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Cite this article: Alves AC *et al*. (2023) All-triploid offspring in the yellowtail tetra *Astyanax altiparanae* Garutti & Britski 2000 (Teleostei, Characidae) derived from female tetraploid × male diploid crosses. *Zygote*. **31**: 123–128. doi: 10.1017/S0967199422000569

Received: 2 April 2022
Revised: 14 September 2022
Accepted: 14 October 2022
First published online: 9 January 2023

Keywords:
Aquaculture; Chromosome manipulation; Embryology; Lambari; Triploids mass production

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All-triploid offspring in the yellowtail tetra *Astyanax altiparanae* Garutti & Britski 2000 (Teleostei, Characidae) derived from female tetraploid × male diploid crosses

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Summary

This study aimed to evaluate the ploidy and survival of larvae resulting from crosses between tetraploid females and diploid males of yellowtail tetra *Astyanax altiparanae*, both females (three diploids and three tetraploids) and males ($n = 3$ diploids). Breeders were subjected to hormonal induction with pituitary gland extract from common carp fish (*Cyprinus carpio*). Females received two doses at concentrations of 0.3 and 3.0 mg/kg $^{-1}$ body weight and at intervals of 6 h. Males were induced with a single dose of 3.0 mg/kg $^{-1}$ applied simultaneously with the second dose in females. Oocytes from each diploid and tetraploid female were fertilized with semen from the same male, resulting in two crosses: cross 1 (diploid male and diploid female) and cross 2 (diploid male and tetraploid female). The procedures were performed with separate females (diploid and tetraploid) and diploid males for each repetition ($n = 3$). For ploidy determination, 60 larvae from each treatment were analyzed using flow cytometry and cytogenetic analyses. As expected, flow cytometry analysis showed that progenies from crosses 1 and 2 presented diploid and triploid individuals, respectively, with a 100% success rate. The same results were confirmed in the cytogenetic analysis, in which the larvae resulting from cross 1 had 50 metaphase chromosomes and those from cross 2 had 75 chromosomes. The oocytes have a slightly ovoid shape at the time of extrusion. Diploid oocytes had a size of 559 ± 20.62 µm and tetraploid of 1025.33 ± 30.91 µm. Statistical differences were observed between eggs from crosses 1 and 2 ($P = 0.0130$). No significant differences between treatments were observed for survival at the 2-cell stage ($P = 0.6174$), blastula ($P = 0.9717$), gastrula ($P = 0.5301$), somite ($P = 0.3811$), and hatching ($P = 0.0984$) stages. In conclusion, our results showed that tetraploid females of the yellowtail tetra *A. altiparanae* are fertile, present viable gametes after stripping and fertilization using the 'dry method', and may be used for mass production of triploids. This is the first report of these procedures within neotropical characins, and which can be applied in other related species of economic importance.

Introduction

For aquaculture purposes, the use of sterile triploid fish improves carcass yield (do Nascimento *et al.*, 2017b; Kizak *et al.*, 2013), growth (Tabata *et al.*, 1999), and meat quality (Turner *et al.*, 2003). However, there are problems associated with early sex maturation, such as high disease susceptibility (Taranger *et al.*, 2010) and with ecological issues due to the escape of fertile fish that could lead to problems of how hybridization, for example, is avoided (Benfey, 2016).

While triploids can naturally occur at low percentages in several species, such as *Astyanax scabripinnis* (1.16–2.5%) (Luis Maistro *et al.*, 1994), *Trichomycterus davisi* (2%) (Borin *et al.*, 2002), and loach (*Misgurnus aguillicaudatus*) (1.2–3.2%) (Zhang and Arai, 1999), artificially induced triploids are achieved at high percentages using thermal, chemical, or pressure shocks (Arai, 2001). These treatments prevent the extrusion of second polar bodies (Benfey, 2016) and result in individuals containing three sets of chromosomes (Arai and Fujimoto, 2018).

