Zygote

cambridge.org/zyg

Reproductive strategies and chromosomal aberrations affect survival in the Rivuliid fish *Hypsolebias sertanejo*

Mariana Machado Evangelista[1], Elizabeth Romagosa[2], Diógenes Henrique Siqueira-Silva[3], George Shigueki Yasui[4], Takafumi Fujimoto[5] and José Augusto Senhorini[4]

[1]Aquaculture Center, São Paulo State University, Jaboticabal, São Paulo, Brazil; [2]Fishery Institute, SAA, SP, Água Branca, São Paulo, SP, Brasil; [3]Group of Studies on the Reproduction of Amazon fish (GERPA/LANEC), Biology Faculty (FACBIO), University of South and Southern of Pará (Unifesspa), Marabá, Pará, Brazil; [4]National Center for Research and Conservation of Continental Fish, Chico Mendes Institute of Biodiversity Conservation, Pirassununga, São Paulo, Brazil and [5]Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Japan

Research Article

Cite this article: Evangelista MM *et al.* (2020) Reproductive strategies and chromosomal aberrations affect survival in the Rivuliid fish *Hypsolebias sertanejo*. *Zygote*. page 1 of 7. doi: 10.1017/S0967199420000362

Received: 7 April 2020
Accepted: 23 May 2020

Keywords
Biotechnology; Fish biology; Fish preservation; Mosaicism; Reproduction

Author for correspondence:
Diógenes Henrique Siqueira-Silva. Group of Studies on the Reproduction of Amazon fish (GERPA/LANEC), Biology Faculty (FACBIO), University of South and Southern of Pará (Unifesspa), Marabá, Pará, Brazil.
E-mail: siqueira.diogenes@gmail.com

Summary

Rivulidae comprises a family of fish largely distributed in Brazil that includes 201 species, of which 125 are considered endangered. This fact emphasizes the need for development of conservation strategies including studies on genetics and reproduction. In this paper, we describe aspects of biology and reproduction of the rivuliid species *Hypsolebias sertanejo*. We outline the reproductive behaviour of this species under laboratory conditions, analyze ploidy status by flow cytometry, describe reproductive behaviour and performance and test dry and wet incubation of eggs. Although *H. sertanejo* showed well known patterns of reproductive behaviour, we verified many peculiarities inherent to its reproductive biology. As expected, most individuals were diploid (87.71%), however 14.29% were considered mosaics. Although no sterility was observed within mosaics, infertility of these fish was not fully evaluated. Hatching rate of the eggs collected was very low following both dry and wet incubation (5.04 and 3.79%, respectively). These results provide interesting information regarding the reproductive success of this species, and suggest that chromosomal abnormalities described may reduce the survival of *H. sertanejo* under natural conditions, limiting the perpetuation of this species, and emphasizing the need for more preservation efforts, including artificial propagation and gene banking.

Introduction

Fish of the Rivulidae family are distributed across southern, North, Central and South America, and comprise about 450 valid species (Fricke *et al.*, 2018). They are sexually dimorphic freshwater fish, with precocious sexual maturation (Errea and Danulat, 2001). Popularly known as killifish, the rivulid species are small bodied animals (usually between 50 to 80 mm total length) presenting a great variety of colour patterns, and making them desirable for the aquarium fish trade (Costa, 2003).

Many rivulids are also known as annual fish, because they inhabit temporary shallow puddles that are formed only during the rainy season in the summer but that become dry for a longer periods during other seasons. Therefore, these fish reproduce and die over a short period of time (Costa, 2002) and eggs survive during this dry season due to the mechanism of diapause. Embryos arrest and hatch only in the next rainy season (Wourms, 2011). Because of this particular reproductive characteristic, many rivulids could become extinct following a short rainy season or an extended dry season, in addition to loss of habitat caused by anthropic actions such as urbanization, deforestation and drainage, which has increased the vulnerability of these species (Rosa and Lima, 2008). Just one genus of the Rivulidae family, *Hypsolebias*, represents at least 32 endangered species (ICMBio, 2018).

In this context, much research effort has been made to preserve rivulids, as knowledge in the fields of biology and ecology is necessary to establish protocols for species conservation and environmental reconstitution action (Volcan *et al.*, 2010, 2011). In this sense, studies on genetic characterization (Nascimento *et al.*, 2014; Kim *et al.*, 2016), ecology (Abilhoa *et al.*, 2010; Keppeler *et al.*, 2013), taxonomy (Costa *et al.*, 2012, Costa and Amorim, 2014) and reproduction (García *et al.*, 2008; Cassel *et al.*, 2013; Passos *et al.*, 2014; Schalk *et al.*, 2014) have already been performed for some Rivulidae species. They present a huge diversity of reproductive strategies that include external (Jara *et al.*, 1995; Volcan *et al.*, 2011) and internal fertilization (Costa *et al.*, 2016), in addition to the self-fertilization (Avise and Tatarenkov, 2015), a uniquely reported case among vertebrates.

