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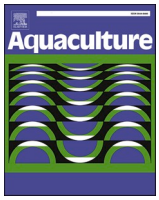

Development of a pressure-induced triploidy protocol and its effects on growth performance and fertility in tambaqui *Colossoma macropomum* (Cuvier, 1816)

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ABSTRACT

We evaluated combinations of post-fertilization timing and shock duration using 8000 psi hydrostatic pressure to develop an efficient protocol for inducing triploidy in tambaqui *Colossoma macropomum* (Cuvier, 1816). Two trials were conducted. In the first (T1), newly fertilized eggs were exposed to 8000 psi for 120 s, initiated at 65 (T1.1), 95 (T1.2), 125 (T1.3), 155 (T1.4), 185 (T1.5), and 312 (T1.6) seconds post-fertilization (spf), with untreated eggs serving as control. All treatments produced high triploidization rates (>93%), but shocks applied at 65 and 95 spf (T1.1 and T1.2) resulted in higher fertilization (FGS >90%) and embryo survival indexes (ES >87%) than later treatments.

Based on these results, a second trial (T2) optimized shock duration at 8000 psi: 65 spf for 60 s (T2.1) and 90 s (T2.2), and 95 spf for 60 s (T2.3) and 90 s (T2.4), with a control group. Triploidy rates were 56% in T2.1 and > 94% in T2.2–T2.4. FGS and ES were highest in T2.3 and T2.4. Growth evaluation of diploid (control; two ponds) and treated (combined T2 groups; two ponds) progenies reared in earthen ponds showed that triploids exhibited significantly greater body weight, height, and total and standard lengths during the first six months. Histological examination of 20-month-old progenies confirmed that triploids were infertile, whereas diploids reached sexual maturation.

This study establishes a reliable 8000 psi pressure-shock protocol for producing sterile triploid *C. macropomum*, supporting its application in sustainable large-scale aquaculture.

1. Introduction

The volume of farmed fish production has increased eight-fold over the last three decades worldwide and the volume of farmed aquatic species has surpassed the volume of aquatic products from capture fisheries (FAO, 2024). The expansion of aquaculture has been primarily driven by the increasing global demand for high-quality protein; however, this growth is accompanied by a range of environmental challenges. One major concern is the potential genetic introgression between cultured and wild fish populations, particularly involving hybrid or genetically modified strains. Such genetic exchanges may occur through escape events or subsequent interbreeding during natural spawning in open-water systems. (Gross, 1998; Ottera et al., 2016; Hashimoto et al., 2012). Further, puberty also results in decreased growth, inferior flesh quality and compromised disease resistance & welfare which lead to huge economic loss for the fish farmers (Taranger et al., 2010).

One way to tackle the negative environmental impacts and economic loss is the production of sterile fish. In fish farming, the production of

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sterile individuals can be extremely advantageous, since sexual maturation is generally associated with reduced weight gain and growth, greater susceptibility to diseases, aggression among them and reduced meat quality (Arai, 2001; Peruzzi et al., 2004; Taranger et al., 2010; Ottera et al., 2016). While new molecular methods such as ablation of primordial germ cells (PGC) by depletion of Dead end (Dnd) are developing, it is in its early stage of R&D (Wong and Zohar, 2015). Triploidization has been used to develop sterility in aquatic species including fish over the several decades (Thorgaard and Gall, 1979). Triploid individual possesses an extra set of chromosomes, as a result of a chemical treatment or physical shock on the newly fertilized egg during the second phase of meiosis. The shock causes a depolymerization of tubulin fibers of the meiotic spindle impeding the extrusion of the second polar body (Dunham, 2011; Piferrer, 2018) and making the zygote possess three sets of chromosomes: two maternal (one from the egg's pronucleus and another from the retained polar body) and one paternal (from the spermatozoa's pronucleus) (Tebaldi and Amaral Jr., 2009).

Physical shocks are more commonly applied for aquaculture species to produce triploids through sudden thermal changes (Sato et al., 2020; Yasui et al., 2020; Silva Júnior et al., 2021; Pereira et al., 2022) or changes in hydrostatic pressure surrounding the newly fertilized eggs (Hyndman et al., 2003; Tiwary et al., 2004; Xu et al., 2008; Piferrer et al., 2009; Peruzzi et al., 2018; Puvanendran et al., 2019). Another alternative method to obtain a triploid zygote is crossing a tetraploid individual with a diploid individual resulting in interploid triploids (Piferrer et al., 2009; Hansen et al., 2025).

In most fishes, the presence of an extra set of chromosomes leads to infertility, as the meiotic division is disrupted by the failure on the chromosomes pairing before crossing over, early in meiosis (Benfey, 1999; Tiwary et al., 2004; Piferrer et al., 2009). However, there are some species in which male triploids can be fertile such as in the Neotropical *Astianax altiparanae* (do Nascimento et al., 2017), the tench *Tinca tinca* (Linhart et al., 2006), pond loach *Misgurnus anguillicaudatus* (Fujimoto et al., 2008) and brown trout *Salmo trutta* (Lahnsteiner and Dünser, 2024). Furthermore, studies indicate that triploid fish have better survival rates, feed conversion and disease resistance (Kerby et al., 2002; Wang et al., 2024), with no difference in performance (Pechsiri and Yakupitiyage, 2005; Wagner et al., 2006) or reduced performance (Aydın et al., 2021; Karayucel et al., 2018; Ottera et al., 2016). However, studies indicate that welfare issues such as the incidence of deformities can appear and need to be dealt with (Fraser et al., 2012; Peruzzi et al., 2018; Hansen et al., 2025). To address the fitness and welfare challenges commonly observed in triploid fishes, Hansen et al. (2025) proposed the use of gynogenesis, which involves the development of offspring from maternal genetic material only. This approach may eliminate lethal alleles associated with biparental inheritance. Furthermore, selective breeding of the best-performing individuals from superior families could contribute to the partial removal of recessive deleterious alleles over successive generations.