However, the application of such shocks can negatively affect survival, increase the percentage of abnormal larvae, and do not totally guarantee triploidization rates (Adamov *et al.*, 2017). In this case, the use of tetraploid individuals by breeders becomes an interesting alternative for

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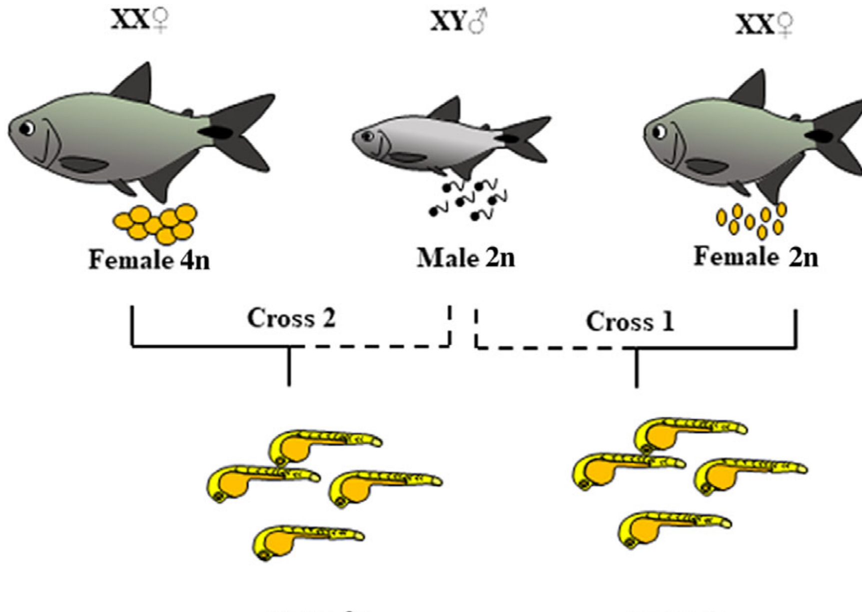

Figure 1. Experimental design evaluating progenies of cross 1 (female 2n × male 2n) and cross 2 (tetraploid females and diploid males) in *Astyanax altiparanae*.

the mass production of triploids and also avoids the deleterious effects caused by the several shocks used for second polar body retention (Arai, 2001; Dunham, 2004). Tetraploid fish, however, are difficult to achieve and limited to a few species (Yoshikawa *et al.*, 2007; Piferrer *et al.*, 2009).

In neotropical regions, for example, tetraploid fishes are described only in two species: the silver catfish *Rhamdia quelen* (Garcia *et al.*, 2018) and the yellowtail tetra *Astyanax altiparanae* (do Nascimento *et al.*, 2020). In the last decade, the yellowtail tetra (*A. altiparanae*) has become an important model organism for basic and applied studies, such as the induction of triploid, tetraploid, and gynogenetic fishes (Adamov *et al.*, 2017; do Nascimento *et al.*, 2017a, 2020, 2021)

The results indicated that triploid females are sterile (do Nascimento *et al.*, 2017a) and present increased performance (do Nascimento *et al.*, 2017b), suggesting that the mass production of such fish is desirable. Triploids (Adamov *et al.*, 2017) and triploid hybrids (Piva *et al.*, 2018) were also produced artificially and checked for sterility and ploidy status (Xavier *et al.*, 2017). The rise of spontaneously occurring triploids was also investigated *in vivo* (dos Santos *et al.*, 2018) and *in vitro* (do Nascimento *et al.*, 2018).

Despite the previous achievements mentioned previously, none of those procedures gave rise to a 100% triploid fish, and this may be achieved using diploid gametes from tetraploid individuals. In this scenario, some studies have been performed to achieve these results. The previous study of do Nascimento *et al.* (2020), for example, showed that high percentages of triploids were produced using tetraploid males. However, as far as we know, viable tetraploid females used for mass production of triploids have never been described in a neotropical species. Therefore, the aim of the present study was to investigate the ploidy of progeny obtained by crossing tetraploid females with diploid males in *Astyanax altiparanae*.

Materials and methods

The experimental procedures were conducted in accordance with the Ethics Committee on Animal Use of the Federal Rural University of Rio de Janeiro (CEUA 009-11-2019). The experiment was performed from January to March 2021 at the Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental/Instituto Chico Mendes de Conservação da Biodiversidade (CEPTA/ICMBio) in Pirassununga, São Paulo State, Brazil.