CAMBRIDGE UNIVERSITY PRESS

The aim of this study was to describe aspects of *Hypsolebias sertanejo* biology, such as ploidy status, reproductive behaviour and performance. Moreover, we also performed experiments to determine the best conditions for incubation and hatching of eggs obtained from fish acclimated in the laboratory. The choice of *H. sertanejo* for such a study was motivated by its absence from the lists of threatened species. For ethical reasons, we avoided using endangered species, but the knowledge acquired here may be used for reproduction and conservation of other Rivulidae species, mainly those of the same genera (*Hypsolebias*, which total at least 32 endangered species (ICMBio, 2018)), as phylogenetic proximity facilitates the application of the protocols developed here.

Materials and methods

Field sampling and maintenance

Adult fish were caught in temporary ponds from Itacarambi city, Minas Gerais State, Brazil (15°4′56.4′′S, 44°5′17.9′′W) (licence number 48541) during March 2014 and March 2015. Sampling was performed using dip-nets (3 mm mesh size) and the sampled fish were distributed into plastic containers (2 litres), kept at 26°C, and taken to the laboratory where the experiments were performed. Fish were maintained in 60-litre glass aquaria in a recirculation system with the temperature set at 27°C, and photoperiod of 12 h of light. They were fed twice a day with earthworms and *Artemia salina nauplii*.

Ploidy analyses

Before the start of the behavioural and biotechnological experiments, to better understand the basic biology of the species, the ploidy status of the fish was checked using flow cytometry and a standard protocol for nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). A piece of the caudal fin from each animal was sampled (Xavier *et al.*, 2017) and analyzed using a Partec CyFlowPloidy Analyzer (Partec GmbH, Münster, Germany). Ploidy status was determined by comparing relative DNA content with a standard haploid DNA content (C) obtained from the sperm of *Astyanax altiparanae* fish species.

Experiment 1. Description of the reproductive behaviour

In this experiment, three couples of diploid animals were placed in three 60-litre glass aquaria containing a 1-litre transparent plastic recipient filled with sand, which was used as substrate for reproduction. The sand was washed and selected using a soil analysis sieve (0.42 mm diameter) to standardize the particle size. Aiming to better describe the reproductive behaviour of the species, video sequences using a DCR-DVD 308 camera (Sony, Japan) were recorded and digital images were obtained from them.

Experiment 2: Evaluation of reproduction

Diploid and mosaic fish were used to perform the reproduction experiment. They were divided into two groups for different treatments: Treatment 1: one diploid male was placed with three mosaic females; Treatment 2: one mosaic male was placed with three diploid females. The experiments took place in 60-litre glass aquaria.

Additionally, histological analysis of testes and ovaries from diploid and mosaic adult fish was performed. Briefly, gonads were removed, cut into transverse and longitudinal sections and fixed in Bouin's solution for 24 h. Samples were dehydrated in increasing concentrations of alcohol solution, embedded in Paraplast (Sigma-Aldrich, P3558), sectioned at 5.0-μm thickness on a microtome equipped with steel blade (LEICA RM 2235), and stained with haematoxylin and eosin. The material was examined using an optical microscope (Nikon, Eclipse Ci-L) and digital images were captured using NIS-Ar Elements software (Nikon, Tokyo, Japan) using a charge coupled device (CCD) camera (Nikon, DS-Fi1).

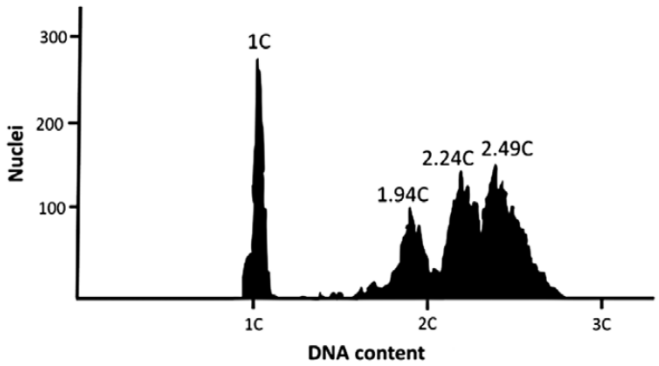

Figure 1. Relative DNA content of *H. sertanejo* compared against a standard haploid DNA content (1C) obtained from *A. altiparanae* sperm and against a standard diploid DNA content (1.94C) obtained from *H. sertanejo*. *H. sertanejo* tail cells had different DNA contents (2.24C and 2.49C).

Experiment 3: Optimizing conditions for hatching

Treatment 1: Hatching eggs under dry conditions

Seven couples of *H. sertanejo* were allocated for reproduction as described in Experiment 2. The resulting eggs (96 samples) were collected from March to June 2014 and transferred to 6-well microplates (35 mm diameter) filled with three different semi-humid substrates (1, coconut fibre; 2, felt made of polyester fibre wool and; 3, dishwasher sponge, mainly made of polyurethane) and kept in a biological oxygen demand (BOD) incubator in the dark at 25°C. After 30 days under those conditions, eggs were collected and counted to determine recovery rate. Eggs were immersed in distilled water, and hatching rate was measured.

