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Triploid or hybrid tetra: Which is the ideal sterile host for surrogate technology?

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ABSTRACT

This work was aimed at developing an effective procedure to obtain sterile ideal host fish in mass scale with no endogenous germ cells in the germinal epithelium, owning permanent stem-cell niches able to be colonized by transplanted germ cells in surrogate technology experiments. Thus, triploids, diploid hybrids, and triploid hybrids were produced. To obtain hybrid offspring, oocytes from a single *Astyanax altiparanae* female were inseminated by sperm from five males (*A. altiparanae, A. fasciatus, A. schubarti, Hyphessobrycon anisitsi, and Oligosarcus pintoi*). Triploidization was conducted by inhibition of the second polar body release using heat shock treatment at 40 °C for 2 min. At 9-months of age, the offspring from each crossing was histologically evaluated to access the gonadal status of the fish. Variable morphological characteristics of the gonads were found in the different hybrids offspring: normal gametogenesis, gametogenesis without production of gametes, sterile specimens holding germ cells, and sterile specimens without germ cells, which were considered "ideal hosts". However, only in the hybrid derived from crossing between *A. altiparanae* and *A. fasciatus,* 100% of the individuals were completely sterile. Among them 83.3% of the male did not present germ cells inside germinal epithelium, having only somatic cells in the gonad. The other 16.7% also presented spermatogonia inside the niches. Such a methodology allows the production of sterile host in mass scale, opening new insights for application of surrogate technologies.

1. Introduction

Germ cell transplantation in fish is an interesting reproductive technology that allows for one fish species to produce gametes from another [1]. Such a technique, also known as surrogate technology, involves the production of heterologous gametes being applicable in aquaculture and conservation since rare strains or species may be produced from farmed or laboratory fish in which reproductive aspects are well established. Genebanking using surrogate technologies is effective since genetic variability and maternal components, such as mitochondrial DNA and germ plasm, are maintained [2].

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In order to perform such a surrogate production, it is first necessary to produce a germline chimera in which the germ cell lineage from a donor species is transplanted to a target sterile host species, whose endogenous gametogenesis is suppressed, to ensure a restrictive maturation, exclusively of the transplanted germ cells.

Hybrid and triploid fish are well known to be sterile for several species [3]. Yamaha et al. [4], for example, used hybrid fish and transplanted PGCs that successfully colonized genital ridges and followed spermatogenesis. Similarly, Okutsu et al. [5] transplanted spermatogonial stem cells from rainbow trout into sterile triploid masu salmon *O. masou,* and only gametes from donor species were obtained after sexual maturation. However, the gonads of some triploid species and hybrids are occupied by endogenous germ cells [6,7]. In such cases, it theoretically may be a limitation for the colonization of the transplanted cells, since an ideal host must present a sterile gonad without germ cells because the niches are necessary for colonization, proliferation, and maturation of the transplanted germ cells. This may increase the colonization success and ensure the production of heterologous gametes.

Thus, the aim of the present study was to establish an appropriate host, which can be produced in large scale for surrogate technologies in threatened fish for example, such as the piracanjuba *Brycon orbgnyanus.* This particular fish is currently listed as critically endangered and at high risk of extinction in the wild in the near future, according to the Red Book of Brazilian Fauna Threatened with Extinction [8]. Given this, five tetra species (*Astyanax altiparanae, A. fasciatus, A schubarti, Oligosarcus pintoi, and Hyphessobrycon anisitsi*) were chosen for their good livestock characteristics, such as ease of handling, small size, early sexual maturity, and intertidal spawning, which make them appropriate models for the development of such a methodological approach.

2. Material and methods

2.1. Origin of broodstock and gamete sampling

All the procedures were performed in line with the Ethical Committee for the Use of Laboratory for the Care and Use of Laboratory Animals in Chico Mendes Institute (CEUA - CEPTA #02031.000033/2015–11).

The yellowtail tetra (*Astyanax altiparanae*) used in this study were collected from the Mogi Guaçu river (21.925706 S, 47.369496 W) and maintained in 1000 m^2 earthen ponds (≈500 fish per tank, SL ≈ 12 cm). As this species spawn spontaneously, F1 offspring were produced within few months and then were used fish in this work. Adult males from *A. schubarti* (≈6 cm), *A. fasciatus* (≈8 cm), and *Oligosarcus pintoi* (≈5 cm) were collected in the Mogi Guaçu River and maintained in 600-L aquariums for sperm sampling. Albino males of *Hyphessobrycon anisitsi* (≈5 cm) were obtained from a commercial fish dealer. Males were identified by the bony hooks on the anal fin, which can be also identified by gently hand-touching. Some of *Oligosarcus pintoi* males did not present bony hooks and were identified only by external morphology, in which the males are smaller and thinner than females.