Induced spawning, gamete collection, and incubation of *Astyanax altiparanae*

Females (three diploids and three tetraploids) and males ($n = 3$ diploids) of *A. altiparanae* with ploidy confirmed using flow cytometry were used. Breeders were subjected to hormonal induction (Yasui *et al.*, 2015) with pituitary gland extract from common carp fish (*Cyprinus carpio*). Females received two doses (applied intraperitoneally) at concentrations of 0.3 and 3.0 mg/kg^{-1} body weight and at intervals of 6 h. The males were induced with a single dose of 3.0 mg/kg^{-1} applied simultaneously with the second dose in females.

After induction, the fish were kept in a 60-litre aquarium with the temperature set at 26ºC. When the spawning behaviour was observed, with the male chasing the female, males and females were anaesthetised with eugenol (Biodinâmica, Ibiporã, Brazil), which was diluted in ethyl alcohol in the proportion of 1 ml of eugenol/10 mL of alcohol (98º GL). Sperm were collected using a 1000-μl pipette (Eppendorf, Hamburg, Germany) and stored in 1.5-ml macrotubes containing 400 μl of modified Ringer's solution (128.3 mM NaCl, 23.6 mM KCl, 3.6 mM $CaCl_2$, 2.1 mM $MgCl_2$). Subsequently, the oocytes were stripped on 90-mm Petri dishes covered with plastic film (Alpfilm, São Paulo, Brazil).

A small sample of oocytes from diploid ($n = 15$) and tetraploid ($n = 15$) females fixed in 2.5% glutaraldehyde was separated to measure the diameter (μm). Oocytes from each diploid and tetraploid female were fertilized with sperm from the same male, resulting in two crosses: cross 1 (diploid male and diploid female) and cross 2 (diploid male and tetraploid female). These procedures were performed with separate females (diploid and tetraploid and male diploid) for each replication ($n = 3$) (Figure 1). Two embryo aliquots (~100) from each cross were selected ($N = 600$) for developmental analysis using a stereomicroscope (Nikon SMZ 18, Tokyo, Japan) and Nis-Ar Elements software (Nikon,

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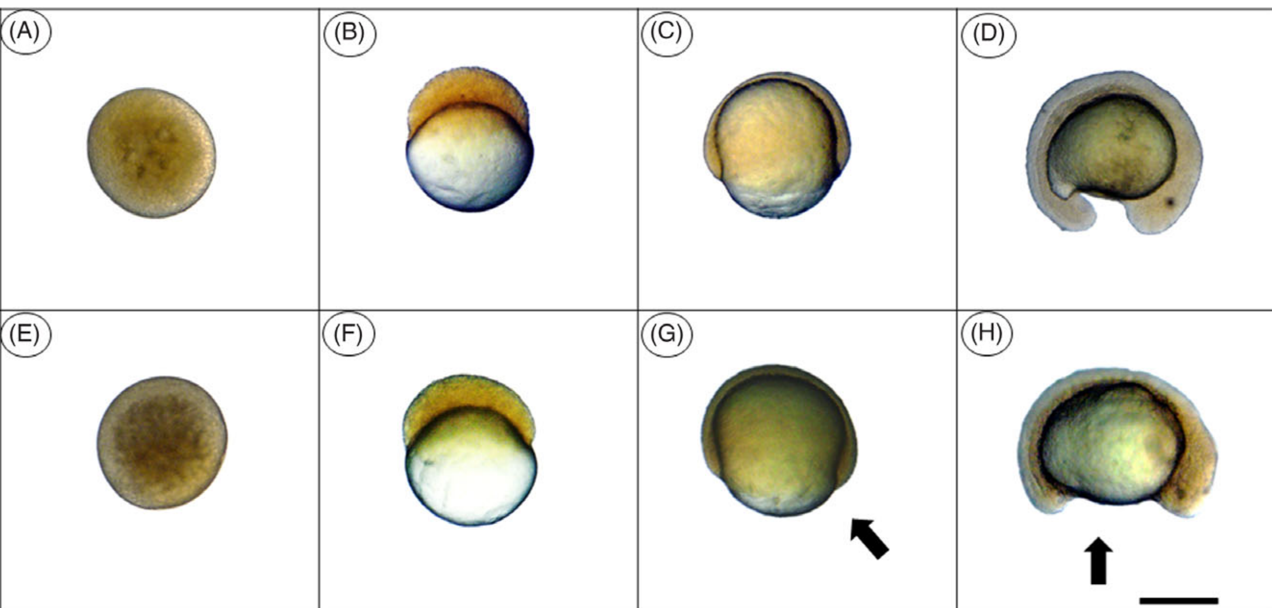