For pressure shock treatment of fish eggs, a specific machine with a stainless-steel pressure chamber, hydraulic electric pump, pressure gauge and air bleed valve are used (Peruzzi and Chatain, 2000). Currently, there are efficient pressure shock protocols to induce triploid for most commercial fish, such as the brook trout *Oncorhynchus gorbuscha* (Benfey et al., 1997; Hyndman et al., 2003), the rainbow trout *O. mykiss* (Lincoln and Scott, 1984), the coho salmon *O. kisutch* (Piferrer et al., 1994), the Atlantic salmon *Salmo salar* (Glover et al., 2020), the brown trout *Salmo trutta* (Preston et al., 2013), the loach *Misgurnus anguillicaudatus* (Gao et al., 2007), the channel catfish *Ictalurus punctatus* (Wolters et al., 1982), the sea bass *Dicentrarchus labrax* L. (Peruzzi et al., 2004), the yellow croaker *Pseudosciaena crocea* (Xu et al., 2008) and the tilapia *Oreochromis aureus* (Hussain et al., 1991). For neotropical species, hydrostatic shock of newly fertilized eggs has yielded 100% triploid in the jundiá *Rhamdia quelen* (Huergo and Zaniboni-Filho, 2006), and temperature shock produced 95–100% triploid in the *Astianax altiparanae* (do Nascimento et al., 2017), curimbatá *Prochilodus lineatus* (Yasui et al., 2020) and matrinxã *Brycon amazonicus* (do Nascimento et al., 2021). However, the effectiveness of either thermal or hydrostatic pressure shock highly depends on the time post fertilization when it is applied, on its intensity and duration (Pandian and Koteeswaran, 1998; Káldy et al., 2021).

Different methods have been used to verify the ploidy in fish. In general, these analyses are based on chromosome counts or DNA content, and less frequently based on the cell size (Abiado et al., 1991; Piferrer et al., 2009). Karyotyping is the most preferred technique, and more recently the flow cytometry has largely been used to quantify the DNA cell content (Thorgaard, 1983; Xu et al., 2008; Silva Júnior et al., 2021; Pereira et al., 2022). The measurement of the size of erythrocyte and/or nuclei in stained blood smear is another approach applied to verify fish ploidy (Benfey and Sutterlin, 1984; Gao et al., 2007; Lu et al., 2009; Flajšhans et al., 2011; Yasui et al., 2020).

The Neotropical fish tambaqui *Colossoma macropomum* (Cuvier, 1816), an Amazonian member of the family Serrasalmidae, is the most farmed native fish species in Brazil. Its cultivation has been steadily intensified over the past decade (Val and Oliveira, 2021), and in 2024 the production of farmed tambaqui exceeded 120 thousand tons (IBGE, 2025). Consequently, in Brazil, a great research effort has been carried out aiming at developing new techniques for the farming of tambaqui to improve the production and economic performance on the effect of different diets at various life stages (de Almeida et al., 2019; dos Santos et al., 2021), feeding regimes (Chellappa et al., 1995; Guilherme et al., 2022), stocking density (da Costa et al., 2019; Rodrigues et al., 2024), and environmental conditions (Aride et al., 2004; Sousa et al., 2017). However, puberty and early maturation have been seen to have negative effects on growth of tambaqui (Almeida et al., 2016; Silva et al., 2023). Given that triploidy induction in fish results in sterility, developing a better triploidy induction protocols can add a great value in the tambaqui industry, allowing greater yield in a shorter period. However, the attempts to date to induce triploidy in tambaqui using temperature shocks were not efficient, generating only 8 to 58.8% of triploids (Sato et al., 2020; Silva Júnior et al., 2021; Pereira et al., 2022). Moreover, the same studies demonstrated that heat shock in tambaqui eggs drastically decreases the fertilization index. Studies comparing the thermal (heat/cold) and hydrostatic pressure shock on triploidization showed that hydrostatic pressure shock methods resulted in better production of triploid fish (Malison et al., 2001; Meng et al., 2023).

To date, no standardized protocol for triploid induction using hydrostatic pressure has been established for *C. macropomum*. Accordingly, the present study aimed to develop and optimize a hydrostatic pressure–based triploidization protocol for this species. To achieve this, we systematically evaluated the effects of post-fertilization timing and pressure shock duration on the efficiency of triploidy induction and the resultant gonadal sterility.

2. Material and methods

The experiments described here complied with the "Brazilian guidelines for the care and use of animals for scientific and educational purposes"–DBCA and were registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge – SISGen (ADBE614) and the Ethics Committee for the Use of Animals - CEUA of Embrapa Fisheries and Aquaculture (protocol n°11/2019).

2.1. Trial 1: Identification of the best time post-fertilization for pressure-shock application

2.1.1. Artificial reproduction and experimental design

Trial 1 was conducted at the facilities of Embrapa Fisheries and Aquaculture (Palmas-TO, Brazil) on December 07 and 08th 2020 using 3.5 years old, earth pond-cultured tambaqui male and females. Mature females ($n = 2$, 6.26 ± 0.82 kg) and spermiating males (n = 2, 5.09 ±

0.07 kg) of *C. macropomum* were selected and treated with carp pituitary extract (CPE, Danube Piscicultura, Brazil) following protocol described in Woynárovich and Van Anrooy (2019). Females were given a preparatory intraperitoneal dose of 0.5 $mg.kg^{-1}$ of body weight (BW) and 12 h later a resolving dose of 5.0 $mg.kg^{-1}$ BW and males were treated with a single intraperitoneal dose of 2.5 $mg.kg^{-1}$ BW. Care was taken to strip good quality eggs at an appropriate time. We closely monitored the fish for the first release of eggs in the tanks after hormonal treatments. When we observed that the females were releasing the eggs at 260 degree-hours and immediately anaesthetized the fish and stripped the eggs (Oliveira et al., 2023). The colour of the eggs was also checked which was homogeneously light green and was devoid of any white spots (presumably dead/over-ripped eggs). For semen collection, males had their urinary tract emptied, urogenital papillae dried, and semen collected using dry sterile 5 mL syringes. After the collection, lack of spermatozoa activation was confirmed under an optic microscope to confirm that any urine/faeces contamination resulted in sperm activation which would lead to lower or no fertilization (Maria et al., 2012; Marques et al., 2021). Equal amounts of semen from two males (2 mL/male) were then pooled and gently mixed prior to fertilization. Likewise, equal amounts of eggs (200 g/female) of the two females were pooled before fertilization.

Trial 1 (T1) was designed to test application of 8000 psi for 120 s (2 min) at six different times (in seconds) post fertilization (spf). The pressure value (8000 psi), total time of the pressure applied (120 s) and the start of the six different times in psf in this trial were selected after carefully analysing the literature and considering possible species-specific differences (Pandian and Koteeswaran, 1998; Peruzzi and Chatain, 2000; Piferrer et al., 2009; de Almeida et al., 2019; Sato et al., 2020; Silva Júnior et al., 2021). Each treatment was carried out in triplicate. Each replicate consisted of 12.5 g of pooled eggs fertilized with 250 μL of pooled semen inside a 50 mL Falcon tube with perforated lids to avoid pressure build up inside the Falcon tube during pressure treatment. Given the short time (65–312 spf and 120 s induction period), fertilization for each treatment was done just before subjecting them to the pressure treatment and this was done in random order. The treatments were: T1.1–65 spf, T1.2–95 spf, T1.3–125 spf, T1.4–155 spf, T1.5–185 spf and T1.6–312 spf (see Fig. 1). For each treatment, triplicates of Falcon tubes containing fertilized eggs were placed together in the stainless chamber of the hydrostatic pressure shock machine (TRC Hydraulics, Dieppe, New Brunswick, Canada) which was filled with water from the incubators (29 °C). The tubes with eggs were placed inside the pressure chamber 10 s prior to the set spf, the lid was closed, and the pressure was raised to 8000 psi in 4 s. This was considered as 0 s and continued for 120 s. Soon after pressure treatment, decompression was instantaneously made using a pressure release valve. Control (CT) replicates were fertilized in Falcon tubes similar as in other six treatments but were not subjected to any pressure. All fertilized eggs in each of the Falcon tubes were incubated in individual 200 L incubators (n = 21).