Ovulation in *A. altiparanae* was induced using the previous protocol of this lab [9]. Briefly, ovulation of females from *A. altiparanae* (SL ≈ 9 cm) and spermiation of males from other species were induced by a single dose of carp pituitary gland (5 mg kg^{-1}). After 10 h at 28 °C, the fish were anesthetized using menthol (Êxodo, Científica) at 100 mg L^{-1}, and the gametes were collected by stripping. The sperm was collected using a 1000 μl micropipette (Eppendorf, Hamburg, Germany). The sampled sperm was transferred to a 1.5 mL tube containing 300 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. The sperm quality was immediately assessed on the microscope after striping using another previous protocol [9], and sperm samples with progressive motility above 80% were used for fertilization trials.

Oocytes were stripped on a plastic Petri dish (90 mm diameter) covered by a polyvinylidene chloride film (saran wrap, Alpfilm, São Paulo, Brazil). Oocytes were covered by a small piece of the same polyvinylidene chloride film to avoid dehydration until the fertilization trials, which was performed right after oocyte extrusion.

2.2. Triploidization and juvenile rearing

Oocytes from a single female (≈8000 oocytes) were divided into 5 Petri dishes and inseminated using 80 μl of sperm from 5 males (*A. altiparanae, A. fasciatus, A. schubarti, H. anisitsi, O. pintoi*), respectively. Gamete activation was achieved by adding 5 mL of water and, then, immediately mixed by gentle hand mixing. Each egg batch was divided into two plastic containers in which the bottom contained a 100 μm nylon mesh and maintained at 22 °C. One egg batch from each was induced to triploidization, and the remaining egg batch were kept intact. Triploidization was performed using a previous protocol of this lab [10], using the timing obtained by cytological observation that showed that extrusion of 2nd polar body occurs at 8 mpf [11]. At 2 min post fertilization (mpf), the eggs were heat shocked at 40 °C for 2 min. After heat shocking, the eggs were immediately transferred to incubation at 25 °C. Hatching took place at 12 h post-fertilization (Fig. 1).

Juveniles were maintained in 40 L aquariums in a recirculation system. The temperature was set at 28 °C and 12 h of light. After 3 days post fertilization, the juveniles were fed three times a day using new-hatched *Artemia salina* nauplii for 10 days, and after this period, the fish were fed with powdered commercial food (4200 kcaL kg-1 and 45% crude protein). As the fish grew up, the feeding was changed to 1 mm-pellets and natural plankton until the end of the experiment.

2.3. Ploidy confirmation

The ploidy status from the adult fish was measured from each cross by flow cytometry using the developed protocol for preservation and analysis from this lab [12]. Briefly, small pieces of fin (≈2 mm^2) were clipped and frozen in 0.9% NaCl for future analysis. Later, the samples were thawed, and the NaCl solution was replaced by a lysing solution (9.53 mM $MgCl_2.7H_20$; 47.67 mM KCl; 15 mM Tris; 74 mM sucrose, 0.6% Triton X-100, pH 8.0) and vortexed. After 30 min, 1.5 mL of staining solution (calcium-free Dulbecco's PBS with 4′,6-diamidino-2-phenylindole - DAPI at 1 μg mL^{-1}) was added and immediately filtered by a 30 μm mesh (Celltrics, Partec, Münster, Germany). Stained samples were then analyzed by a Partec CyFlow Plody Analyzer (Partec GmbH, Münster, Germany) with a specific filter set for DAPI excitation (358 nm). The relative DNA content was determined based on the histograms in which diploid *A. altiparanae* were used as controls.

