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Stereological analysis of gonads from diploid and triploid fish yellowtail tetra *Astyanax altiparanae* (Garutti & Britski) in laboratory conditions

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Summary

This study aimed to examine the gonadal morphology of diploid and triploid fish through stereological analysis. Triploid individuals were obtained after temperature shock (40°C for 2 min) at 2 min post-fertilization and reared until 175 days post-fertilization (dpf). Intact eggs were used to obtain the diploids. Gonads were collected for histological analysis at 83, 114, 144 and 175 dpf. Diploid females and males presented normal oogenesis and spermatogenesis through all the experimental period. Conversely, stereological analysis revealed that triploid females were sterile and oogonia were the prevalent cell type in the ovaries. Triploid males presented increased amounts of spermatocyte cysts and a large area of lumen when compared with diploids and in addition the amount of spermatozoa was lower than that observed for diploids. However, some triploid males presented spermatogenesis similar to diploids. Therefore, we concluded that triploidization is an interesting alternative to produce sterile individuals in *A. altiparanae*.

Keywords: Chromosome set manipulation, Fish reproduction, Germ cells, Growth, Sterility

Introduction

The rearing of triploid sterile fish is an interesting alternative to aquaculture (Piferrer *et al.*, 2009; Arai, 2001), as the deleterious aspects of early maturation (e.g. decreased growth and survival) may be avoided (Taranger *et al.*, 2010). In sterile fish, the energy used for gonadal development in diploids (especially females) are deviated for somatic tissue in triploids, with increased growth and carcass yield (Dunham, 2004; Golpour *et al.*, 2016; Nascimento *et al.*, 2017). As the reproductive capacity of sterile triploids is extremely reduced, it also may reduce the impacts of accidental escapes into the wild population (Benfey, 2015). Additionally, the novel surrogate technologies, including germ-cell transplantation, requested a sterile host, and then triploid fish may be used (Yamaha *et al.*, 2007), as previously observed by the production of trout offspring from triploid salmon (Okutsu *et al.*, 2007).

In the neotropical region, however, very few studies have focused on triploid induction in fish but some protocols exist like for the silver catfish *Rhamdia quelen* (Huergo & Zaniboni-Filho, 2006) and the

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yellowtail tetra *Astyanax altiparanae* (Adamov *et al.*, 2016). *Astyanax altiparanae* is a small characin that presents intertidal spawning, bred throughout a year and presents early sexual maturity at approximately 4 months (Garutti, 2003; Porto-Foresti *et al.*, 2010). Additionally, as it presents great importance to aquaculture, it becomes an interesting model for both basic and applied studies (de Siqueira-Silva *et al.*, 2015; Yasui *et al.*, 2015).

With this purpose, we first developed an *in vitro* fertilization (IVF) protocol (Yasui *et al.*, 2015) for *A. altiparanae*. The protocol supported important studies on early development such as the moment of the second-polar body extrusion (dos Santos *et al.*, 2016), which made it possible to determine the adequate timing for subsequent induction of triploid individuals (Adamov *et al.*, 2016). Recently, Nascimento *et al.* (2017) showed that triploid females are sterile and presents increased carcass yield. However, little difference was observed within males. Despite such work, the authors have performed histological analysis, as more detailed studies on gonad morphology are important for a better understanding of the biology and to confirm the sterility in triploid fish. Although previous works have also attempt to study to evaluate the reproductive biology in other related species (De Carvalho *et al.*, 2009; Dala-Corte & Azevedo, 2010; Galvão *et al.*, 2016), there is not a reference in order to assess reproductive ability in this species. Additionally, the pattern of stereological observation within diploids and triploids may be an interesting approach to evaluate sterilization quantitatively in fish. Therefore, the aim of this study was to examine the gonad morphology of diploid and triploid fish through stereological analysis.

Materials and Methods

Ethics

All the procedures were performed in line with the Guide for the Care and Use of Laboratory Animals in São Paulo State University (UNESP/CEUA 07919/14).