2.1.2. *Survival at final gastrula stage and embryo survival (hatching) indices*

To estimate the final gastrula survival (FGS) and embryo survival (ES), a random triplicate samples of 5 mL water sample containing embryos (at final gastrula stage) were taken from each incubator at 6 h post fertilization (hpf) for FGS estimation and 11 hpf for ES estimation and were placed in a petri dish to form a single layer of embryos. To estimate FGS, the number of fertilized and unfertilized eggs at 6 hpf were counted under a binocular microscope and FGS was calculated as FGS = (Number of fertilized eggs / total number of eggs in the sample) * 100. To estimate the ES, the number of moving embryo inside the egg (viable) and whitish eggs or with dead embryos (nonviable) were counted at 11 hpf, and the ES was calculated as ES = (Number of viable eggs / total number of eggs) *100. The stage of viable embryos at 11 hpf is considered as a good proxy for the number of hatched larvae (Galo et al., 2015). Larval hatching started approximately 12 h after fertilization at 28 °C (336 degree-hours). The gastrula and embryo survival

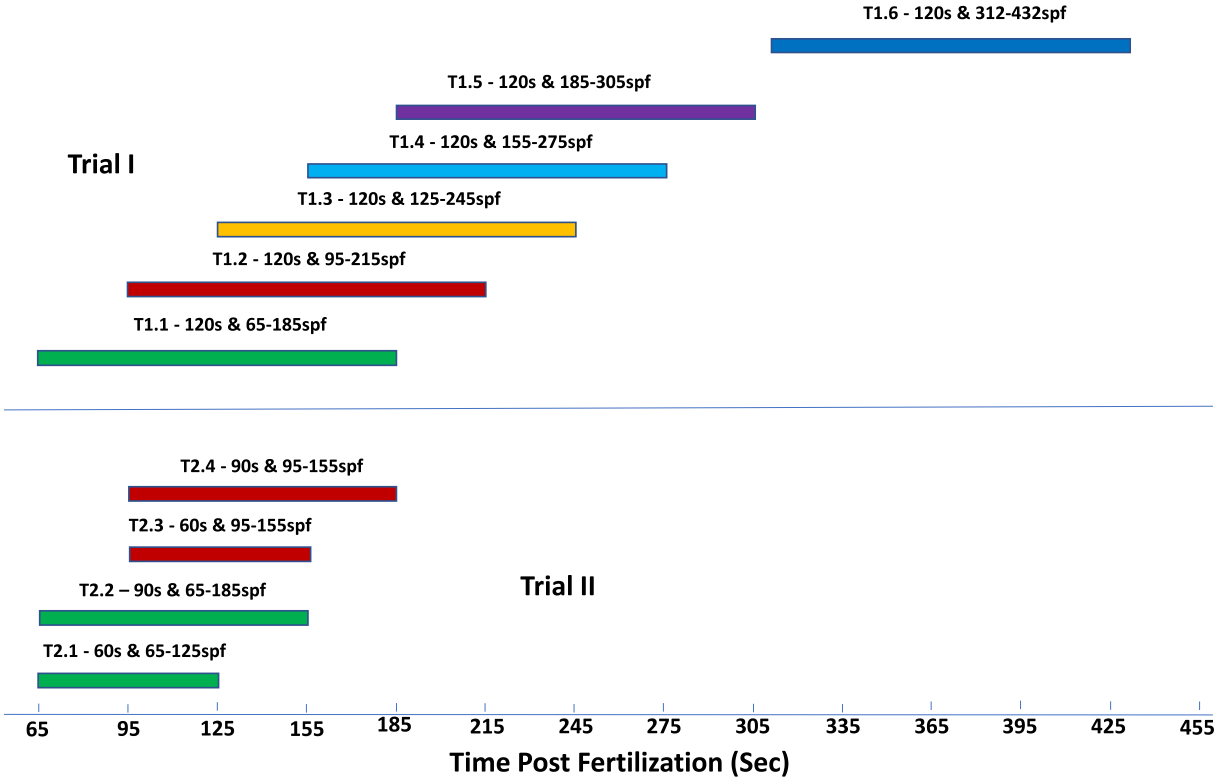

Fig. 1. Schematic representation of both trials set-up to induce triploidy in tambaqui *C. macropomum*. Each line represents one treatment and shows the time post fertilization (*sec*) when fertilized eggs were subjected to pressure shock (8000 psi) and the duration of shock (above each line).

indexes may be underestimated, as early embryonic mortality and egg disintegration before gastrulation were not accounted for. The total number of fertilized eggs immediately after fertilization could not be recorded due to the high number of treatment groups and replicates. No larval rearing was carried out for Trial 1.

2.1.3. Flow cytometry determination of larval ploidy

After 54 hpf, 20 larvae from each incubator were netted, euthanized in cold water (0 °C), dried on a paper towel, placed in 200 μL microtubes (1/incubator), then dip frozen in liquid nitrogen. Samples were then stored at −80 °C and shipped on dry ice to Embrapa Amazonia Ocidental (Manaus-AM) where they were kept at −80 °C until being processed for flow cytometry analysis.

Flow cytometry analysis was carried out at FIOCRUZ (Fundação Oswaldo Cruz - FIOCRUZ Amazônia, Manaus). Because all larval samples from each incubator were placed in one single microtube, the thawing process impeded a proper separation of the individual larva and some larvae were lost. After thawing, only 14 larvae were intact in T1.5–185 spf while all other treatments had 20 larvae. Using these larvae cell lysis was made following protocol from Xavier et al. (2017), where individually thawed larvae were immediately immersed in lysis 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) for 120 min at room temperature. After cell lysis, the sample was filtered through a 30 μm mesh (Celltrics, Partec Gmbh, Germany), stained with 50 μL of propidium iodide at 1 mg (Sigma Aldrich, Saint Louis, MO, EUA) and individually processed in a flow cytometer (FACSCanto™ II, Erebodegem, Belgium). The equipment was operated using 6 colours within 2 lasers (Blue, 488 nm + red, 633 nm, being 4 colours in the blue laser and 2 colours in the red laser), power forward Scatter - FSC of 229, side scatter SSC of 330 and fluorescence intensity FL 330, and 50,000 acquired events were performed.

