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In vivo storage of oocytes leads to lower survival, increased abnormalities and may affect the ploidy status in the yellowtail tetra *Astyanax altiparanae*

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Research Article

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Summary

In this study we analyzed whether the *in vivo* storage of oocytes (time after ovulation until fertilization) affects the survival and the ploidy status of the yellowtail tetra *Astyanax altiparanae*. Fish were induced to spawn and, after ovulation, a small aliquot was stripped and immediately fertilized (positive control group). Subsequently, aliquots (~150 oocytes) were stripped and fertilized at various time points of 60, 120, 180 or 240 min. Developmental stages, abnormalities, survival and the ploidy status of the hatched larvae were examined. As expected, in the control group, 100% of the larvae were diploid. Conversely, triploid individuals were observed just at the 60 min treatment time point (0.6%). *In vivo* storage of oocytes also influenced the survival rates ($P < 0.05$); the 180 and 240 min samples, respectively, presented lower survival rates at gastrula ($50.10 \pm 6.26\%$ and $40.92 \pm 5.32\%$), and somite ($17.80 \pm 5.14\%$ and $4.41 \pm 2.76\%$) stages and lower hatching rates ($12.01 \pm 4.04\%$ and $4.41 \pm 2.76\%$). A higher percentage ($99.27 \pm 0.40\%$) of normal larvae and only a few abnormal larvae ($0.73 \pm 0.40\%$) were observed in the control group ($P = 0.0000$). This observation did not differ from that observed at the 60 min treatment point ($P = 0.9976$). A significant increase in the percentage of abnormalities was observed in the other treatments, and, after 240 min, the highest percentage of abnormal larvae was seen ($P = 0.0024$; $83.33 \pm 16.67\%$). In conclusion, we showed that oocyte ageing had a significant effect on survival and may affect the ploidy status in *A. atiparanae*.

Introduction

The yellowtail tetra *Astyanax altiparanae* has recently been used as model organism for basic and applied studies such as early embryology (Pereira-Santos *et al.*, 2016) and chromosome set manipulation (Adamov *et al.*, 2017; Nascimento *et al.*, 2017b; Piva *et al.*, 2018). This small characin (8–14 cm SL) can breed throughout the year, is resistant to suboptimal environmental conditions, presents rapid growth and reaches sexual maturity at approximately 4 months (Garutti, 2003; Porto-Foresti *et al.*, 2010), all of which facilitate its use for laboratory conditions. In this species, studies on the production of triploids were conducted by our group. A protocol for *in vitro* fertilization was established previously that permits control of timing of fertilization (Yasui *et al.*, 2015). The timing for extrusion of the second polar body has also been evaluated by Pereira-Santos and colleagues (2016), supporting high percentages of produced triploids (Adamov *et al.*, 2017). First feeding within triploids has also been established for laboratory studies, permitting the evaluation of triploids in adult stages. In addition, evaluation of germ cells (Nascimento *et al.*, 2017a), performance of triploids (Nascimento *et al.*, 2017b) and hybrid triploids (Piva *et al.*, 2018) have all been examined previously. However, in the genus *Astyanax*, some spontaneous triploids may have arisen under natural conditions (Centofante *et al.*, 2001), this event was generally linked with thermal shock in the environment or production of unreduced gametes during interspecies hybridization (Alves *et al.*, 2001). The origin of the natural triploids was not clearly established, but this phenomenon may affect studies of induced triploidization as increase in the number of triploids may cause a change in triploid percentages.

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Some studies have also associated ageing of oocytes with problems in gametes, such as aneuploidy and production of triploidy (Aegerter and Jalabert, 2004; Flajšhans *et al.*, 2007). Oocyte ageing can be defined as the time between ovulation and fertilization and occurs *in vivo* or *in vitro* (Mohagheghi Samarin *et al.*, 2015). After ovulation, oocytes retain their capacity for fertilization for lengths of time that vary depending on fish species and temperature (Rizzo *et al.*, 2003; Mohagheghi Samarin *et al.*, 2015). As oocyte ageing occurs, a reduction in survival rate (Mohagheghi Samarin *et al.*, 2015) and increased abnormal larvae are described (Sohrabnezhad *et al.*, 2006). This process of ovulation without spawning can lead to oocyte 'over-ripening', which is responsible for morphological, physiological, hormonal and molecular alterations and is considered to be the main factor affecting survival of early embryos (Mohagheghi Samarin *et al.*, 2015).