Figure 2. Embryonic development of *Astyanax altiparanae* was obtained through crosses between (female 2n × male 2n) and (female 4n × male 2n). (A, E) Animal pole differentiation; (B, F) blastula; (C, G) gastrula; (D, H) segmentation. Arrow indicates a small delay in yolk covering and embryonic shield formation. Arrowhead indicates delay in cephalic and caudal region formation.

Figure 3. External morphology of (A) larvae and (B) abnormal *Astyanax altiparanae* obtained through cross 1 (female 2n × male 2n) and cross 2 (female 4n × male 2n). Arrow indicates an anomaly in the final portion of the tail.

Tokyo, Japan). Survival rates (%) were measured during the cleavage, blastula, gastrula, somite, and hatching stages with subsequent normal and abnormal larvae according to dos Santos *et al.* (2016).

Confirmation of ploidy status

For ploidy determination, 60 larvae from each treatment were analyzed using flow cytometry and cytogenetic methods. Flow cytometry was performed according to the protocol developed by Xavier *et al.* (2017). The samples (each larva) were transferred to microtubes containing 120 µl of cell lysis solution (9.53 mM $MgSO_4.7H_2O$, 47.67 mM KCl, 15 mM Tris, pH 8.0, 74 mM of sucrose, and 0.8% of Triton X-100) for 10 min. Afterwards, nuclei staining was performed by adding 800 µl of 4′,6-diamidino-2-phenylindole (DAPI; Sigma #D5773, St. Louis, USA). The resultant solution was passed through a 30-µm filter and analyzed using a flow cytometer (CyFlow Ploidy Analyzer, Partec, GmbH, Germany).

Procedures for chromosome preparations (cytogenetics) were performed according to Foresti *et al.* (1993). Briefly, 20 embryos from each replicate ($n = 60$) were separated at the somite stage. Embryos were maintained in colchicine (0.007 %) for 4 h, and then the chorion was removed using a Pronase solution (0.03%). The larvae were anaesthetised in eugenol solution (1 ml of eugenol/10 ml of alcohol 98º GL) and individually dissociated (whole) in a Petri dish containing 7 ml of hypotonic KCl solution (0.075 M). Samples were maintained at 37°C for 21 min, fixed in methanol and acetic acid (3:1), and then stained with Giemsa solution (5%).

Results are presented as the mean ± standard error. The data were previously checked for normality and homogeneity using the Lilliefors and Levine's tests, respectively. Afterwards, one-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed. The software STATISTICA v.10.0 (Statsoft, Tulsa, USA) was used and significance was set at $P < 0.05$.

Results

Early development

The oocytes presented a slightly ovoid shape at the time of extrusion, in which diploid oocytes had a size of 559 ± 20.62 µm and tetraploid of 1025.33 ± 30.91 µm. Statistical differences were observed between eggs from crosses 1 and 2 ($P = 0.0130$).

The embryos from cross 2 (triploids) showed some delay (45 min) from the epibolism movements when compared with the diploid embryos of cross 1 (Figure 2H). Most larvae from cross 2 presented irregular formations at the final portion of the tail (Figure 3B).

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Table 1. Ploidy and survival (%) of yellowtail tetra *A. altiparanae* in percentage (±SE) resulting from cross 1 (female 2n × male 2n) and cross 2 (female 4n × male 2n)

Treatments	Ploidy of parents		Eggs	Fertilization (%)	Blastula (%)	Gastrula (%)	Somite (%)	Hatch (%)	Normal (%)	No. of larvae analysis	Ploidy (%)	
	♀	♂									2n	3n
Cross 1	2n	2n	100	93.78 ± 2.43[a]	89.53 ± 4.86[a]	85.23 ± 6.64[a]	81.96 ± 7.52[a]	77.38 ± 8.22[a]	93.64 ±2.24[a]	60	100	0.00
Cross 2	4n	2n	93	94.92 ± 0.64[a]	89.30 ± 4.02[a]	79.60 ± 4.82[a]	71.33± 7.76[a]	55.01 ±6.41[a]	58.39 ± 2.74[b]	60	0.00	100
P-value			–	0.6174	0.9717	0.5301	0.3811	0.0984	0.0005			

[a,b]Data were obtained from different gamete sources resulting in three replications. Different superscript letters within a column indicate statistical differences using the Tukey multiple range test (ANOVA; $P < 0.05$). SE, standard error.