Ploidy of presumptive triploid fish was confirmed by comparison with diploid control fish. For the analysis of relative DNA content, we first analysed the control samples ($n = 12$) to establish a standard amount of DNA present in nuclei suspension of diploid (2n) tambaqui larvae. The fluorescent cytometry histograms obtained from diploid and triploid fish are illustrated in Fig. 2. Relative fluorescence was evaluated using software FlowJo v. 10 (TreeStar Inc., Ashland, OR, USA).

Finally, we estimated the triploid yield (TY), which is a good indicator of the overall success of the triploidization. It was calculated using embryo survival (ES) of triploid and control and triploidization rate, as follows: TY = (ES of triploid group/ES of control group) x triploidization rate (Teskeredžić et al., 1993).

2.2. Trial 2: Optimization of pressure shock duration

2.2.1. Experimental design

Based on triploidization, TY, FGS and ES from Trial 1, a second trial (T2) was designed to optimize pressure shock duration. The experiment was conducted at a commercial hatchery located at Brejinho de Nazaré-TO (Fazenda São Paulo; 11°1′57.81"S, 48° 35′22.05"W) using the same pressure shock machine used in Trial 1. On 8th March 2022, 7 kg mature

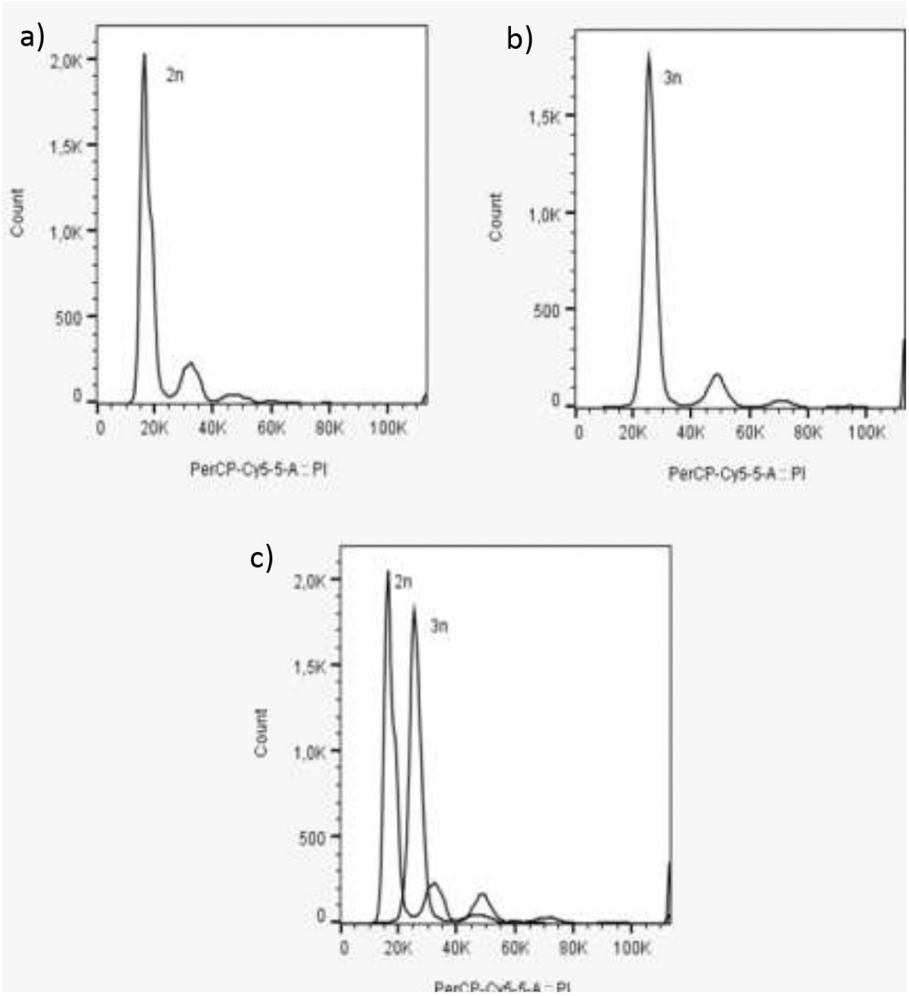

Fig. 2. Flow cytometry histograms (from trial 1) for the nuclei DNA relative content of tambaqui *C. macropomum* larvae. a) In diploid fish (from unshocked egg) used as standard, b) triploid fish (eggs treated with hydrostatic pressure) and c) merged data from 2n and 3n fish.

females ($n = 2$) and 7.5 kg spermiating males ($n = 3$) were selected and ovulation/spermiation was induced using CPE protocol (Woynárovich and Van Anrooy, 2019). Eggs and semen were collected as previously described. The semen was pooled (2 mL from each male). The trial aimed to test two durations (60s and 90s) of pressure (8000 psi) application in eggs (12.5 g eggs fertilized with 250 μL semen) at 65 spf and 95 spf. All four combinations (treatments) were done at 8000 psi in four replicates T2.1 - 60s at 65 spf; T2.2 - 90s at 65 spf; T2.3 - 60s at 95 spf and T2.4 - 90s at 95 spf (see Fig. 1). The water temperature for the whole trial was 27.5 °C. Control (CT) replicates were fertilized exactly as treatments but not subjected to a pressure shock. After pressure shock, fertilized eggs were incubated in twenty, 200 L incubators. FGS and ES were estimated as previously described for Trial 1, at 6 and 11 hpf, respectively.

2.2.2. Rearing conditions, feeding regime and biometrics

From 3 dph, larvae were fed with *Artemia* nauplii *ad libitum* every two hours from 8:00 AM to 04:00 PM and then at 09:00 PM. After seven days post-hatching (dph), larvae were transported in plastic bags saturated with O_2 from Fazenda São Paulo (Brejinho de Nazaré-TO) to the facilities of Embrapa Fisheries and Aquaculture (Palmas-TO). Replicates were acclimated into individual 1000 L tanks (total of 20 tanks) at an approximate density of 2 larvae L^{-1} maintained in a recirculation aquaculture system (RAS) and kept under constant aeration. Bottom of the tanks were siphoned daily, and water quality was kept at the optimal conditions (6 mg L^{-1}; pH – 7,5) for the species (Hilsdorf et al., 2022). The average temperature was 29 °C, 12 h continuous light per day (12 L:12D) and L $min.^{-1}$ water flow were provided.