Treatment 2: Hatching eggs at wet conditions

Seven males and 21 females were distributed into aquaria (1 male and three females in each aquarium) as previously described (see above). Eggs collected from May to July 2015 (26 samples) were transferred to 6-well microplates (35 mm diameter) filled with 15 ml ultrapure water, and kept in BOD incubator in the dark at 25°C. The microplates were checked daily to remove dead eggs and change the water. Hatching rates were measured for each egg batch.

Results

Ploidy analyses

Ploidy analysis indicated that 85.71% of the fish were diploid and 14.29% (one male and three females) showed chromosome mosaicism. In these animals, some of the analyzed somatic cells presented a DNA content of 2N and some showed a DNA content between diploidy and triploidy (Fig. 1).

Table 1. Stages of the reproductive behavior in *H. seertanejo*

Stage	Behavior	Image
I	Courtship display. Usually, the female stays close to the spawning area and waits for male pairing. In some cases, the roles are reversed and the male waits for the female in the spawning area	Fig. 1*A*
II	Invitation to submerge. The male accepts the invitation to spawn and pairs with the female. Then, followed by the female, the male starts to swim near the bottom and initiates an excavation by putting its snout down in the substrate	Fig. 1*B–D*
III	Submerging. The female couples with the male and they dig and submerge into the sand together	Fig. 1*E*
IV	Spawning and fertilization. The female is pressed against the bottom of the substrate by male and gametes are released and fertilized into the substrate	Figs. 1*F–H*
V	Emerging. After spawning, the male and female leave the substrate and separate. No parental care of the offspring occurs	Figs. 1*I–L*

Description of reproductive behaviour (Experiment 1)

Reproductive behaviour occurred spontaneously and was divided into five stages based on the description by Belote and Costa (2002) (Table 1, Fig. 2).

Evaluation of reproduction (Experiment 2)

When mosaic females were put together with a diploid male, spawning and fertilization occurred. Conversely, the mosaic male did not breed with diploid females and no reproductive behaviour was observed.

Histologically, ovaries and testes from diploid and mosaic animals did not show differences. In ovaries, most of the oocytes were perinucleolar oocytes, which are in primary growth (Fig. 3A). They had a central nucleus with an irregular border and many peripheral spherical nucleoli, basophilic cytoplasm, surrounded by zona radiata and follicle cells (Fig. 3B). oogonia (Fig. 3D) and the post-ovulatory follicle complex (POC) (Fig. 3C), which is formed by hypertrophied follicular cells; basement membrane and theca protruding towards the lumen after ovulation were also observed

Testes were lobular with spermatogonia restricted to the lobule periphery and spermatogenesis was cystic (Fig. 4A–C). A primary growth oocyte was found in the testicle of one male (Fig. 4D).

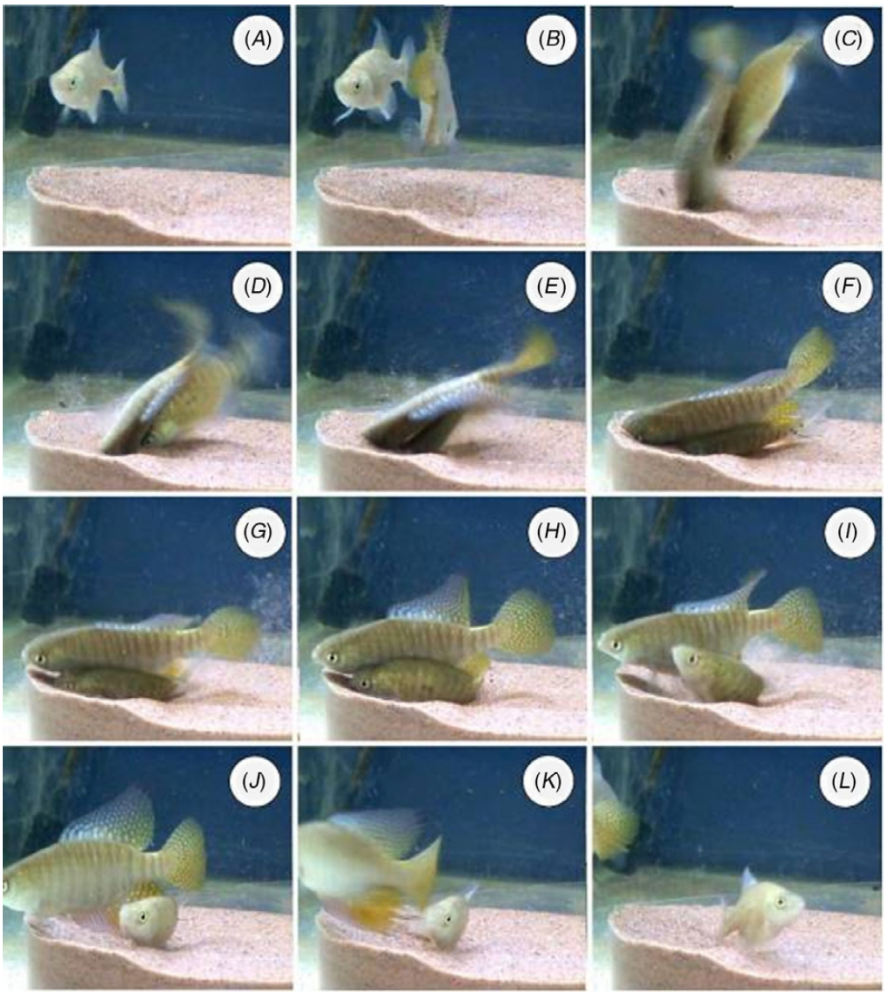

Figure 2. Spawning behavior of *H. sertanejo* divided in five stages. (*A*) Courtship displays. (*B*, *C*) Invitation to submerge. (*D*) Excavation. (*E*) Submerging. (*F–H*) Spawning and fertilization. (*I–L*) Emerging.