2.4. Histological analysis

For histological analysis, the specimens were anesthetized using 2-Phenoxy-Ethanol ($C_6H_{10}O_2$ – SIGMA-ALDRICH), their gonads were removed, cut into transverse and longitudinal sections, and fixed with Bouin's fixative (Picric Acid P.A., Ethyl alcohol 96% or 99%, Formaldehyde P.A., Glacial Acid Acetic – Cinetica; Adria laboratories, Brazil) for 24 h. Samples were dehydrated in grade ethanol solution, embedded in paraffin – polyisobutylene mixture (Paraplast®, SIGMA-ALDRICH), sectioned at 3.0 μm on a microtome (Leica RM2235, Germany) equipped with steel blade (Leica 818), and sections were then stained with hematoxylin and eosin. All

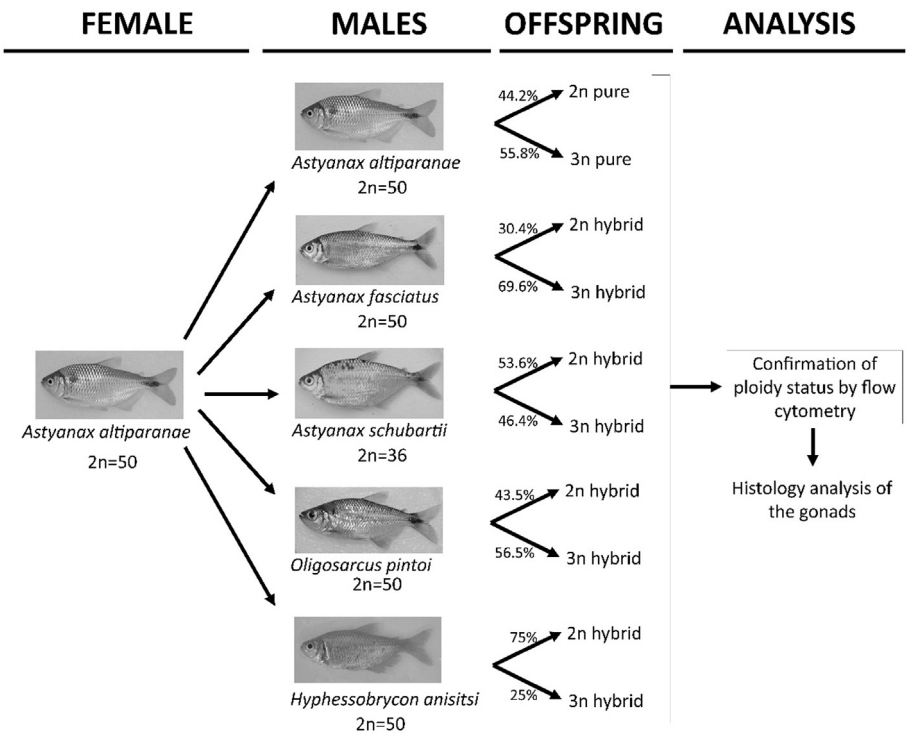

Fig. 1. Draft of the experimental design for hybridization and triploidization experiments. Offispring numbers means the percentage of individuals that hatch as diploid or triploid after the crosses.

material was microscopically examined on a microscope (Nikon-Eclipse CI, Japan), digital images were captured with a CCD camera (Nikon DSF1, Nikon, Tokyo, Japan), and analyzed with NIS-Elements AR software (Nikon, Tokyo, Japan). Four morphological types of gonads were observed among the offspring: 1) Normal gametogenesis (specimens showing normal gametogenesis; 2) No gametes (specimens showing normal gametogenesis without production of gametes; 3) Sterile with germ cells (specimens showing mainly oogonia, in case of female, and spermatogonia, in case of male, into their gonads); and 4) Sterile without germ cells (specimens showing only somatic cells into their gonads).

3. Results

Table 1 summarizes embryo development and survival rates of offspring resulting from the different crosses of specimens originating from hybridization and triploidization processes. The number of hatching larvae presenting normal shape was very similar among most of the treatments, except the crosses involving triploid of *H. anisitsi* that had a higher number of individuals (282) and *O. pintoi* diploid and triploid that showed the lowest number of surviving specimens 77 and 67, respectively.

In Table 2, it is possible to observe that *A. altiparanae* crosses did not cause deviation in the 1:1 sex ratio for both diploid ($x^2 = 1.316$) and triploid ($x^2 = 2.666$) fish. However, in other crosses involving *A. fasciatus* diploids ($x^2 = 7$) and triploids ($x^2 = 4$), *A. schubarti* triploid ($x^2 = 6.231$), and *O. pintoi* triploids ($x^2 = 9.308$) resulted in an increased number of male.

In Fig. 2, is possible to observe the main morphological characteristics of the gonads in the resultant offspring.

Table 1
Embryo development and survival rate of the offspring from each cross between *Astyanax altiparanae* female and the male of the other studied species.