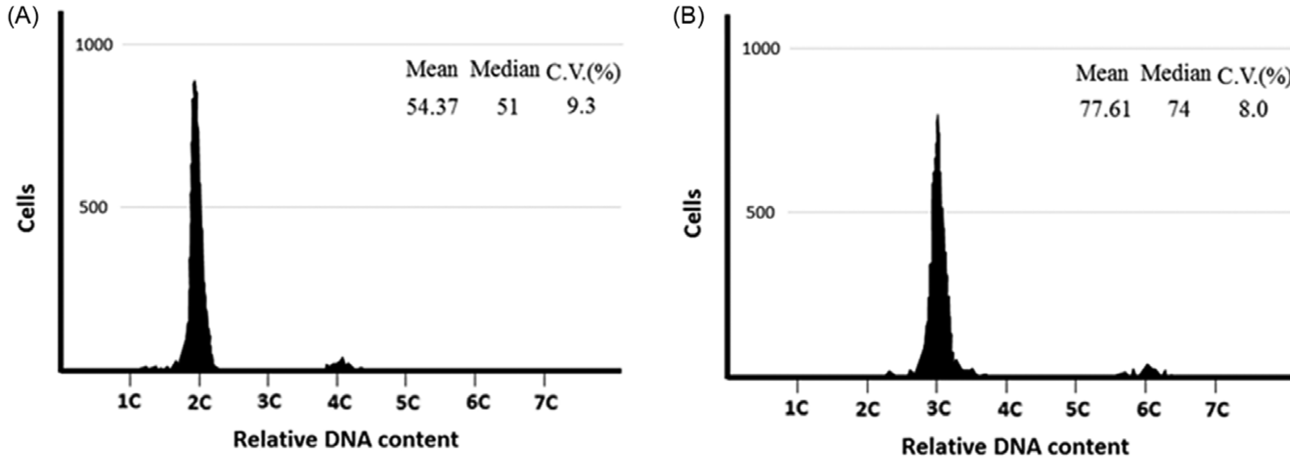

Figure 4. Flow cytometry histogram showing the relative DNA content of larvae from (A) cross 1 (female 2n × male 2n) diploid group and (B) cross 2 (female 4n × male 2n) triploid group *Astyanax altiparanae*.

Data on early development into 2-cell stage, blastula, gastrula, somite, and hatching of abnormal and normal larvae, as well as the respective ploidy status, are detailed in Table 1. Despite morphological differences during critical stages of development, cross 2 embryos showed similar survival when compared with cross 1 embryos. No differences between treatments were observed for survival at the 2-cell ($P = 0.6174$), blastula ($P = 0.9717$), gastrula ($P = 0.5301$), somite ($P = 0.3811$), and hatching ($P = 0.0984$) stages.

Confirmation of ploidy status

As expected, flow cytometry analysis showed that progenies from crosses 1 and 2 presented 100% diploid and triploid individuals, respectively (Table 1; Figures 3B, 4A). The same results were confirmed in the cytogenetic analysis. The larvae resulting from cross 1 had 50 metaphase chromosomes, and those from cross 2 had 75 chromosomes (Figure 5).

Discussion

In this study, for the first time in a neotropical species, triploid fish were obtained using tetraploid females and diploid males of *A. altiparanae*. These results are very interesting for fish aquaculture, as triploid females of yellowtail tetra present increased growth parameters when compared with diploids, such as carcass yield (do Nascimento *et al.*, 2017b) and sterility (do Nascimento *et al.*, 2017a). Therefore, the large-scale production of triploid (in special females) using tetraploids in this species could guarantee increased production and also reduce the risks of environmental impacts, as the possible escapes of sterile fish reduce the risks of introgression through hybridization (Benfey, 2016).