In our previous study with *A. altiparanae*, we found that *in vitro* storage of oocytes may increase the percentage of triploids without any of the treatments commonly used to induce them such as temperature, pressure or chemical procedures (Pereira-Santos *et al.*, 2018). This finding indicated that ageing of ovulated oocytes may give rise to triploids. If such a phenomenon also occurred *in vivo*, this may be a good explanation for the rise of natural triploids in *Astyanax*. Therefore, the aim of this study was to analyze the effect of *in vivo* storage of oocytes on early survival in *A. altiparanae* and measure its ploidy status.

Materials and methods

Ethics

This study was performed in accordance with the Care and Use of Laboratory Animals of the National Research and Conservation of Continental Fish (CEUA/CEPTA #02031.000033/2015-11).

Broodstock induction

Three adult couples of *A. altiparanae* were used in this study. The fish were previously maintained in 1000 m^2 ponds at the National Center for Research and Conservation of Continental Fish/Chico Mendes Institute of Biodiversity Conservation (CEPTA/ICMBio) in Pirassununga City, São Paulo State, Brazil. Procedures for artificial fertilization were performed according to Yasui *et al.* (2015) and Nascimento *et al.* (2017b). The fish were anesthetized in eugenol solution (100 mg l^{-1}) and hormonally induced with a single injection of pituitary gland from carp fish (3 mg kg^{-1} body weight). At 8 h after induction, reproduction behaviour was observed, in which the males started following the females, the fish were then separated for gamete collection. Males were anesthetized as describe above and sperm collected using a micropipette (Eppendorf, Hamburg, Germany) and immediately transferred to 1.5 ml tubes containing 400 µl of modified Ringer's solution (NaCl 128.3 mM, KCl 23.6 mM, $CaCl_2$ 3.6 mM, $MgCl_2$ 2.1 mM) and maintained refrigerated (4°C) for later use. Only males with sperm motility higher than 80% were used. Females were immediately separated for fertilization trials.

Fertilization trials

During fertilization trials, females were individually maintained in a covered aquarium (40 l) with the temperature set at 26°C and under constant aeration. Samples of oocytes were stripped on a 90-mm Petri dish covered with a polyvinylidene chloride film (SaranWrap) and fertilized immediately after ovulation (positive control group) and after 60, 120, 180 or 240 min. Every time, females were anesthetized and 20 µl of oocytes (~150 eggs) were inseminated with 30 µl of diluted sperm (see above). Activation of gametes was performed by adding 5 ml of distilled water. These procedures were performed in triplicate with different couples. Development of embryos, and survival at the 2-cell, blastula, gastrula, and somite stages, as well hatching and subsequent normal and abnormal larvae, were observed using a stereomicroscope (Nikon SMZ 1500, Tokyo, Japan). Digital images were obtained using a charged coupled device (CCD) camera and Nis-Ar Elements software (Nikon, Tokyo, Japan).

Ploidy confirmation

At hatching, 20 larvae from each treatment group ($n = 60$) were analyzed by flow cytometry. Samples were processed using the protocol developed by Xavier *et al.* (2017) using the nuclear stain 4,6-diamidino-2-phenylindole (DAPI) in a Partec CyFlow Ploidy Analyzer (Partec GmbH, Münster, Germany). All samples were analyzed using *A. altiparanae* spermatozoa from confirmed diploid males, and used as a reference for 1C DNA content.