From 20 dph onwards, fingerlings were also fed *ad libitum* with powdered commercial ration (50% crude protein, Aqua line SUPRA, São Leopoldo-RS, Brazil) along with *Artemia* nauplii, for weaning. Larvae were completely weaned onto a dry diet at 25 dph. When juveniles were approximately 2 months old (May 10th of 2022), 30 fish per replicate of each treatment ($n = 120$/treatment and 480 in total) were transferred to one 600 m^2 earthen pond. Likewise, fish from control groups were also polled and transferred to another 600 m2 earth pond ($n = 480$). The remaining fish were discarded and euthanized with Benzocaine due to the limited number of earthen ponds available for the grow-out phase. From this moment up to 20 months-old, all fish were fed with commercial ration (CP 45%, Aqua line SUPRA, São Leopoldo-RS, Brazil) offered daily at 10% of total estimated biomass.

To perform biometric measurements, fifty fish from each group (control and treatment) were randomly captured from the earthen ponds and transferred to a 1000 L anaesthetic tank (Eugenol at 100 mg. L-1) and total length (TL in cm), standard length (SL in cm), body height (BH in cm) and body weight (BW in g) were measured at three, four, five and six months post-hatch (mph) on 6th June, 15th July, 17th August and 16th September, respectively.

2.2.3. Flow cytometry analysis for triploidization

To avoid sample loses during thawing of very small newly hatched larvae, the sampling of Trial 2 was done at 15 dph, and 15 larvae from each tank were sampled (60 per treatment), euthanized in cold water (0 °C), and individually placed in 200 μL microtubes for cryopreservation in liquid nitrogen until flow cytometry analysis. Sample preservation and transportation were done as in Trail 1. The following number of larvae were analysed from the treatments: CT2 = 31, T2.1 – 60s at 65 spf = 37, T2.2 - 90s at 65 spf = 42, T2.3 - 60s at 95 spf = 45 and T2.4 - 90s at 95 spf = 39 (see Fig. 2). In Trial 2, the triploid yield was also estimated as in Trial 1.

2.3. Blood smear and karyotype

At 12 months, 30 fish of each pond (treated and control) received individual transponders and were sedated with Benzocaine for blood sampling. The blood was collected in heparinized syringes, for the preparation of blood smears, which were then air-dried and stained with Giemsa. Pictures of the erythrocytes were taken in an optical microscope at magnification of 100× and the images were used for the measurement of 30 cells per slide. The longest diameter of each erythrocyte was measured in the software Image G (v. 1.8), and the average (in μm) was estimated.

The karyotype of 6 triploids and 6 control fish was performed as described in Sato et al., 2020.

2.4. Histology analysis of the gonads

To evaluate the gonad development of triploid tambaqui at 20 months of age, 10 triploids (based on the average size of the erythrocytes) and 19 diploid fish were anaesthetised with 0.01% benzocaine (Acros®, New Jersey, USA) and sacrificed by cerebral concussion. The gonads were dissected and placed in Karnovsky solution for 24 h, dehydrated, infiltrated and embedded in glycol methacrylate (Leica®; Heidelberger, Germany). The 5 μm thick sections were stained with blue toluidine (1%), borax (1%), and mounted on slides for further analysis.

When fish were three years old, four females from the control group and one from the triploid group were sampled. Fish were weighed and dissected, and the weight of the ovary was used to estimate the gonadosomatic index (GSI).

2.5. Data and statistical analysis

FGS and ES of both trials were checked for normal distribution by the Shapiro Wilk test, subjected to one-way ANOVA, followed by Tukey *post hoc* test for multiple comparisons. The erythrocyte diameter data passed the Shapiro Wilk normality test and Levene's Test for homogeneity of variance and were subjected to unpaired *t*-test to compare triploid and diploid averages. All biometric data passed the Shapiro Wilk normality test and were subjected to multiple unpaired t-test to compare the values of control and triploid groups in each month. Data are represented as mean ± SEM. GraphPad Prism 9 (GraphPad Software, Inc.) was used for statistical analysis.

3. Results

3.1. Trial 1: Triploidization, final gastrula survival (FGS) and embryo survival (ES) (hatching) indices

All six treatments resulted in relatively higher triploidization ranging from 93 to 100% with T1.1–65 spf and T1.2–95 spf had 93 ± 11.54% triploidiation and T1.3–125 spf, T1.4–155 spf, T1.5–185 spf and T1.6–312 spf resulted 100% (Fig. 3a). Control group (86.85 ± 4.7%), T1.1–65 spf (94.64 ± 2.8%), T1.2–95 spf (89.95 ± 3.3%) and T1.3–125 spf (86.02 ± 5.3%) had significantly higher FGS compared to T1.4–155 spf (64.34 ± 13.9%), T1.5–185 spf (52.33 ± 6.7%) and T1.6–312 spf (54.50 ± 14.01%) (Fig. 3b; $P < 0.05$). The ES of control group (79 ± 1%), T1.1–65 spf (89 ± 3%) and T1.2–95 spf (87 ± 5%) were significantly higher than the ES of T1.3–125 spf (58 ± 1%), T1.4–155 spf (49 ± 2%), T1.5–185 spf (43 ± 4%) and T1.6–312 spf (31 ± 1%), while T1.1 and T1.2 had significantly higher ES than the control group (Fig. 3b; $P < 0.05$). The results showed that treatments T1.1 and T1.2 had higher triploid yield (89.48% and 86.07%, respectively) and were considered as the most effective protocols, while T1.3, T1.4, T1.5 and T1.6 ranged from 60.24% to 30.45%; Fig. 3c). We then considered T1.1 and T1.2 for further development (Trial 2). Although no quantitative assessment of larval deformities was performed, no visible abnormalities were observed among hatched larvae in any treatment.