Species		Proportion 2n/3n	Number of eggs used	Blastula stages (%)	Gastrula stages (%)	Segmentation stages (%)	Hatching (%)	Normal larvae (%)	Normal larvae
Astyanax altiparanae	Control	57/3	359	86,39 ± 4,07	79,91 ± 6,10	76,44 ± 7,40	64,17 ± 9,58	77,42 ± 9,39	176
	Heat shock	8/52	413	81,98 ± 1,98	81,39 ± 1,72	77,97 ± 2,87	75,54 ± 2,90	76,27 ± 10,15	223
Astyanax fasciatus	Control	55/2	304	86,05 ± 6,07	84,74 ± 5,87	83,07 ± 5,51	75,57 ± 9,00	90,91 ± 4,26	209
	Heat shock	1/39	360	92,24 ± 0,78	89,30 ± 1,50	84,55 ± 2,87	67,40 ± 5,18	66,01 ± 16,35	149
Astyanax schubarti	Control	35/3	292	81,59 ± 3,98	80,04 ± 2,43	79,01 ± 1,40	76,43 ± 1,18	92,04 ± 0,26	115
	Heat shock	9/31	452	75,55 ± 9,35	70,62 ± 7,24	68,54 ± 7,97	61,76 ± 4,01	86,14 ± 6,55	194
Oligosarcus pintoi	Control	28/0	283	79,63 ± 9,73	67,90 ± 2,31	64,71 ± 0,88	61,52 ± 4,07	81,69 ± 15,03	77
	Heat shock	4/31	220	77,99 ± 15,34	74,29 ± 14,05	70,44 ± 16,22	59,61 ± 5,39	79,74 ± 0,26	67
Hyphessobrycon anisitsi	Control	60/0	339	78,40 ± 10,40	76,74 ± 9,66	76,16 ± 9,47	71,95 ± 8,19	85,31 ± 10,71	202
	Heat shock	13/42	520	76,87 ± 10,08	74,70 ± 10,00	71,09 ± 11,52	66,13 ± 11,93	81,90 ± 8,12	282

Table 2
Number of individuals by sex in each cross between *Astyanax altiparanae* female and the male of the other studied species. Asterisks indicate a deviation from the expected 1:1 sex ratio by the chi-square test ($\chi^2<0.05$).

	Astyanax altiparanae		*Astyanax fasciatus*		*Astyanax schubarti*		*Oligosarcus pintoi*		*Hyphessobrycon anisitsi*	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Diploid	7	12	7	0*	11	4	6	4	8	13
Triploid	8	16	12	4*	11	2*	12	1*	6	1

3.1. *A. altiparanae x A. altiparanae crossing*

As expected, diploid *A. altiparanae* progeny were not sterile. Males presented the entire germ cell lineage including spermatogonia, spermatocytes, spermatids, and fully-grown spermatozoa (S1, Table 3). Similarly, females presented previtellogenic and vitellogenic oocytes (Normal gametogenesis) (S1, Table 3). However, triploid *A. altiparanae* progeny presented some gametogenesis abnormality. All the male specimens presented apparently normal spermatogenesis, but no spermatozoa were visible in the lumen (No gametes). Furthermore, seminal fluid, which normally appear in diploid fish testes dyed by eosin (S1), was not found in the lumen in these triploid fish testes (S1, Table 3). Regarding the females, 14 out of 16 were sterile, presenting ovaries full of oogonia with rare previtellogenic, but there were no vitellogenic oocytes (S1, Table 3). The other two females, similar to diploid specimens, presented normal oogenesis (Table 3).

3.2. *A. altiparanae x A. fasciatus crossing*

Diploid progeny of *A. altiparanae* x *A. fasciatus* crossing were all male. One out of the seven individuals showed normal spermatogenesis (S2, Table 3), five of them were sterile presenting the epithelium filled only by spermatogonia (S2, Table 3), and one specimen showed no germ cells into the seminiferous compartment, which was composed only by somatic cells (S2, Table 3). In relation to triploid progeny, all of them were sterile, being that two out of 12 males presented only spermatogonia inside the germinal epithelium, similar to the diploid sterile, and the other 10 individuals held no germ cells (S2, Table 3). Female ovaries were very thin, showing only oogonia in the epithelium (S2, Table 3).