Origin of broodstock and triploid induction

We used three separately couples of *A. altiparanae* provided from different ponds at the Instituto Chico Mendes de Conservação a Diversidade (ICMBio/CEPTA), Pirassununga City, São Paulo State, Brazil. The procedures of artificial fertilization were performed according to Yasui *et al.* (2015). Briefly, mature females and males were injected with a single dose of carp pituitary gland at 3 mg/kg for both males and females. Ten hours afterwards, fish were anesthetized in menthol (~100 mg l^{-1}, Êxodo Científica, Brazil) and the semen was collected using a 1000 µl pipette (Eppendorf, Hamburg, Germany) and immediately transferred to a 1.5 ml tube containing 400 µl of modified Ringer solution (128.3 mM NaCl, 23.6 mM KCl, 3.6 mM $CaCl_2$, 2.1 mM $MgCl_2$), mixed by gently pipetting and stored at 2.5°C. Oocytes from females were stripped onto a Petri dish (90 mm diameter) covered by polyvinylidene chloride film (Saran wrap, Alpfilm, São Paulo, Brazil). For fertilization, 70 µl of the diluted semen was added on the oocytes and the gametes were activated by addition of 5 ml of water. This procedure was performed separately from each couple, generating three batches of eggs that was considered as replicates.

Triploid induction and rearing

Each fertilized group of eggs was divided into two aliquots. One was kept intact and served as a control group and the other was heat shocked to induce triploid at 40°C, for 2 min at 2 min post-fertilization (Adamov *et al.*, 2016). The eggs from each cross were incubated separately in six 40-l aquarium (three for diploids and three for triploids) for hatching and subsequent larval rearing. The resultant larvae were initially fed exclusively with *Artemia franciscana* nauplii until 30 days post-fertilization. At this moment, each group of fish were transferred to larger aquariums (125 L) in a recirculation system, with the temperature set at 28°C and 12 h of light. The stocking density was adjusted to 120 fishes per aquarium that were fed twice a day with a commercial pellet (1 mm) containing 45% of crude protein (until apparent satiation), until the end of the experiment.

Samples and histological analysis

At 83, 114, 144 and 175 days post-hatching (dph), 10 fish from each aquarium were randomly collected and euthanized in menthol (~100 mg l^{-1}, Êxodo Científica, Brazil). The gonads were dissected, fixed in Bouin's fixative for 24 h and stored in 70% ethanol prior to histological processing. Samples were subsequently dehydrated trough increasing concentrations series of ethanol, cleared in xylene, embedded into paraffin blocks, sectioned at 5 µm on a microtome (Leica RM2235, Nussloch, Germany) equipped with steel blade (Leica 818, Nussloch, Germany), and sections were then stained with hematoxylin and eosin.

Stereological analysis

Stereological analyzes used an 825-intersection grid (ImageJ software) on a section from the midgonad

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region. Each grid was considered a field, and three fields (2475 points) were randomly selected and examined under ×200 magnification on a microscope (Nikon-Eclipse Ni, Tokyo, Japan) for each fish. Digital images were captured with a CCD camera (Nikon DSRi2, Nikon, Tokyo, Japan) and analyzed with NIS-Ar Elements software (Nikon, Tokyo, Japan). Spermatogonia, spermatocytes, spermatid, spermatozoa, interstitial tissue, and lumen without cells were counted for males. Oogonia, primary growth oocyte, secondary growth oocyte, vitellogenic oocyte, interstitial tissue and atresic oocyte were counted for females. Different cell types were identified based on the study by Schulz *et al.* (2010) for male and Quaggio-Grassiotto *et al.* (2011) for female.

Flow cytometry

Flow cytometric analysis from somatic tissue (dorsal fin) were performed in order to confirm the ploidy status of each individual. The relative DNA content and ploidy status of each fish was estimated by comparison with diploids controls, according to Nascimento *et al.* (2017). The samples were placed into a 1.5 ml macrotube containing 100 µl of lysis solution (9.53 mM MgSO4.7H2O, 47.67 mM KCl, 15 mM Tris, 74 mM sucrose, 0.8% Triton X-100) for 10 min, and then stained using 800 µl of 4′,6-diamidino-2-phenylindole dihydrochloride-DAPI (1 µg ml^{-1} of DAPI in Dulbecco's phosphate buffered saline). The samples were filtered through 30-µm nylon mesh and analyzed by flow cytometry (CyFlow Ploidy Analyzer, Partec, GMBh, Germany).