Statistical analysis

Data were presented as mean ± standard error (SE). Data were checked for normality and homogeneity using the Liliefors and Levene test (5%), respectively, and submitted to analysis of variance (ANOVA) followed by Tukey test. The software STATISTICA (Version 10.0, Statsoft, Tulsa, USA) was used and significance was set at a P-value < 0.05.

Results

Early development and flow cytometry

In vivo storage of oocytes influenced the survival rates in all treatments (Table 1). No difference among treatments was observed in fish in the non-fertilized ($P = 0.7681$), 2-cell ($P = 0.7681$) and blastula ($P = 0.2459$) stages (Table 1). However, except for the control group (Fig. 1A), some morphological alterations were observed at the blastula stage, mainly in the 180 min and 240 min groups. Cells with irregular patterns of cleavage were the most common malformations described (Fig. 1B, C). Treatments for 180 and 240 min presented significant reduction in survival rates at gastrula ($P = 0.0008$), somite ($P = 0.0008$) and hatching ($P = 0.0000$) stages (Table 1). These cells did not present a regular pattern of cleavage and several morphological alterations were observed as irregular epiboly movements (Fig. 1E, F), which led to an increased number of abnormal larvae.

At hatching, the control group presented higher percentages (99.27 ± 0.40%) of normal larvae (Fig. 1G) and just a few abnormal larvae (0.73 ± 0.40%). This observation did not differ from that observed in the 60 min treatment group ($P = 0.9976$). A significant increase in the percentage of larval abnormality was observed in the other treatment groups, and the 240 min treatment group presented the highest number of abnormal larvae (83.33 ± 16.67%). Larvae malformations were mainly observed in the tail region (Fig. 1H), resulting in irregular swimming and consequent mortality.

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Table 1. Survival rates (%) during development in *Astyanax altiparanae* after *in vivo* storage of oocytes

Treatments	Unfertilized (%)	2-Cell (%)	Blastula (%)	Gastrula (%)	Somite (%)	Hatch (%)	Normal (%)	Abnormal (%)	Ploidy	
									2n	3n
Control	4.77 ± 3.07	95.23 ± 3.07	94.92 ±3.03	90.53 ±3.73^{a}	87.74 ± 2.97^{a}	87.74 ± 2.97^{a}	99.27 ± 0.40^{a}	0.73 ± 0.40^{a}	60/60	0
60	5.94 ± 3.07	94.06 ± 3.07	91.11 ± 3.50	87.95 ± 5.00^{a}	84.38 ± 6.43^{a}	84.07 ± 6.73^{a}	96.93 ± 3.07^{a}	3.07 ± 3.07^{a}	59/60	1/60
120	4.55 ± 2.64	95.45 ± 2.64	88.98 ± 5.13	84.53 ± 7.15^{a}	70.65 ± 11.94^{a}	69.45 ± 12.97^{a}	89.35 ± 2.80^{ab}	10.65 ± 2.80^{ab}	60/60	0
180	6.28 ± 3.17	93.72 ± 3.17	86.00 ± 3.96	50.10 ± 6.26^{b}	17.80 ± 5.14^{b}	12.01 ± 4.04^{b}	38.53 ± 21.11^{bc}	61.47 ± 21.11^{bc}	38/38	0
240	13.49 ± 9.07	86.51 ± 9.07	77.77 ± 7.88	40.92 ± 5.32^{b}	4.41 ± 2.76^{b}	4.41 ± 2.76^{b}	16.67 ± 16.67^{c}	83.33 ± 16.67^{c}	17/17	0

Data are show as mean ± standard error. Distinct superscript letters indicate significant difference (ANOVA, $P < 0.05$).