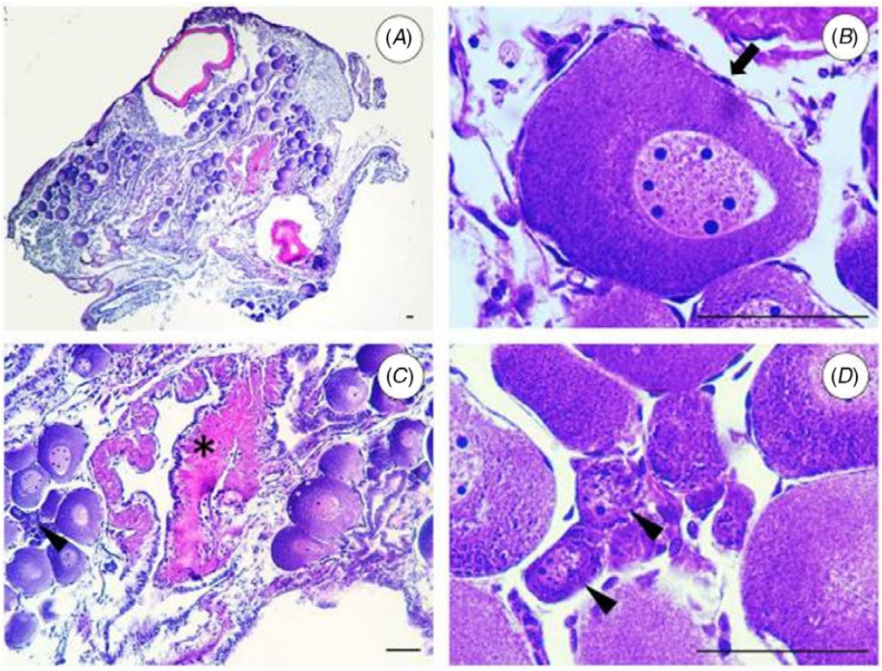

Figure 3. Morphology of *H. sertanejo* ovaries. (*A*) Overview. (*B*) Pre-vitellogenic oocyte in primary growth; arrow: follicular cell. (*C*) Asterisk: post-ovulatory follicle complex; arrowhead: oogonia. (*D*) Arrowheads: oogonia. Scale bars represent 50 µm.

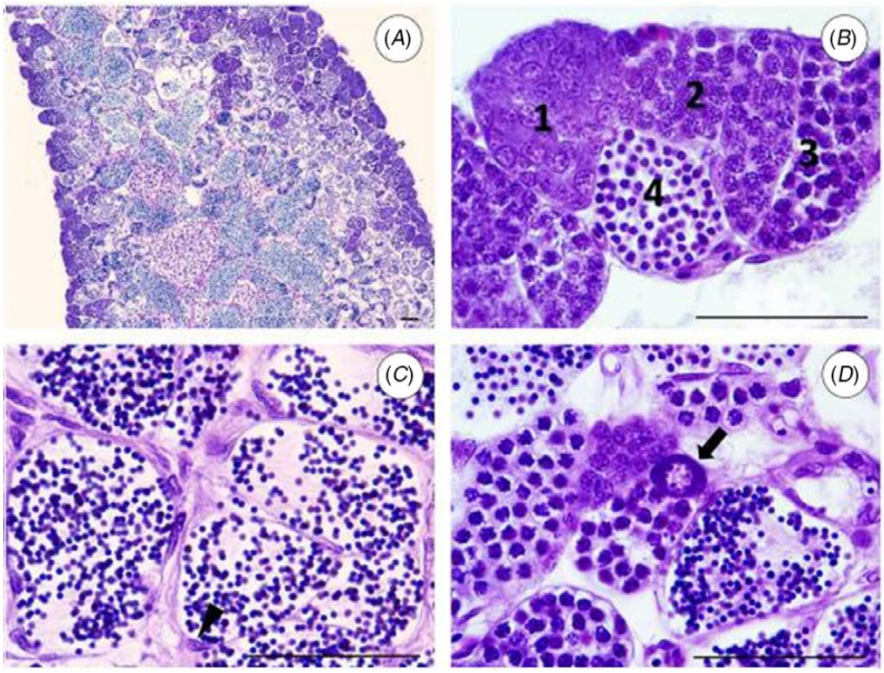

Figure 4. Morphology of *H. sertanejo* testes. (*A*) Overview. (*B*) 1. Spermatogonia. 2. Spermatocytes in pachytene phase. 3. Spermatocytes in diplotene phase. 4. Spermatids. (*C*) Spermatozoa into the lumen, arrowhead: Sertoli cell. (*D*) Arrow: oocyte in primary growth. Scale bars represent 50 µm.