Statistical analysis

The results are expressed as mean ± standard error. Data were checked for normality using the Lilliefors test (5%). Data expressed as percentages were transformed in order to fit the assumptions of statistical variance homogeneity using the Levene test (Brown & Forsythe, 1974) and then compared by paired *t*-test (5%), considering the effect of ploidy in each time separately. Analysis was performed using the software STATISTICA (Version 10.0, Statsoft, Tulsa, USA).

Results

Females

As expected, diploid females presented normal oogenesis during all the experimental period (Figs 1 and 2*A*). However, triploid females presented impaired gonads (Figs 1 and 2*B*). Triploid females present greater numbers of oogonia at 83 dph ($P = 0.0436$; 27.88 ± 22.88%), 114 dph ($P = 0.0000$; 82.32 ± 1.79%), 144 dph ($P = 0.0000$; 45.65 ± 8.82%) and 175 dph ($P = 0.0000$; 76.42 ± 5.78%); when compared with diploids (14.61 ± 5.39%, 4.95 ± 5.51%, 0% and 1.86 ± 1.17%, respectively). The numbers of oocytes in primary growth were significantly higher in diploid females at 83 dph ($P = 0.0000$; 61.99 ± 5.39%) and 114 dph ($P = 0.0013$; 7.83 ± 1.78%); when compared with triploid fish (36.01 ± 52.49% and 2.27 ± 0.77%, respectively). Diploid females present higher percentages of secondary growth oocytes at 83 dph ($P = 0.0266$; 2.92 ± 0.94%) than that observed for triploid fish (0%). Vitellogenic oocytes numbers were significantly higher in diploid females at 83 dph ($P = 0.0133$; 7.54 ± 2.69%), 114 dph ($P = 0.0000$; 79.61 ± 5.66%), 144 dph ($P = 0.0000$; 83.04 ± 3.50%) and 175 dph ($P = 0.0000$; 86.07 ± 3.93%); when compared with triploid fish (0%, 1.14 ± 0.97%, 31.16 ± 8.66% and 5.04 ± 3.64%, respectively). Triploid females present higher area occupied by interstitium at 83 dph ($P = 0.0000$; 36.12 ± 24.19%), 114 dph ($P = 0.0000$; 13.50 ± 1.46%), 144 dph ($P = 0.0180$; 11.46 ± 1.84%) and 175 dph ($P = 0.0000$; 12.51 ± 2.27%); when compared with diploid fish (12.94 ± 2.35%, 6.04 ± 0.97%, 10.52 ± 2.42% and 4.59 ± 0.70%, respectively). Besides the prevalence of oogonia, four triploid females presented sporadic vitellogenic oocytes.

Males

While diploid males presented a regular spermatogenesis (Figs 2*C* and 3), triploid males present an impaired gonad development (Figs 2*D* and 3). The numbers of spermatogonia were significantly higher in diploid males at 175 dph ($P = 0.0005$; 5 ± 0.58%) than that observed for triploid fish (2.30 ± 0.27%). Triploid males present significantly higher amounts of spermatocytes at 114 dph ($P = 0.0000$; 69.36 ± 1.71%), 144 dph ($P = 0.0062$; 59.05 ± 3.86%) and 175 dph (55.87 ± 3.51%); when compared with diploid fish (27.54 ± 38.66; 5.23% and 28 ± 2.45%). Spermatid cells were significantly greater in diploid males at 114 dph ($P = 0.0000$; 6.43 ± 0.68%), 144 dph ($P = 0.0002$; 2.49 ± 0.50%) and 175 dph ($P = 0.0000$; 4 ± 0.38%); in comparison with triploid fish (1.05 ± 0.30%; 0.99 ± 0.25% and 0.52 ± 0.13%). Diploid males present significantly increased area occupied by spermatozoa at 83 dph ($P = 0.0000$; 12.22 ± 2.22%), 114 dph ($P = 0.0000$; 46.38 ± 3.57%), 144dph ($P = 0.0000$; 42.29 ± 5.30%) and 175 dph ($P = 0.0000$; 47 ± 3.60%); when compared with triploid fish (1.44 ± 0.45%; 2.80 ± 1.16%; 4.34 ± 2.20% and 16.33 ± 4.53%). Increased area occupied by interstitium were observed for diploid males at 114 dph ($P = 0.0012$; 6.72 ± 1.24%) than that verified in triploid fish (3.55 ± 0.38%). However, at