In aquaculture, tetraploid fish are an interesting alternative for the mass production of triploid fish, as observed in other studies (Nam and Kim, 2004; Weber *et al.*, 2014; do Nascimento *et al.*, 2020). Additionally, the protocols for triploid induction established by Adamov *et al.* (2017) and those currently used for triploid induction in *A. altiparanae* do not guarantee 100% of triploids. Therefore, the current protocol overcomes the deleterious effects of heat shock, and 100% triploidy fish were obtained.

However, lower percentages of normal larvae were observed in the triploid group. This unexpected result, conversely, does not limit the large-scale production of triploids by these methods because high hatching rates were still achieved. In the previous work of Weber *et al.* (2014), for example, the deformity was much lower in triploids derived from the cross of a tetraploid with a diploid than in shock-induced conditions. Therefore, we can attribute the results to other variables such as differences in reproductive performance in tetraploid and diploid females. Additionally, some studies have also shown that the first generation of tetraploids has lower reproductive potential (Chourrout 1984; Chourrout *et al.*, 1986; Myers, 1986; Blanc *et al.*, 1987, 1993; Hershberger and

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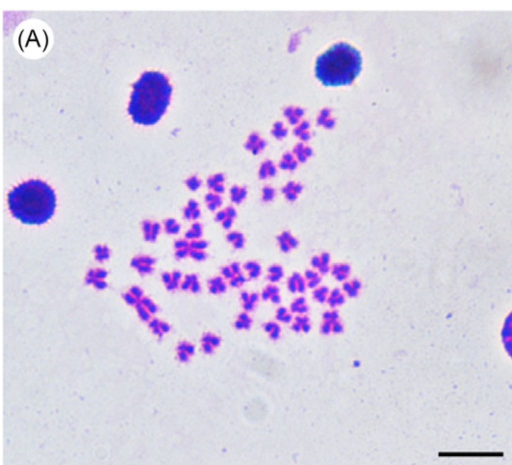

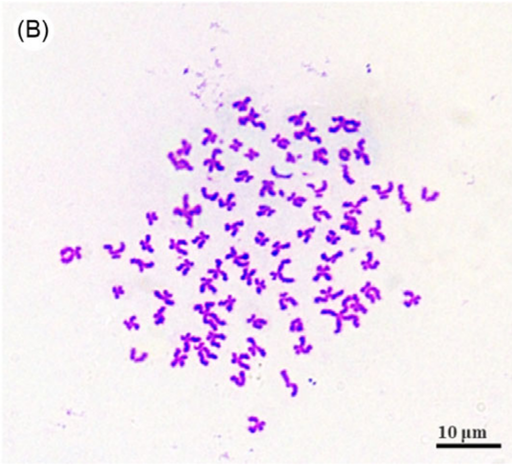

Figure 5. Karyotypes of larvae from (A) cross 1 (female 2n × male 2n) diploid group and (B) cross 2 (female 4n × male 2n) triploid group of *Astyanax altiparanae*.

Hostuttler 2005, 2007) and, subsequently, higher normality and egg quality were observed in the second generation of tetraploids. Therefore, the same results may be observed in our study and these effects must be addressed in future studies.

Fish ploidy was determined using flow cytometry and chromosome preparations (Allen, 1983; Xavier *et al*., 2017). As these methods present specific advantages (Piferrer *et al*., 2009), the combination of both techniques ensures the accuracy of the current study. Therefore, the cytogenetic method could be an interesting alternative when a flow cytometer is not available, due to the low cost. In conclusion, our results showed that tetraploid females of the yellowtail tetra *A. altiparanae* are fertile, present viable gametes after stripping and fertilization using dry methods, and may be used for mass production of triploids. This is the first report of these procedures within neotropical characins. These results are innovative and can be applied to other related species of economic importance.

Data availability statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements. The authors are grateful to CAPES #88882.426164/2019-01 and CTG (China Three Gorges Corporation) for the financial support of this research. We also acknowledge CEPTA/ICMBio for kindly providing the facilities and experimental fish.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines ethical standards on the care and use of laboratory animals.

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https://doi.org/10.1017/S0967199422000569 Published online by Cambridge University Press