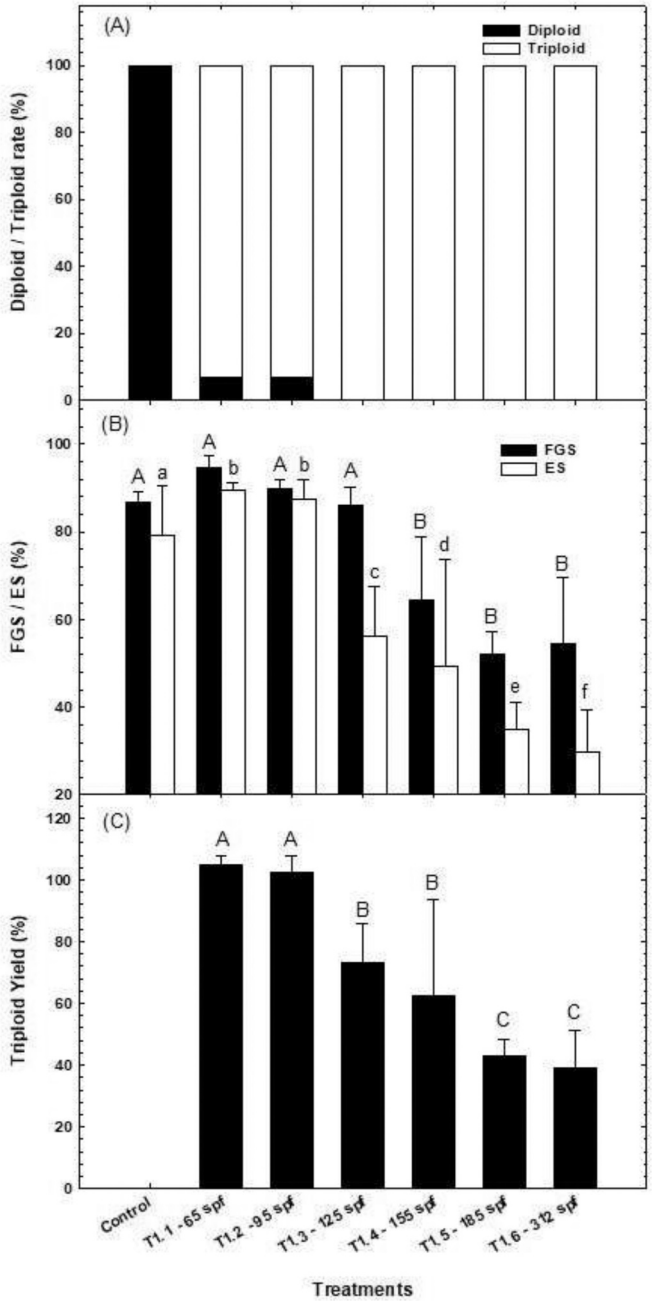

Fig. 3. Triploidy induction (a), final gastrula survival (FGS), embryo survival index (ES) and triploid yield (c) in tambaqui *C. macropomum* treated and non-treated with pressure shock in tiral 1. Data are presented as mean ± SD. Treatment groups labelled with same letter do not differ significantly ($P < 0.05$), lower case refer to FI and upper case to ESI.

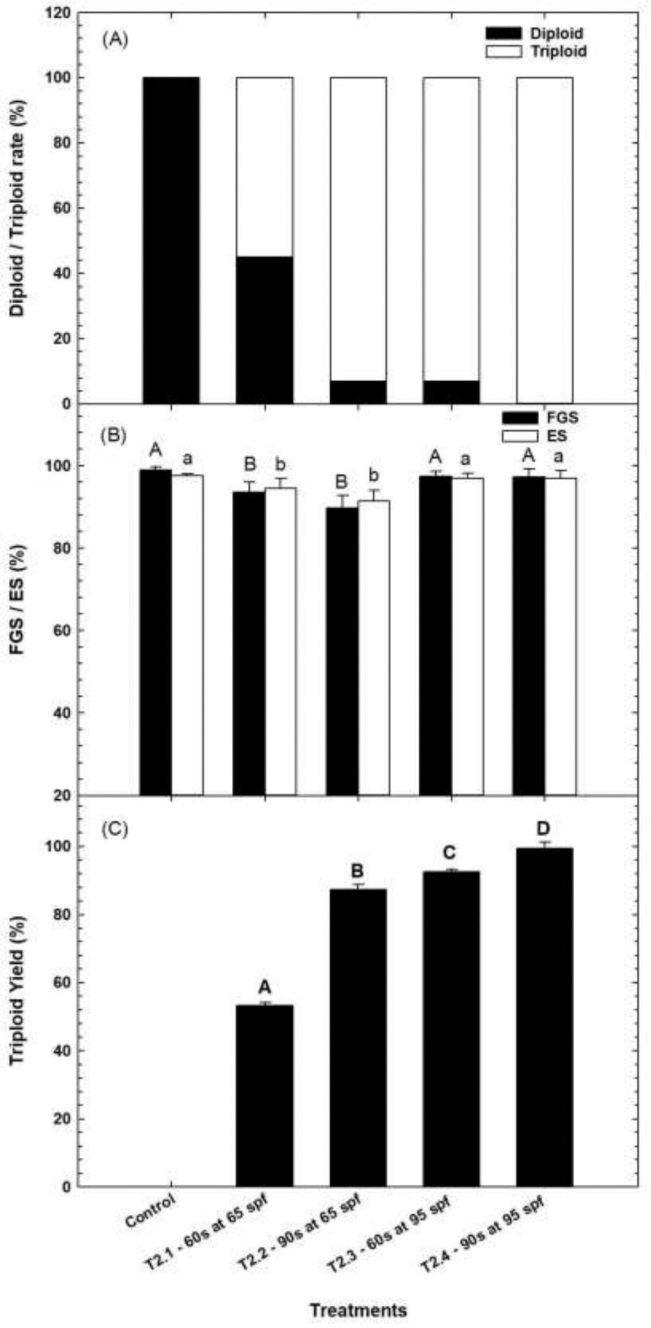

Fig. 4. Triploidy induction (a), final gastrula survival (FGS), embryo survival index (ES) and triploid yield (c) in tambaqui *C. macropomum* treated and non-treated with pressure shock in trial 2. Data are presented as mean ± SD. Treatment groups labelled with same letter do not differ significantly ($P < 0.05$), lower case refer to FI and upper case to ESI.

3.2. Trial 2: Optimization of pressure shock duration

3.2.1. Triploidization, final gastrula survival (FGS) and embryo survival (ES) (hatching) indices

T2.1 – 60s at 65 spf resulted in significantly lower triploidization compared to T2.2 - 90s at 65 spf, T2.3 - 60s at 95 spf and T2.4 - 90s at 95 spf at 55 ± 3.4%, 93 ± 2.4%, 93 ± 9.2% and 100%, respectively (Fig. 4a). No significant differences in FGS among control group (98.9 ± 0.9%) and T2.3 (97.4 ± 1.2%) and T2.4 (97.3 ± 1.9%), while T2.1 (93.6 ± 2.5%) and T2.2 (89.7 ± 3.1%) had significantly lower FGS compared to control group, T2.3 and T2.4 (Fig. 4b; $P < 0.05$). Similarly, control (97.5 ± 0.6%), T2.3 (96.9 ± 1.2%) and T2.4 (96.9 ± 1.8%) had significantly higher ES compared to T2.1 (94.5 ± 2.4%) and T2.2 (91.5 ± 2.6%) (Fig. 4b; $P < 0.05$). The overall triploid yield was 53.3%, 87.27%, 92.40% and 99.35% for T2.1, T2.2, T2.3 and T2.4, respectively (Fig. 4c). Although no quantitative assessment of larval deformities was performed, no visible abnormalities were observed among hatched larvae in any treatment.