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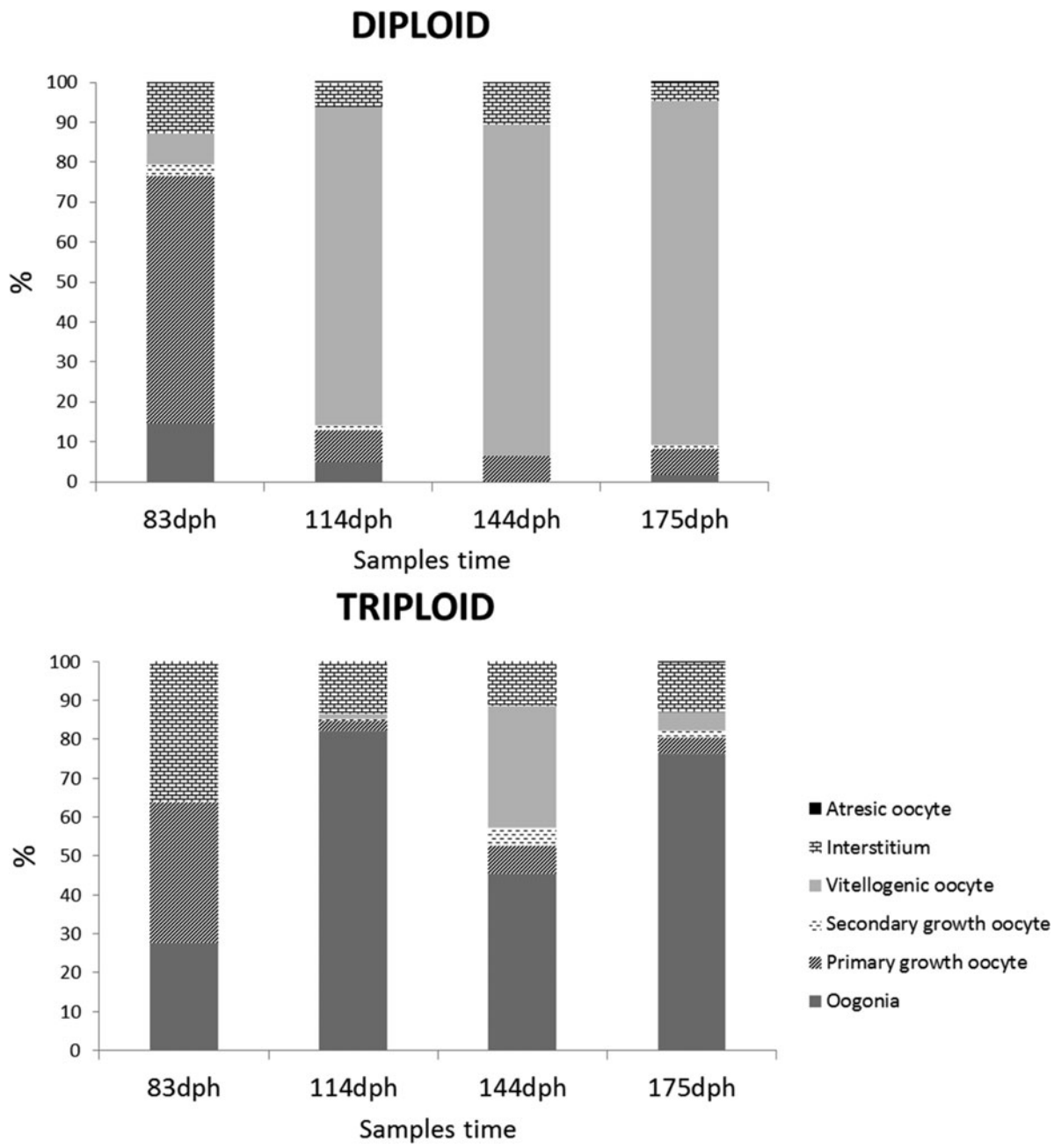

Figure 1 Stereological analysis of *A. altiparanae* ovarium from diploid (2n) and triploid (3n) fish. Triploid females showed impaired gonad development with prevalence of oogonia.

