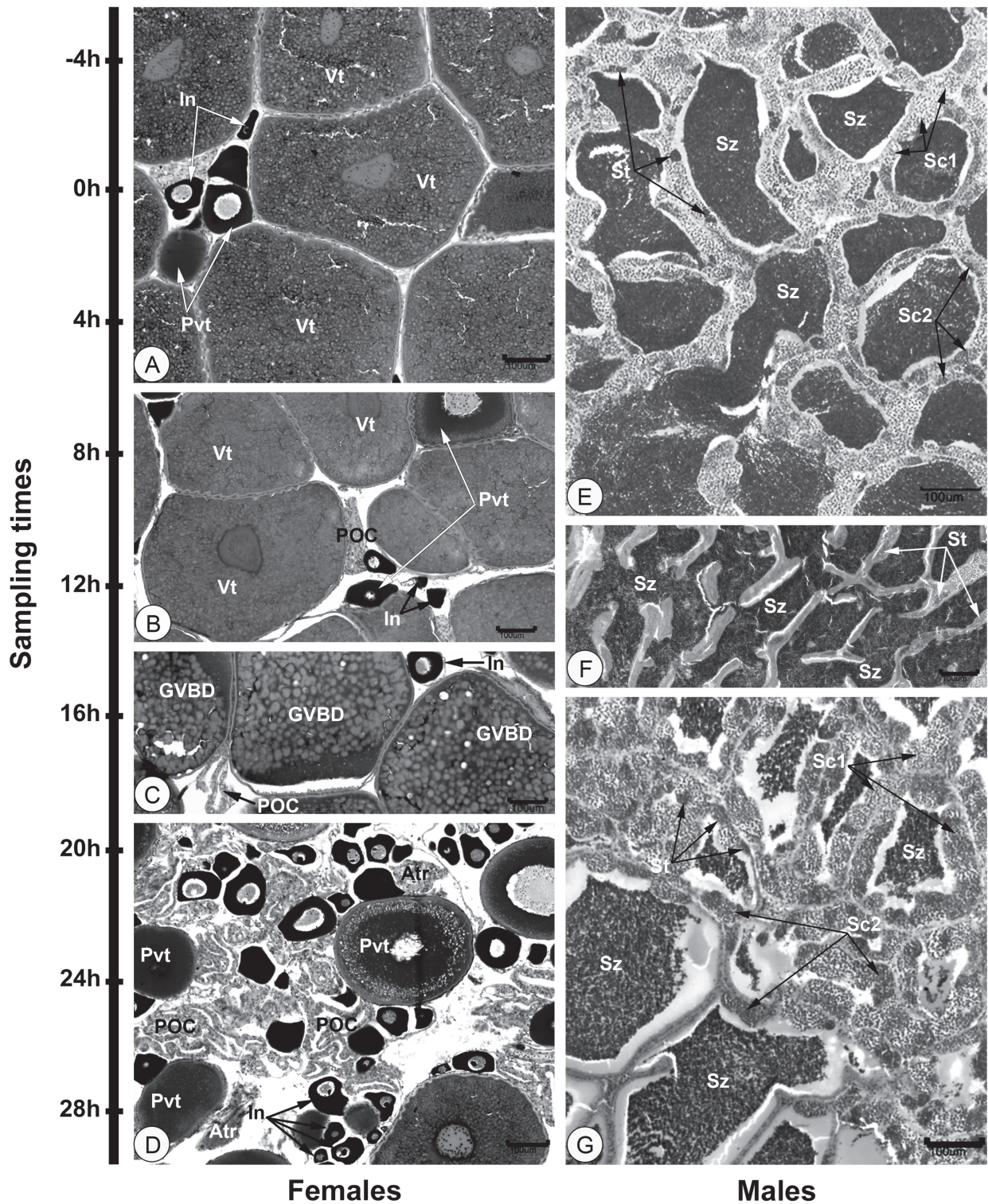


Fig. 1. Photomicrographs of gonads of *Astyanax altiparanae* over the sampling times (stained with hematoxylin-eosin). (A) Ovary in spawning capable phase from -4h to 4h; (B) Ovary in the same phase but with post-ovulatory complexes presents in 8h and 12h; (C) Ovary in the advanced actively spawning subphase of the spawning capable phase at 16h; (D) Ovary during regressing phase from 20h to 28h; (E) Testis in spawning capable phase from -4h to 8h; (F) Testis in the advanced actively spawning subphase of the spawning capable phase at 12h; (G) Testis returning the primary spawning capable phase from 16h to 28h. In - initial oocytes; Pvt - pre-vitellogenic oocytes; Vt - vitellogenic oocytes; POC - post-ovulatory complexes; GVBD - mature oocytes (oocytes with germinal vesicle breakdown); Atr - atresias; Sc1 - primary spermatocyte; Sc2 - secondary spermatocyte; St - spermatids; Sz - spermatozooids.