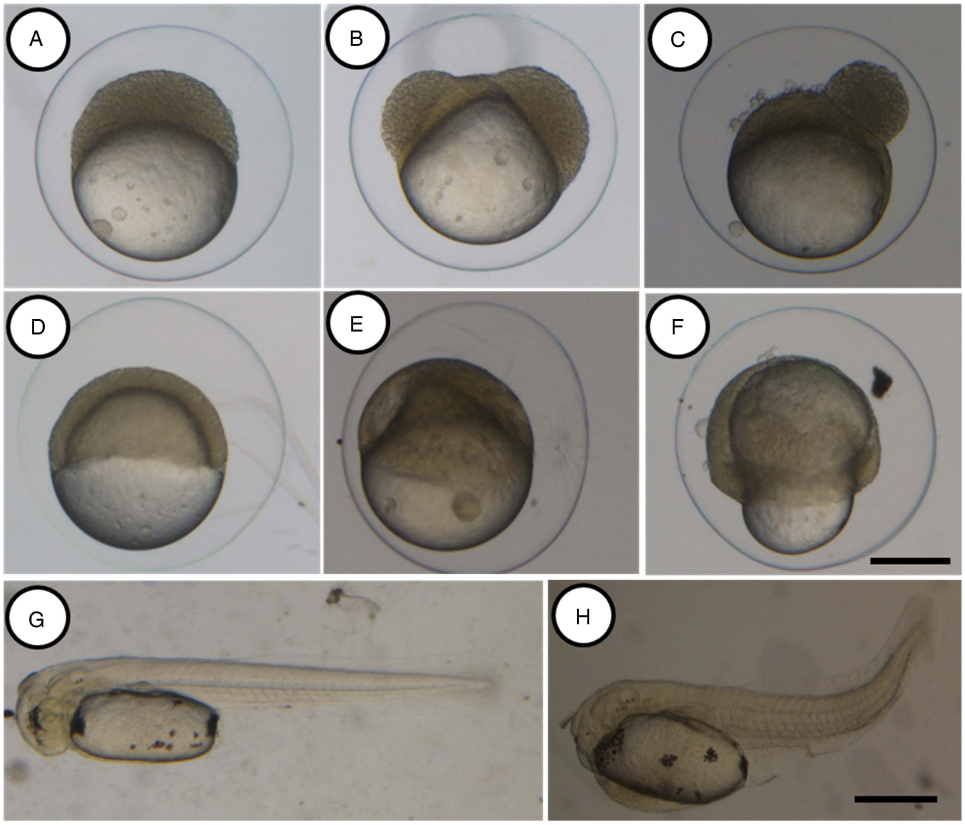

Figure 1. Morphological alterations during the early development of *Astyanax altiparanae* after *in vivo* storage of oocytes. Embryos from the control group at blastula (*A*), gastrula (*D*) and hatching (*G*) stages showed normal development, embryos after 180 and 240 min of storage showing irregular patterns of division at the blastula stage (*B* and *C* respectively) and irregular epiboly movements (*E* and *F*, respectively). Abnormal larvae after 240 min of storage (*H*). Scale: *A–F*, 200 µm (scale bar in *F*); *G*, *H*, 500 µm (scale bar in *H*).

Ploidy analysis

Ploidy analysis is detailed in Table 1. As expected, in the control group, all individuals were diploid (100%). After 60 min of storage, 0.6% triploid larvae were observed. However, in the other treatments, all samples were diploid (100%).

Discussion

In this study, we observed that *in vivo* storage of oocytes affected their survival and could influence the ploidy status of *Astyanax altiparanae*, giving rise to triploid fish. Similar results were observed under *in vitro* conditions for the same species (Pereira-Santos *et al.*, 2018). In both studies, we observed a low percentage of triploid individuals and these results were similar to those observed by Fauaz *et al.* (1994) who detected one triploid fish from 64 individuals in *Astyanax scabripinnis*. For the same species, Maistro *et al.* (1994) detected various frequencies of natural triploids, such as 1/86 and 1/40. However, Da Silva *et al.* (2011) detected increased numbers of triploid individuals in *Rhamdia quelen* under natural conditions. As natural triploidy can vary with species and the local sampling environment (Tsuda *et al.*, 2010), we believe that the absence of triploidy in the other treatments could be due the random collection process, which

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means that if more samples were analyzed, more triploid fish would be obsereved. Additionally, as the temperature affects the triploid larval yields during oocyte ageing (Flajšhans *et al.*, 2007), different storage temperatures must be tested in the future for *A. altiparanae*.