175 dph, triploid males present higher area occupied by interstitium ($P = 0.0000$; 4.42 ± 0.39%) when compared with diploid fish (3.00 ± 0.24). Triploid males present higher percentages of luminal area at 114 dph ($P = 0.0000$; 19.48 ± 1.17%), 144 dph ($P = 0.0000$; 28.70 ± 3.73%) and 175 dph ($P = 0.0015$; 20.55 ± 2.07%); when compared with diploid fish (10.20 ± 0.88%, 8.74 ± 2.60% and 13 ± 1.38%). Besides the impaired gonad development, seven triploids males present identical histology compared with diploid fish, with the full of spermatozoa in the lumen.

Discussion

In this study we have shown that triploidy impaired gonadal development in both *A. altiparanae* females and males. In triploid fish, sterility is more evident in females (Piferrer *et al.*, 2009), as observed for Atlantic cod (*Gadus morhua*; Feindel *et al.*, 2011), grass puffer (*Takifugu niphobles*; Hamasaki *et al.*, 2013) and rainbow trout (*Oncorhynchus mykiss*; Han *et al.*, 2010). In *A. altiparanae*, the gonads from triploid females were immature and full of oogonia, therefore we

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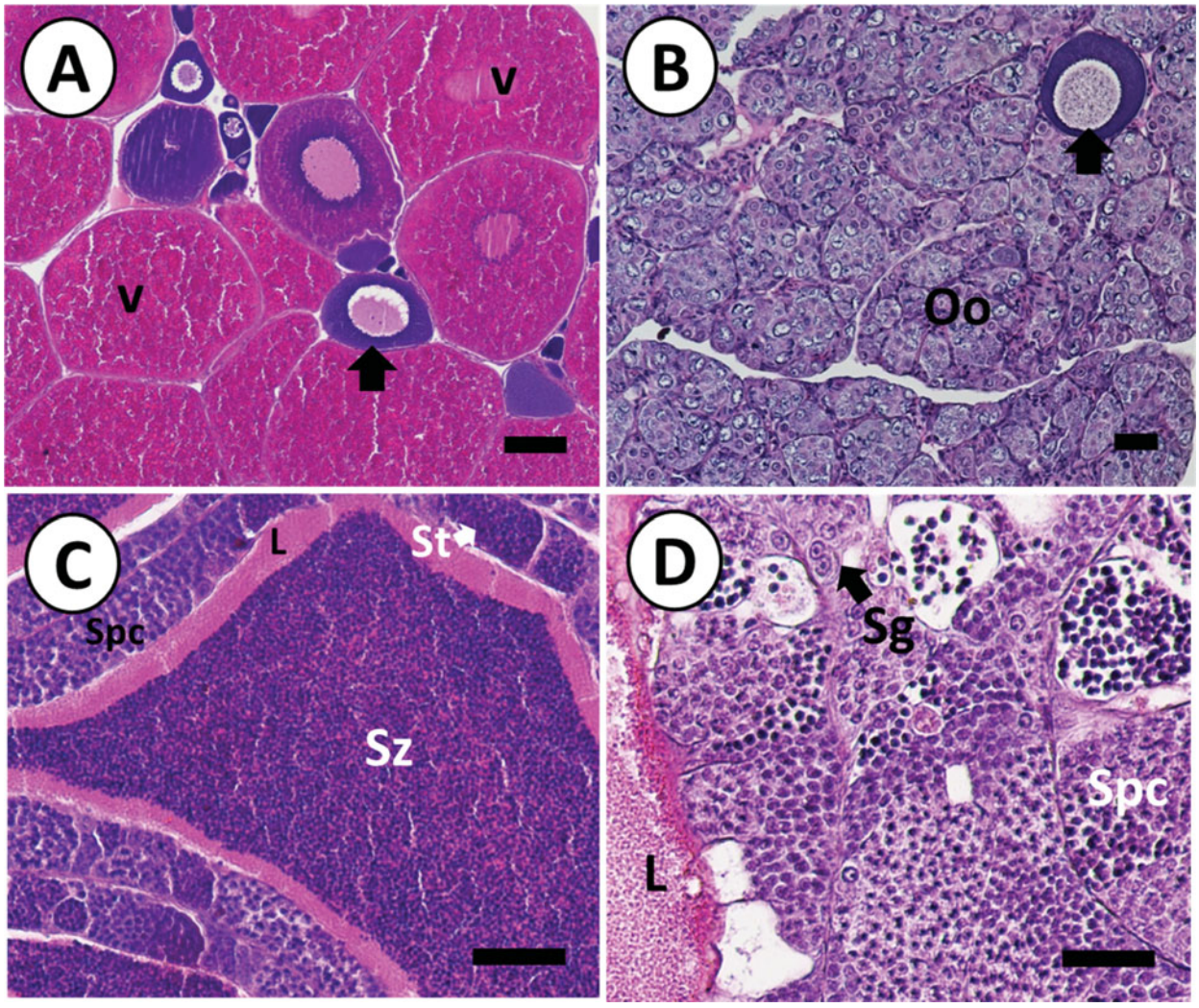