3.3. *A. altiparanae x A. schubarti crossing*

In diploid hybrids resulting from the *A. altiparanae* and *A. schubarti* crossing, one out of four females was sterile, having ovaries full of oogonia and rare previtellogenic and vitellogenic oocytes (S3, Table 3). The other three specimens showed normal oogenesis (S3, Table 3). Among the males, six specimens were sterile, presenting filiform testes filled only by spermatogonia (S3, Table 3), and five of them, such as the triploid *A. altiparanae,* presented apparently normal gametogenesis, but there were no fully grown spermatozoa inside the lumen. However, different from that of triploid *A. altiparanae*, these individuals had seminal fluid inside the luminal compartment (S3, Table 3). Among triploid female, only two individuals arise, one showing normal oogenesis and another one was sterile. Their ovaries presented characteristics that were very similar to that of diploid females. Among triploid males, six out of 11 specimens presented normal spermatogenesis without spermatozoa inside the lumen. For the diploid ones, three out of them were sterile, presenting some endogenous spermatogonia inside the germinal compartment (S3, Table 3), and the other two individuals had no germ cells inside the testes (S3, Table 3).

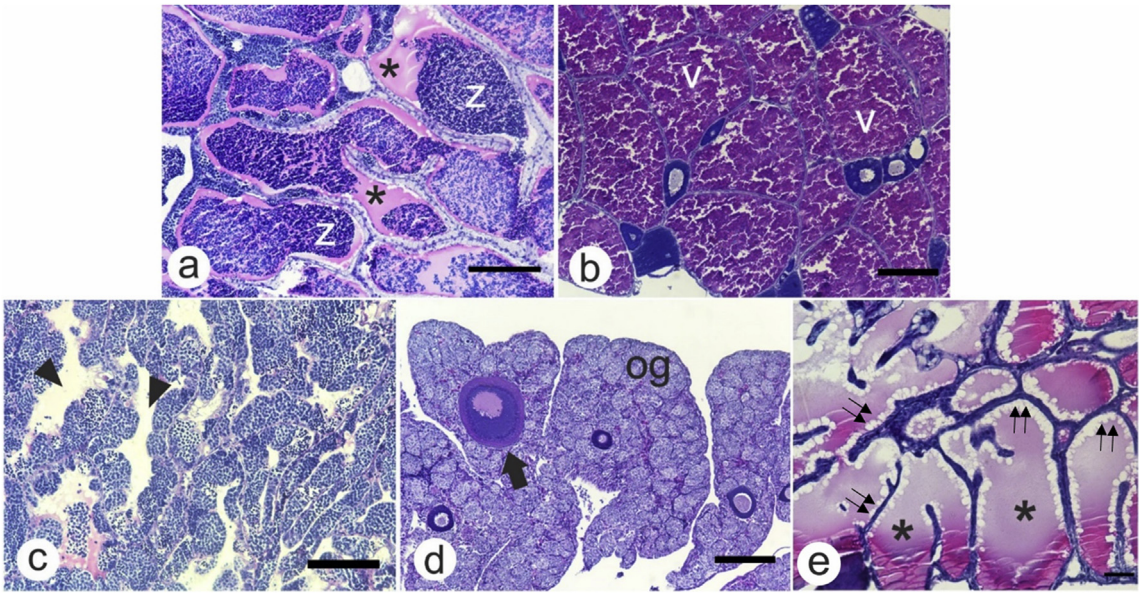

Fig. 2. a) testes of diploid fish with normal gametogenesis, showing spermatozoa **(Z)** and seminal fluid inside the lumen; **b)** Ovary of diploid fish showing normal oogenesis with many vitellogenic oocytes **(v)**; **c)** Sterile testes of triploid fish showing abnormal spermatogenesis and no spermatozoa or seminal fluid inside the lumen (arrowhead); **d)** Sterile ovary with abnormal oogenesis, full of oogonia (Og), rare previtellogenic (arrow) and no vitellogenic oocyte; **e)** testis of a sterile fish showing the total absence of germ cell, presenting a prominent lumen (asterisks), and free space for transplanted germ cell colonization (double arrow).

Table 3
Specimens resulting from artificial fertilization between *Astyanax altiparanae* female and male of the other studied species. (n = number of individuals).