3.2.2. Growth performance of triploid vs diploid fish

No mortality was registered during the growth phase of the study in both ponds. The control fish were significantly smaller in size compared to the triploid fish from two mph until the end of the experiment at six

mph (Fig. 5). Due to their smaller size, fish were not weighed at two mph. In both groups, the weight steadily increased from 3 mph until the end of the experiment, and the biomass in the triploid group was significantly higher ($P < 0.001$ in all parameters analysed). At six mph, the fish from the control group were 342.9 ± 67 g, 25.3 ± 1.7 cm in total length, 24.1 ± 1.7 cm in standard length and 10.9 ± 0.8 cm in body height. And the triploid group was 416.08 ± 79.73 g, 27.4 ± 1.6 cm in total length, 26.2 ± 1.5 cm in standard length and 11.9 ± 0.7 cm in body height (Fig. 5).

3.3. Blood smear and karyotype

The erythrocytes of diploid fish presented an average size of 12.7 ± 0.6 μm, while the erythrocytes of fish induced to triploidy had an average of 15.8 ± 0.9 μm ($P < 0.001$). The karyotype of triploid fish presented cells in metaphase with more than 70 visible chromosomes. The erythrocytes of all 6 triploid fish used in the karyotype presented an average nuclear size above 15 μm (Fig. 6).

3.4. Histology analysis of the gonads

From the 19 control fish, eight were females and six of them had developing ovaries at primary growth stage, while two had immature ovaries. Ovaries in primary growth had pre-vitellogenic and atretic oocytes, besides a higher number of primary oocytes arrested in prophase I of meiosis and surrounded by a single layer of flattened granulosa cells (Fig. 7a and b). This germ cell line was arranged within typical ovarian lamellae. Among the ten triploid fish, five were females and all displayed immature ovaries without the usual ovarian lamella morphology. In these triploid females, no germ cells were observed in meiosis or post-meiosis and there was an apparent increased cellularity in the interstitium. Instead, the germinal epithelium was either devoid of germ cells or containing only oogonia as germ cell (Fig. 7c and d, respectively).

Eleven diploid males were evaluated, and they were either in late maturation, with seminiferous tubules filled with free spermatozoa, or presented resting testis (Fig. 7e and f), with spermatogonia as the main germ cell besides some residual spermatozoa. The resting testis could also be easily identified by the presence of thick, post spawning or absorbed, somatic stroma. This indicates that all control males have attained puberty at this age. The five triploid males were immature with testis containing only early and isolated type A spermatogonia in the germinal epithelium (Fig. 7g). No presence of more advanced spermatogenic cells was observed in any triploid male.

Female diploid tambaqui at 3-years old showed well developed ovaries compared to the triploid female. The GSI of the triploid female was 0.01 while the diploid females had a GSI of 7.9 ± 1.01 (Fig. 8).

4. Discussion

Results from our experiments showed that applying the hydrostatic pressure to induce triploidy can be successfully achieved in tambaqui. To our knowledge, no attempts were made to induce triploidy in tambaqui using a pressure shock. This is a breakthrough for the developing aquaculture industry in South America for tambaqui. The earlier attempts to produce triploidy in tambaqui using thermal shock were not successful because of lower triploidization (maximum 58.8%) and/or reduced fertilization index and embryo survival index (ES) (Sato et al., 2020; Silva Júnior et al., 2021; Pereira et al., 2022). In in our study, we have achieved 100% triploidization using 8000 psi pressure at 95 s post fertilization (spf) for 90 s. Studies have also shown that time after the post fertilization the shock applied, and the duration of the shock are very important factors affecting triploidization in fish (Káldy et al., 2021) while the magnitude of the hydrostatic pressure of approximately 8000 psi usually gives a satisfactory triploidy (Zajicek et al., 2011; Fetherman et al., 2015). In both trials in this study, we used a

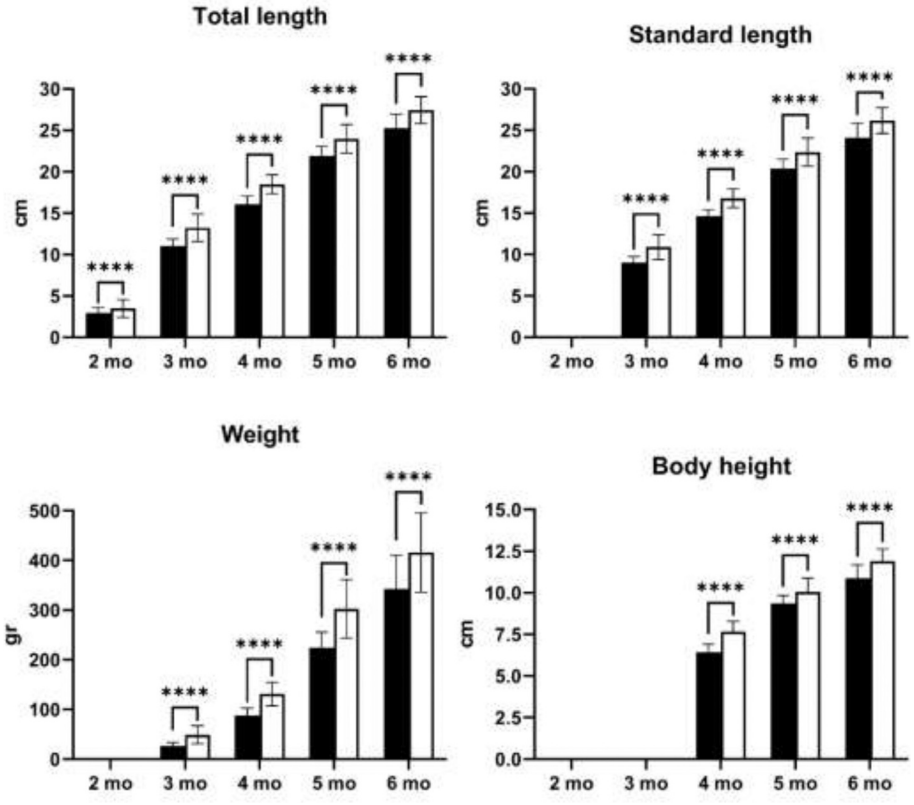

Fig. 5. Monthly biometrics of tambaqui *C. macropomum* from 2 to 6 months of age. Black bars represent the control (unshocked; 2n) group; white bars represent the treated group, mostly composed by 3n fish. Data are presented as mean ± SD ($p < 0.001$).