Figure 2 Ovarian and testis histology of diploid and triploid *Astyanax altiparanae* at 175 dph. (*A*) Mature ovaries from diploid females. (*B*) Ovaries from triploid females. (*C*) Testis of diploid males. (*D*) Testis of triploid males. Oo: oogonia nests; arrows: pre-vitellogenic oocytes; v: vitellogenic oocytes. Sg: spermatogonia; Spc: spermatocytes; St: spermatids; Sz: spermatozoa. Scale bars (*A*, *C*, *D*) 100 μm; (*B*) 40 μm.

proposed that this stage may be used as a reference to assume sterility in the yellowtail tetra. A few sporadic vitellogenic oocytes were verified in gonads of some females, such conditions do not ensure subsequent ovulation and reproduction, therefore we confirmed that triploid females were then sterile as stated by Nascimento *et al.* (2017). Additionally, such oocytes are probably aneuploid (Piferrer *et al.*, 2009) and non-viable, as observed for grass puffer (*Takifugu niphobles*) (Hamasaki *et al.*, 2013).

In triploid males, we observed a strong impairment of gonad development, with the prevalence of lumen and spermatocyte cells. Similar results were observed in *Heteropneustes fossilis* (Tiwary *et al.*, 2000) and sea bass *Dicentrarchus labrax* (Felip *et al.*, 2001). In males of sea bass *Dicentrarchus labrax* (Felip *et al.*, 2001) and seabream *Sparus aurata* (Haffray *et al.*, 2005), triploidy severely affect meiosis II, when the secondary spermatocytes differentiate into spermatids. It is probable that the same process occurs in *A. altiparanae*, because most triploid males showed prevalence of spermatocytes, which is the last diploid germ-cell lineage during spermatogenesis.

Some triploid males presented with the same morphology of diploids, with large amounts of spermatozoa, as observed in other species of teleost such as *Tinca tinca* (Linhart *et al.*, 2006) and *Misgurnus anguillicaudatus* (Fujimoto *et al.*, 2008). The spermatozoa of triploid males are generally aneuploid and non-motile (Peruzzi *et al.*, 2009; Feindel *et al.*, 2010). Further studies are necessary to evaluate the ploidy status and fertility capacity of triploid males. Therefore, contrary with the results from females, the sterility of *A. altiparanae* triploid males could not be confirmed.