Optimizing conditions for hatching (Experiment 3)

Treatment 1: Hatching eggs at dry conditions

During the experimental period 1403 eggs were collected, and females presented spawning peaks (Fig. 5). Eggs stored in felt and sponge substrates were not able to be recovered, whereas 18% of the eggs kept in coconut fibre were recovered. In total, 5.04% of the eggs hatched when immersed in distilled water.

Treatment 2: Hatching eggs at wet conditions

During the experimental period, 2137 eggs were collected and hatching rate was 3.79% (81 hatchings). Average time of incubation before hatching was 75.84 ± 27.23 days, varying from 29 to 128 days.

The occurrence of conjoined embryo was verified (Fig. 6A, B). Histological analysis showed that organs such as gills, guts, urinary and reproductive system were independent in each larvae (Fig. 6C, D), which were joined by connective tissue. Both larvae had gonads already differentiated in ovaries with pre-vitellogenic oocytes (Fig. 6E, F). Flow cytometric analysis from tails of the larvae showed that relative DNA content from cells of each tail was different (Fig. 7). Larvae 1 had DNA content as diploid (2C), whereas larvae 2 showed higher relative DNA content (2.15C).

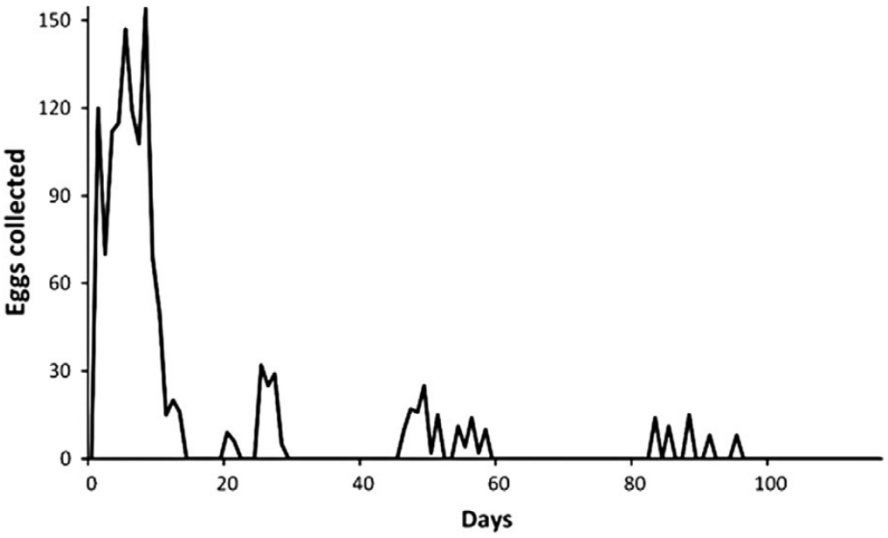

Figure 5. Number of *H. sertanejo* eggs collected daily during the experimental period.

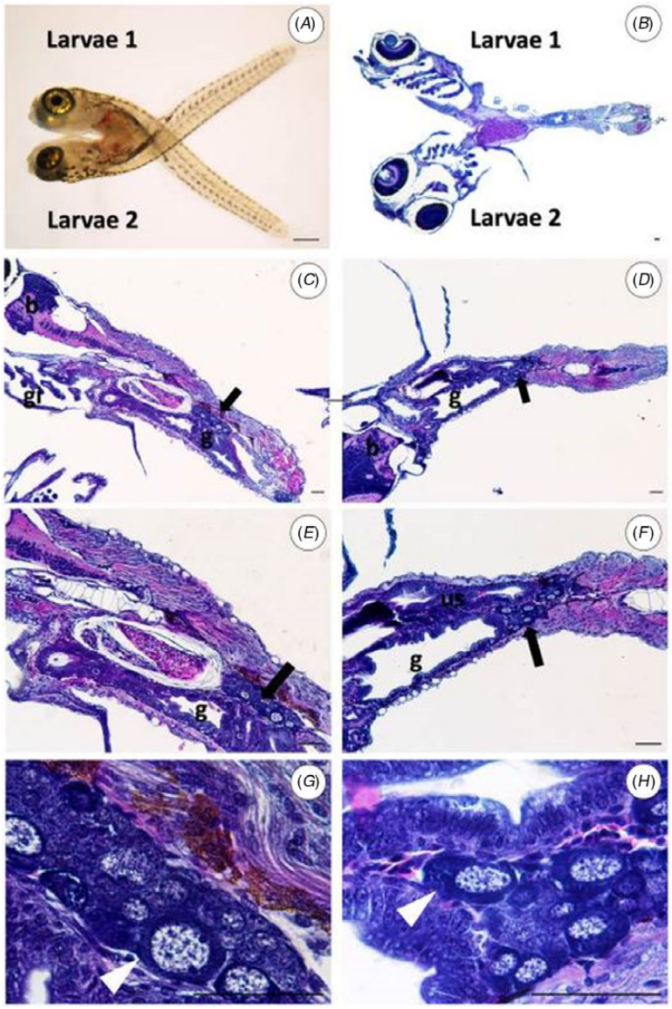

Figure 6. *Hypsolebias sertanejo* larvae connected. (*A*, *B*) Larvae 1 (l1) and 2 (l2). (*C*) Larvae 1, b: brain, g: gut, gi: gill. (*D*) Larvae 2, b: brain, g: gut. (*E*, *F*) us: urinary system. Arrows: ovary. (*G*, *H*) Arrowheads: pre-vitellogenic oocytes. Scale bars represent 50 μm.