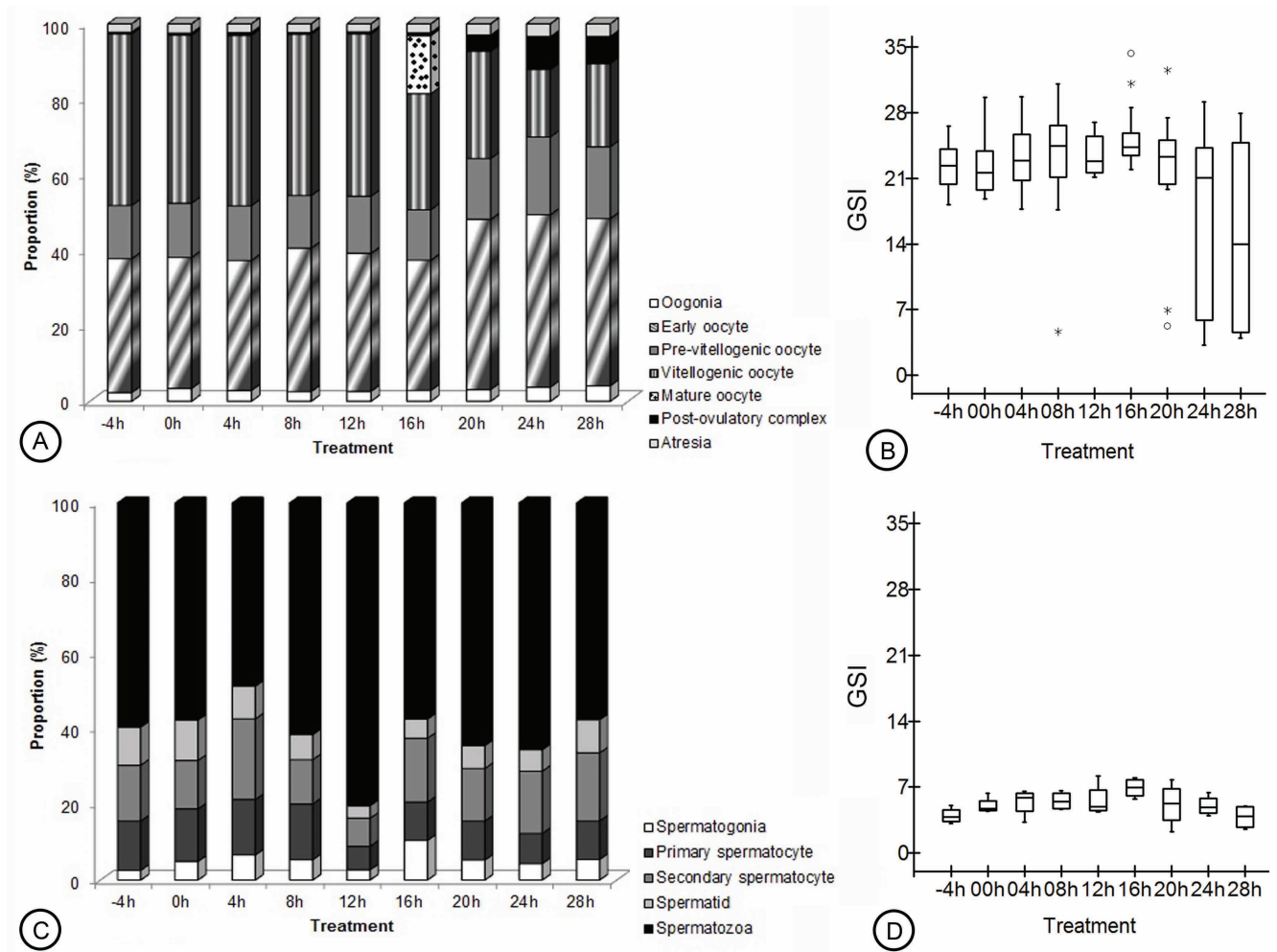


Fig. 2. Quantitative analyses of the gonads. (A) Plot of female germ cells proportion through sampling times; (B) Variation in female gonadosomatic index (GSI) over the sampling times ($H=16.388$, $p=0.037$); (C) Plot of male germ cells proportion through sampling times; (D) Variation in male gonadosomatic index (GSI) over the sampling times ($F=1.806$, $p=0.120$).

This energetic demand is apparently stronger in females, especially because ovarian maturation and fecundity of the fish are directly linked to energy reserves (Milton *et al.*, 1994). The availability of energy can cause variations in egg production (Coward & Bromage, 1999; Marshall *et al.*, 1999) with probable impact on ovarian allometry (Somarakis *et al.*, 2004) and, therefore, in the gonadosomatic index (GSI) of the specimens. This fact may explain why the variation in the GSI happened in females, but not in males.

In addition to the GSI, specimens from both sexes did not present a complete emptying of the gonad, although spawning has been observed. This may be related to the reproductive strategy of *Astyanax altiparanae*. These strategies comprise a set of characteristics that the species must have to succeed in reproduction (Vazzoler, 1996). However, to the genus *Astyanax* the descriptions of reproduction are different among authors in the literature. For example, specimens of *Astyanax bimaculatus* from Ivaí River, upper Paraná River basin, exhibit spawning between November and February (Agostinho *et al.*, 1984).

However, Nomura (1975) studying this species in the Mogi Guaçu River, São Paulo, noted that it shows total spawning between November and December. According to Rodrigues *et al.* (1992), *Astyanax bimaculatus* (= *A. altiparanae*) from Ibitinga dam has a total spawning, and reproduction is discontinuous, periodic and annual, with greater reproductive intensity from December to January.

For specimens of *Astyanax altiparanae* analyzed by Lizama & Ambrosio (2002), by means of condition factor, three reproductive periods were found: August/September, November/January and March. However, observing the variations at more accurate levels, as histological characterization, it is possible to notice that this species, in captive conditions, presents split spawning, and may be classified as asynchronous in the reproductive season (Mylonas & Zohar, 2007). This finding corroborates the observations in *A. scabripinnis*, which presented split spawning, with asynchronous development and showed the highest reproductive activity from November to April (Pereira-Filho *et al.*, 2011).

After all the analysis it can be inferred that the process of inducing reproduction by water level drawdown was satisfactory, since males and females showed a reduction in the number of mature gametes at the end of the sampling period. Regarding the induction time, it was observed that males of *Astyanax altiparanae* responded previously to the stimulus. Finally, according to the qualitative and quantitative analysis, we suggest that *Astyanax altiparanae* has an asynchronous development within the reproductive period for both sexes.

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