The triploid individual observed in our study probably arose due to spontaneous retention of the second polar body, as observed in other studies. This affirmation is supported by Linhart *et al.* (1995) who obtained spontaneous gynogenesis using overmaturated ova fertilized with irradiated spermatozoa (without any diploidization procedure). The authors generally stated that natural triploid fish may be produced by thermal shock in the environment (Centofante *et al.*, 2001). Based in our previous studies in the genus *Asyanax* (Adamov *et al.*, 2017; Nascimento *et al.*, 2017a, 2017b; Piva *et al.*, 2018), high temperatures (~40°C) and an adequate timing for heat shock are necessary to induce triploid individuals. This combination of conditions does not occur very often in nature, therefore we have excluded this explanation for the rise of natural triploids in the *Astyanax*.

Astyanax species are asynchronic intertidal spawners (Vazzoler, 1996; Mazzoni *et al.*, 2005; De Carvalho *et al.*, 2009) in which groups of oocytes mature and then are fertilized by a male. During spawning, one or more males follow the female over a long period, and then groups of ovulated oocytes are released into the external medium and are immediately fertilized by the male. Fertilization is highly successful due to oocyte morphology, as described in our recent study (Pereira-Santos *et al.*, 2018). We hypothesized that some of the ovulated oocytes may remain inside the ovary and become aged and fertilization of such oocytes may give rise to triploids. Another explanation for the increase in the number of triploids could be the production of unreduced gametes due interspecies hybridization (Alves *et al.*, 2001; Fujimoto *et al.*, 2008; Arias-Rodriguez *et al.*, 2009); this aspect may be investigated for *Astyanax* in future studies.

Previous studies on the genus have described the rise of spontaneous triploids in fish, including *Astyanax schubartii* (Morelli *et al.*, 1983); *Astyanax scabripinnis* (Maistro *et al.*, 1994; Machado *et al.*, 2012); and *Astyanax* (Fauaz *et al.*, 1994; Kantek *et al.*, 2007). These studies did not focus on hybrids, emphasizing a need for future studies on triploids. Other studies have demonstrated the effects of oocyte ageing on aneuploidy rate and triploid larvae in fish (Aegerter & Jalabert, 2004; Flajšhans *et al.*, 2007). This process can also occur in nature. Salmonids, for example, release ovulated eggs only when natural conditions are optimal (Barlaup *et al.*, 1994) and this delay might affect the ploidy status of the offspring. Therefore, we believe that *in vivo* storage of oocytes may be an important cause of natural triploidy in fish.

Our results showed that *in vivo* storage of oocytes may lead to non-viable embryos. Consequently, the decreased survival among all treatment groups may be due to oocyte ageing and 'over-ripening', a phenomenon that leads to alteration in eggs for example in their morphology and physiology (Lahnsteiner *et al.*, 2008; Mohagheghi Samarin *et al.*, 2015). Therefore, in captivity, in which stripping of oocytes is performed manually, this event needs to be controlled.

Conversely, short-term storage of oocytes might be necessary. In some cases, the male may not be available at the same time as the female or, for particular procedures such as chromosome set manipulation, short-term storage could be essential. For this aspect, the length of time that the oocytes remain viable can vary among species, for example 2 h for Asian catfish (*Pangasius hypophthalmus*) (Legendre *et al.*, 2000) and 9 h for the South American catfish (*Rhamdia sapo*) (Ros *et al.*, 1984). In our study, for up to 60 min of *in vivo* storage, non-significant effects were observed for survival during early development. Therefore, determination of the time for storage for each species is necessary to optimize seed production.

In conclusion, we showed that oocyte ageing had a significant effect on survival and may affect the ploidy status of *A. atiparanae*. These data provide new information that would be applicable for basic and applied sciences in the field of chromosome set manipulation and karyological studies.

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Conflicts of interest. None

Ethical standards. Not applicable

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