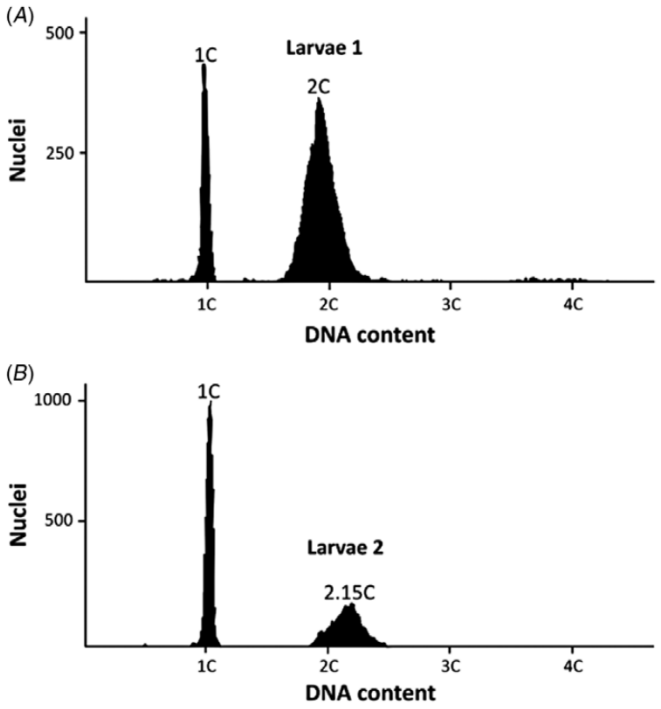

Figure 7. Relative DNA content of connected *H. sertanejo* larvae compared against a standard haploid DNA content (1C) obtained from *A. altiparanae* sperm. Larvae 1 (*A*) shows the DNA content as diploid (2C), in comparison larvae 2 (*B*) has a relatively higher DNA content (2.15C).

Discussion

The breeding protocol used here has proven to be successful for *H. sertanejo* and may be used as a model for other related species, including threatened ones. Although the reproductive behaviour of *H. sertanejo* presented similarities to other species of related genera, such as *Austrolebias reicherti* (García *et al.*, 2008.), *Cynolebias albipunctatus* (Belote and Costa, 2002) and *Simpsonichthys boitonei* (Shibatta, 2006), many peculiarities inherent to its reproductive biology could be observed.

Our data suggested that several chromosome abnormalities occur in this species such as mosaicism, polyploids and morphological abnormalities, including conjoined fish presenting different ploidy status. Mosaicism did not affect males regarding maturation of germ cells (sterility), as our results showed, but suggested infertility. The origin of this alteration is still unknown but naturally occurring polyploidy may arise from hybridization and production of unreduced gametes (Fujimoto *et al.*, 2008; Yasui *et al.*, 2009), thermal treatments after fertilization (Adamov *et al.*, 2017; Garcia-Abiado *et al.*, 2001; Kalbassi *et al.*, 2009; Xavier *et al.*, 2017) and delayed egg fertilization (Aegerter and Jalabert, 2004; Flajšhans *et al.*, 2007; Samarin *et al.*, 2015, 2016). The occurrence of such chromosomal abnormalities can lead to formation of aneuploidy gametes (Hamasaki *et al.*, 2013; Linhart *et al.*, 2006), which results in inviable eggs and may be related to the low hatching rates.

Hypsolebias sertanejo inhabits a limiting and selective environment that accelerates the speciation process. Such a phenomenon may explain a wide number of species even if the fish are morphologically similar and separated by small distances, as is the case for *H. sertanejo* and other species of the *H. flavicaudatus* complex such as *H. janaubensis*, as described by Costa and colleagues (Costa *et al.*, 2012). Such similarity suggested that hybridization may occur especially regarding interpopulation hybridization because, during the rainy season, ponds could be connected and then generate massive introgression. High temperatures may also affect the ploidy status in this species because fertilized eggs naturally enter diapause and embryos can remain for long periods in small wetlands at extreme temperatures (Markofsky and Matias, 1977). According to Costa and colleagues (Costa *et al.*, 2012), species of this genera are commonly found in temporary ponds that are more exposed to sunlight.