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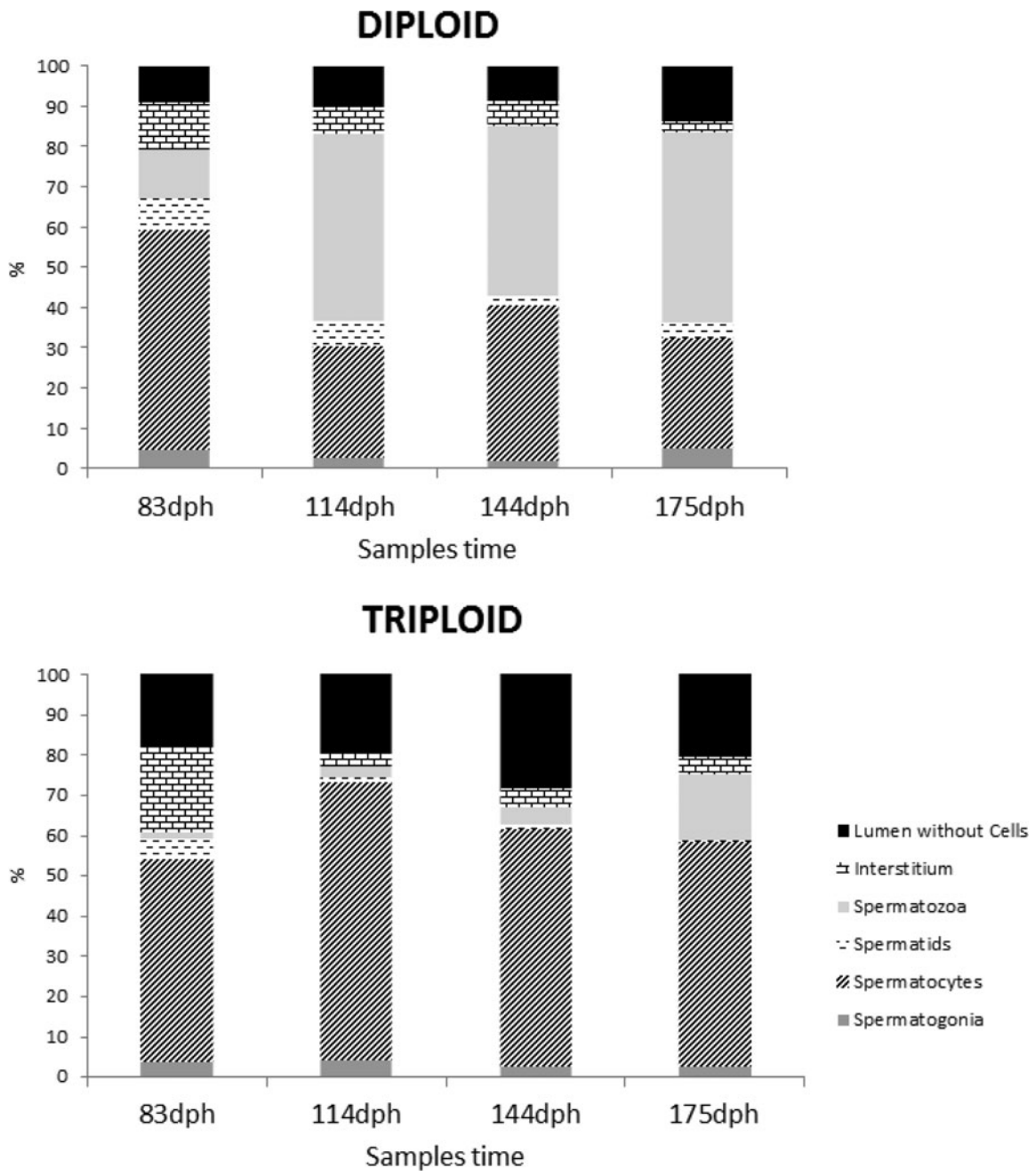

Figure 3 Stereological analysis of *A. altiparanae* testis from diploid (2n) and triploid (3n) fish. Triploid males showed impaired gonad development with prevalence of spermatocytes.

Sterile fish might be used as the host in germ-cell transplantation approaches, as shown by Okutsu *et al.* (2007), by successful production of rainbow trout offspring from sterile triploid salmon. Our results indicated that triploid female *A. altiparanae* fish may be used in such types of experiments. Additionally, due to the increased carcass yield (Nascimento *et al.*, 2017), triploid fish could be interesting for use in aquaculture and will also guarantee a more sustainable production, as the effects of fish escaping are reduced. Therefore, as females are sterile and males present with reduced gonad development, we concluded that the *A. altiparanae* triploid fish is an interesting alternative choice for aquaculture production and germ-cell transplant experiments.

Moreover, considering that *A. altiparanae* females are larger than males and present increased growth performance, the production of monosex sterile triploid female will be a useful procedure for aquaculture. In conclusion, an effective method that confirms the

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sterility in *A. altiparanae* females was developed in this study. The applicability of this technique for both basic and applied sciences needs to be considered in future studies.

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