Diploid specimens	*Astyanax altiparanae*		*Astyanax fasciatus*		*Astyanax schubarti*		*Oligosarcus pintoi*		*Hyphessobrycon anisitsi*	
Gametogenesis	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Normal	7	12	1	0	0	3	1	1	0	0
No gametes	0	0	1	0	5	0	5	0	8	0
Sterile with germ cells	0	0	4	0	6	1	0	3	0	13
Sterile without germ cells	0	0	1	0	0	0	0	0	0	0
Triploid specimens										
Gametogenesis	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Normal	0	2	0	0	0	1	2	0	4	1
No gametes	8	0	0	0	6	0	10	0	2	0
Sterile with germ cells	0	14	2	4	3	1	0	1	0	0
Sterile without germ cells	0	0	10	0	2	0	0	0	0	0

Normal = Specimens showing normal gametogenesis.
No gametes = Specimens showing normal gametogenesis without production of gametes.
Sterile with germ cells = Specimens showing mainly oogonia, in case of female, and spermatogonia, in case of male, into their gonads.
Sterile without germ cells= Specimens showing only somatic cells into their gonads.

3.4. *A. altiparanae x Oligosarcus pintoi crossing*

Among diploid male hybrid between *A. altiparanae* and *Oligosarcus pintoi,* one out of six individuals showed normal spermatogenesis (S4, Table 3), and the other five specimens presented normal gametogenesis without any spermatozoa in the lumen (S4, Table 3). Seminal fluid was also observed. Among diploid females, one presented normal oogenesis (S4, Table 3), and the other three were sterile, showing very thin ovary, filled with oogonia and scarce previtellogenic oocytes (S4, Table 3). Among triploid males, two out of 12 had normal spermatogenesis, and ten fish showed no spermatozoa inside the lumen (similar to the diploid one). Among females, one out of four had normal oogenesis, and the other three had ovaries that were sterile but had germ cells (S4, Table 3).

3.5. *A. altiparanae x Hyphessobrycon anisitsi crossing*

Male diploid hybrids between *A. altiparanae* and *Hyphessobrycon anisitsi* presented all testes with normal spermatogenesis but no spermatozoa inside the lumen (S5, Table 3), and all the females were sterile, presenting some previtellogenic oocytes and many oogonia (S5, Table 3). Among triploid males, four out of six had normal spermatogenesis (S5, Table 3), and the other two presented apparently normal spermatogenesis without spermatozoa inside the lumen. The single female presented normal oogenesis (S5, Table 3).

4. Discussion

In this study, a combination of various hybrids associated with triploidization was evaluated in order to achieve ideal sterile fish to be used as host in surrogate technology programs.

Surprisingly, some triploid females of *A. altiparanae* presented vitellogenic oocytes at 9-months-age, indicating that triploidization does not ensure permanent sterilization in this species. In previous works from this lab, a histological analysis of the same kind of triploids revealed 100% sterile females at 6-months of age. It suggests that aging may recover fertility within *A. altiparanae* triploids, as similarly observed in other teleost [13,14]. *Astyanax altiparanae* triploid males presented apparently normal gametogenesis, showing the entire lineage of germ cells (spermatogonia, spermatocytes, spermatid). There were no fully-grown spermatozoa inside the epithelium, showing that the differentiation of spermatid into spermatozoa during spermiogenesis is affected by triploidization process. Thus, triploids may not be effective for surrogate technologies because females recover fertility, and there is no space on male gonads epithelium.

On the other hand, all of the 13 female hybrids that resulted from *A. altiparanae* and *H. anisitsi* crossing were sterile, and the triploid hybrids offspring arising from *A. altiparanae* and *A. fasciatus* were 100% sterile (n = 16) for both males and females, in addition to presenting not fertility recovery with aging. However, some of them presented endogenous germ cells that occupied the stem cell niche, and this fact theoretically may reduce the colonization success of the transplanted cells. In order to develop exogenous (transplanted) germ cells in a host gonad, several factors related to physiological and histological compatibility are necessary.

The testes of most of the triploid hybrid offspring between *A. altiparanae* x *A. fasciatus* (10 out of 12) did not present germ cells, although all the remaining supporting cells including Leydig, Sertoli, myomeres, vases, and other somatic cells were present. Since, supposedly transplanted germ cells may find adequate space and supporting cells in order to proceed gametogenesis, these offspring specimens were considered as ideal hosts.

In conclusion, four types of diploid hybrids and triploid hybrids were evaluated, and the production of a permanent sterile fish was achieved within triploid hybrids. Such a technique depends however on the combination of the parental crosses and only one cross produced such an ideal sterile fish. As established for the first time, an effective procedure applicable for mass production of host species presenting permanent stem-cell niches that may be colonized by the transplanted cells was achieved. Such a methodology opens new insights for application of surrogate technologies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.theriogenology.2017.12.013.

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