The conjoined embryo found in this study was of the same sex, but had a different ploidy status. Sex may be explained by the monozygotic origin that suggested a common genetic sex determination. Additionally, both embryos were maintained together under the same conditions and environmental sex determination was the same. The explanation for different ploidy status conversely is still unknown, but asychronic development within the blastoderm was observed in some embryos. This development may be related to partitioning of the blastoderm, generating these morphological and chromosomal differences in the conjoined embryos.

In conclusion, several reproductive, chromosomal and morphological problems arose in our study species. In addition, a very low percentage of the collected eggs survived as far as the hatching stage, suggesting that these deleterious effects may reduce the long-term survival of *H. sertanejo* under natural conditions, which limits the perpetuation of this species and emphasizes the need for more preservation efforts, including artificial propagation and gene banking for the killifish species.

Acknowledgements. The authors acknowledge CEPTA/ICMBio for gently providing experimental fish and the facilities.

Financial support. AES Tietê/ANEEL project 4690000174 and São Paulo Research Foundation (FAPESP, 2010/17429-1 and 2011/11664-1) for financial support.

Conflicts of interest. The authors declare no conflict of interest

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

References

Abilhoa V, Vitule JRS and Bornatowski H (2010). Feeding ecology of *Rivulus luelingi* (Aplocheiloidei: Rivulidae) in a coastal Atlantic rainforest stream, southern Brazil. *Neotrop Ichthyol* **8**, 813–8.

Adamov NSM, Nascimento NF, Maciel ECS, Pereira-Santos M, Senhorini JA, Calado LL, Evangelista MM, Nakaghi LSO, Guerrero AHM, Fujimoto T and Yasui GS (2017). Triploid induction in the yellowtail tetra, *Astyanax altiparanae*, using temperature shock: tools for conservation and aquaculture. *J World Aquacult Soc* **48**, 741–50.

Aegerter S and Jalabert B (2004). Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss Aquaculture* **231**, 59–71.

Avise JC and Tatarenkov A (2015). Population genetics and evolution of the mangrove rivulus *Kryptolebias marmoratus*, the world's only self-fertilizing hermaphroditic vertebrate, *J Fish Biol* **87**, 519–38.

Belote DF and Costa WJEM (2002). Reproductive behavior patterns in the neotropical annual fish genus *Simpsonichthys* Carvalho, 1959 (Cyprinodontiformes, Rivulidae): description and phylogenetic implications: Oficina Grafica da Universidade do Brasil.

Cassel M, Mehanna M, Mateus L, and Ferreira A (2013). Gametogenesis and reproductive cycle of *Melanorivulusaff punctatus* (Boulenger, 1895) (Cyprinodontiformes, Rivulidae) in Chapada dos Guimarães, Mato Grosso, Brazil. *Neotrop Ichthyol* **11**, 241–4.

Costa WJ (2002). The neotropical seasonal fish genus *Nematolebias* (Cyprinodontiformes: Rivulidae: Cynolebiatinae): taxonomic revision with description of a new species. *Ichthyol Explor Fresh* **13**, 41–52.

Costa WJ (2003). Family Rivulidae (South American annual fishes). In *Check List of the Freshwater Fishes of South and Central America* (eds Roberto E. Reis, Sven O. Kullander, and Carl J. Ferraris, Jr), p. 729. Edipucrs, Porto Alegre, Brazil.

Costa WJ and Amorim PF (2014). Integrative taxonomy and conservation of seasonal killifishes, *Xenurolebias* (Teleostei: Rivulidae), and the Brazilian Atlantic Forest. *Syst Biodivers* **12**, 350–65.

Costa WJ, Amorim PF and Mattos JL (2012). Species delimitation in annual killifishes from the Brazilian Caatinga, the *Hypsolebias flavicaudatus* complex (Cyprinodontiformes: Rivulidae): implications for taxonomy and conservation. *Syst Biodivers* **10**, 71–91.

Costa WJEM, Amorim PF and Mattos JLO (2016). Molecular phylogeny and evolution of internal fertilization in South American seasonal cynopoeciline killifishes. *Mol Phylogenet Evol* **95**, 94–9.

Errea A and Danulat E (2001). Growth of the annual fish, *Cynolebias viarius* (Cyprinodontiformes), in the natural habitat compared to laboratory conditions. *Environ Biol Fishes* **61**, 261–8.

Flajšhans M, Kohlmann K and Rab P (2007). Autotriploid tench *Tinca tinca* (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing *in vitro* and *in vivo*. *J Fish Biol* **71**, 868–76.

Fricke R, Eschmeyer WN and Van der Laan R (2018). *Catalog of fishes: genera, species, references.* California Academy of Sciences, San Francisco, CA, USA. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp.

Fujimoto T, Yasui GS, Yoshikawa H, Yamaha E and Arai K (2008). Genetic and reproductive potential of spermatozoa of diploid and triploid males obtained from interspecific hybridization of *Misgurnus anguillicaudatus* female with *M. mizolepis* male. *J Appl Ichthyol* **24**, 430–7.

García D, Loureiro M and Tassino N (2008). Reproductive behavior in the annual fish *Austrolebias reicherti* Loureiro & García 2004 (Cyprinodontiformes: Rivulidae). *Neotrop Ichthyol* **6**, 243–8.

Garcia-Abiado MAR, Lynch Jr WE, Dabrowski K and Hartman T (2001). Use of thermal and pressure shocks to induce triploid hybrid saugeyes. *N Am J Aquacult* **63**, 243–8.

Hamasaki M, Takeuchi Y, Miyaki K and Yoshizaki G (2013). Gonadal development and fertility of triploid grass puffer *Takifugu niphobles* induced by cold shock treatment. *Mar Biotechnol* **15**, 133–44.

ICMBio (2018). *Livro Vermelho da Fauna Brasileira Ameaçada de Extinção*: Vol. I / 1. edn. Brasília, MMA, 492 pp.

Jara F, Soto D and Palma R (1995). Reproduction in captivity of the endangered killifish *Orestias ascotanensis* (Teleostei: Cyprinodontidae). *Copeia* 1995, 226–8.

Kalbassi MR, Dorafshan S, Pourkazemi M and Amiri BM (2009). Triploidy induction in the Caspian salmon, *Salmo trutta caspius*, by heat shock. *J Appl Ichthyol* **25**, 104–7.

Keppeler FW, Lanés LEK, Rolon AS, Stenert C and Maltchik L (2013). The diet of *Cynopoecilus fulgens* Costa, 2002 (Cyprinodontiformes: Rivulidae) in Southern Brazil wetlands. *Ital J Zool* **80**, 291–302.

Kim BM, Lee BY, Lee JH, Rhee JS and Lee JS (2016). Conservation of *Hox* gene clusters in the self-fertilizing fish *Kryptolebias marmoratus* (Cyprinodontiformes; Rivulidae). *J Fish Biol* **88**, 1249–56.

Linhart O, Rodina M, Flajšhans M, Mavrodiev N, Nebesarova J, Gela D, and Kocour M (2006). Studies on sperm of diploid and triploid tench, *Tinca tinca* (L.). *Aquac Int* **14**, 9–25.

Markofsky J and Matias JR (1977). The effects of temperature and season of collection on the onset and duration of diapause in embryos of the annual fish *Nothobranchius guentheri*. *J Exp Zool* **202**, 49–56.

Nascimento WSD, Bezerra JG, Lima-Filho PA, Yamamoto ME, Chellapa S and Molina WF (2014). Karyotype patterns of *Hypsolebias antenori* (Cyprinodontiformes: Rivulidae): an endangered killifish of the semiarid region of Brazil. *Sci World J* **2014**, 862434.

Passos C, Tassino B, Reyes F and Rosenthal GG (2014). Seasonal variation in female mate choice and operational sex ratio in wild populations of an annual fish, *Austrolebias reicherti*. *PLoS One* **9**, e101649.

Rosa RS and Lima FCTL (2008). Os peixes brasileiros ameaçados de extinção, In *Livro Vermelho Da Fauna Brasileira Ameaçada de Extinção*, p. 1420.

Samarin AM, Policar T and Lahnsteiner F (2015). Fish oocyte ageing and its effect on egg quality. *Rev Fish Sci Aquac* **23**, 302–14.

Samarin AM, Blecha M, Uzhytchak M, Bytyutskyy D, Zarski D, Flajšhans M and Policar T (2016). Post-ovulatory and post-stripping oocyte ageing in northern pike, *Esox lucius* (Linnaeus, 1758), and its effect on egg viability rates and the occurrence of larval malformations and ploidy anomalies. *Aquaculture* **450**, 431–8.

Schalk CM, Montaña CG and Lisbon ME (2014). Reproductive strategies of two neotropical killifish, *Austrolebias vandenbergi* and *Neofundulus ornatipinnis* (Cyprinodontiformes: Rivulidae) in the Bolivian Gran Chaco. *Rev Biol Trop* **62**, 102–11.

Shibatta OA (2006). Comportamento social do pirá-brasília, *Simpsonichthys boitonei* Carvalho (Cyprinodontiformes, Rivulidae). *Rev Bras Zool* **2** 23, 375–80.

Volcan MV, Lanés LEK and Gonçalves AC (2010). Threatened fishes of the world: *Austrolebias univentripinnis* Costa and Cheffe 2005 (Cyprinodontiformes: Rivulidae). *Environ Biol Fishes* **87**, 319–20.

Volcan MV, Gonçalves AC and Lanés LEK (2011). Distribution, habitat and conservation status of two threatened annual fishes (Rivulidae) from southern Brazil. *Endanger Species Res* **13**, 79–85.

Wourms JP (2011). The developmental biology of annual fishes. III. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes. *Endanger Species Res* **13**, 79–85.

Xavier PL, Senhorini JA, Pereira-Santos M, Fujimoto T, Shimoda E, Silva LA, Dos Santos AS and Yasui GS (2017). A flow cytometry protocol to estimate DNA content in the yellowtail tetra *Astyanax altiparanae*. *Front Genet* **8**, 131.

Yasui GS, Arias-Rodriguez L, Fujimoto T and Arai K (2009). A sperm cryopreservation protocol for the loach *Misgurnus anguillicaudatus* and its applicability for other related species. *Anim Reprod Sci* **116**, 335–45.