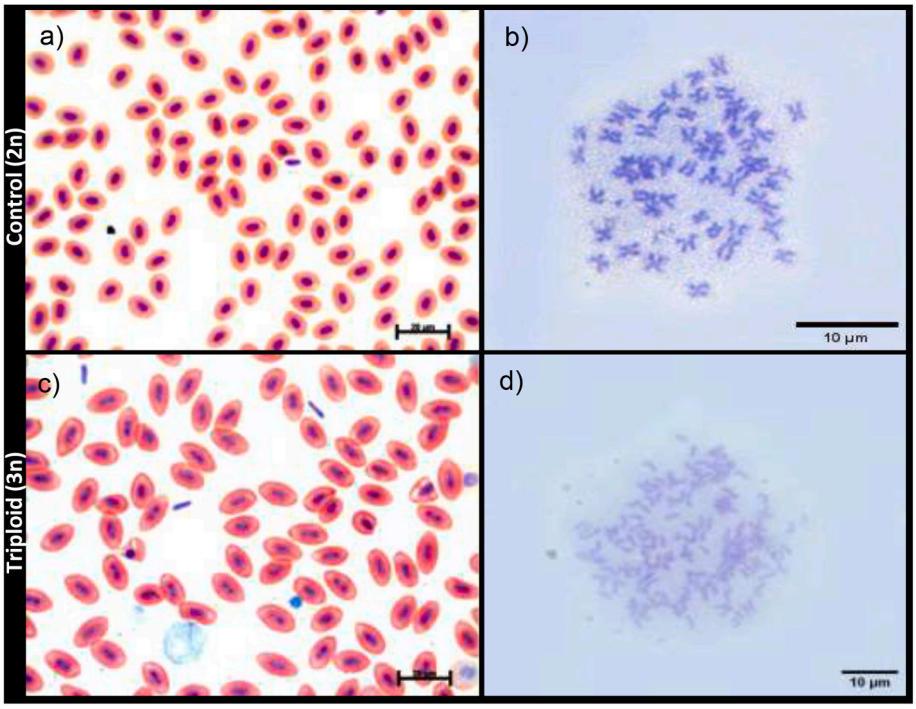

Fig. 6. Erythrocytes and karyotype of diploid (a and b) and triploid (c and d) tambaqui *C. macropomum.*

hydrostatic pressure of 8000 psi while a wide range of different times of spf (65–312 s in Trial I) and selected spf (65 and 95 spf) and duration of the shock (60 and 90 s) were used. In general, both trials gave higher triploidization but resulted in variable final gastrula survival FGS and ES (Figs. 3 and 4).

Application of the hydrostatic pressure needs to be applied on the fertilized eggs before the release of the second polar body to achieve a successful triploidization (Piferrer et al., 2009). In our experiments, higher rate of triploidization (93–100%) in Trial 1 confirmed that there was an extended time available between 65 and 312 s after fertilization and during this time interval, the release of the second polar body during meiosis can be withheld. Although resulted in higher triploidization, the estimated FGS and ES were significantly lower in four of the longer spf (125, 155, 185 and 312 s) compared to 65 and 95 spf (Fig. 3b). The reduction in FGS and ES in T1.3 - T1.6 (125, 155, 185 and 312 spf, respectively) in trial 1 indicates that to get an optimal result, the pressure treatment should start earlier than 125 spf. Similar trends have been reported in turbot where the triploidization was higher in all treatments, but the ES was variable (Meng et al., 2023) or the TY was variable in brown trout, *Salmo trutta* (Preston et al., 2013). It is important to consider that even though our method, due to the experimental procedures, did not account for early embryonic mortality or egg disintegration before gastrulation, hence possibly leading to an underestimation of the actual fertilization and embryo survival indices, it was sufficient to allow reliable comparisons among the treatments applied.

When comparing different pressure treatments in newly fertilized eggs of different fish species, there is a range of pressure values (from 6000 to 9500 psi), timing to apply the shock (up to 40 min after fertilization) and length of the shock (up to 5 min in Atlantic salmon) to produce triploid fish (Chourrout, 1984; Hyndman et al., 2003). This indicates that the intensity of pressure and duration of shock for retention of the second polar body is species specific and could be related to the sensitivity of eggs and embryonic development time of each species. Fish with longer embryonic development time would require relatively longer duration for the release of the second polar body (40 mins as in rainbow trout; Chourrout, 1984) and fish species with shorter embryonic development time would require shorter duration as shown in our study for tambaqui. Hence, in the Trial 2 we tested two times after fertilization (65 and 95 spf) and pressure duration (60 and 90 s). Treating the eggs at 95 spf for either 60 or 90 s induced from 93 to100% of triploidy without affecting the FGS and ES (Fig. 4a, b). Although we used only one hydrostatic pressure value (8000 psi), a high triploidization up to 100% has justified the selection of 8000 psi and the time selections in our experiments.

Our study produced higher triploidy induction rates compared to studies in other fish species; African catfish (*Clarias gariepinus*; Babaheydari et al., 2016), rainbow trout (*Oncorhynchus mykiss*; Chatchaiphan et al., 2016), big-head catfish (*Clarias microcephalus*; Lawson and Ishola, 2010), and even with recent studies with tambaqui (Sato et al., 2020; Pereira et al., 2022; Silva Júnior et al., 2021). However, higher triploid induction efficiency of 100% with high rates of fertilization index and ES have also been reported in some commercial species such as Atlantic salmon *Salmo salar* (Graham et al., 1985), trout *Oncorhynchus mykiss* (Chourrout, 1984), channel catfish *Ictalurus punctatus* (Wolters et al., 1981), coho salmon *Oncorhynchus kisutch* (Teskeredžić et al., 1993), European catfish *Silurus glanis* (Linhart et al., 2001), and the Neotropicals jundiá *Rhamdia quelen* (Huergo and Zaniboni-Filho, 2006; Silva et al., 2007) and curimbatá *Prochilodus lineatus* (Yasui et al., 2020).

Triploid tambaqui juveniles in our study were heavier and larger than diploids counterparts from two to six mph. Due to the limited number of earthen ponds available for the grow-out phase, it was not possible to maintain separate ponds for all treatment groups, and therefore, individuals were pooled into a single pond, while the control replicates were kept separately in equal number. This approach likely introduced a small proportion of diploid fish into the treated pond, as the triploidy rate among replicates ranged from 93% to 100%. Nevertheless, the zootechnical performance of this group remained consistently superior to that of the control fish even with this small proportion of diploid individuals, reinforcing the positive effects of triploidy in the growth of tambaqui. Differences in growth performance between individuals of different ploidies reared under the same conditions are related to age and species (Ihssen et al., 1990). In *Pseudosciaena crocea*, the growth difference between diploid and triploid fish was evident only from 10 mph with triploid individuals were bigger and heavier than

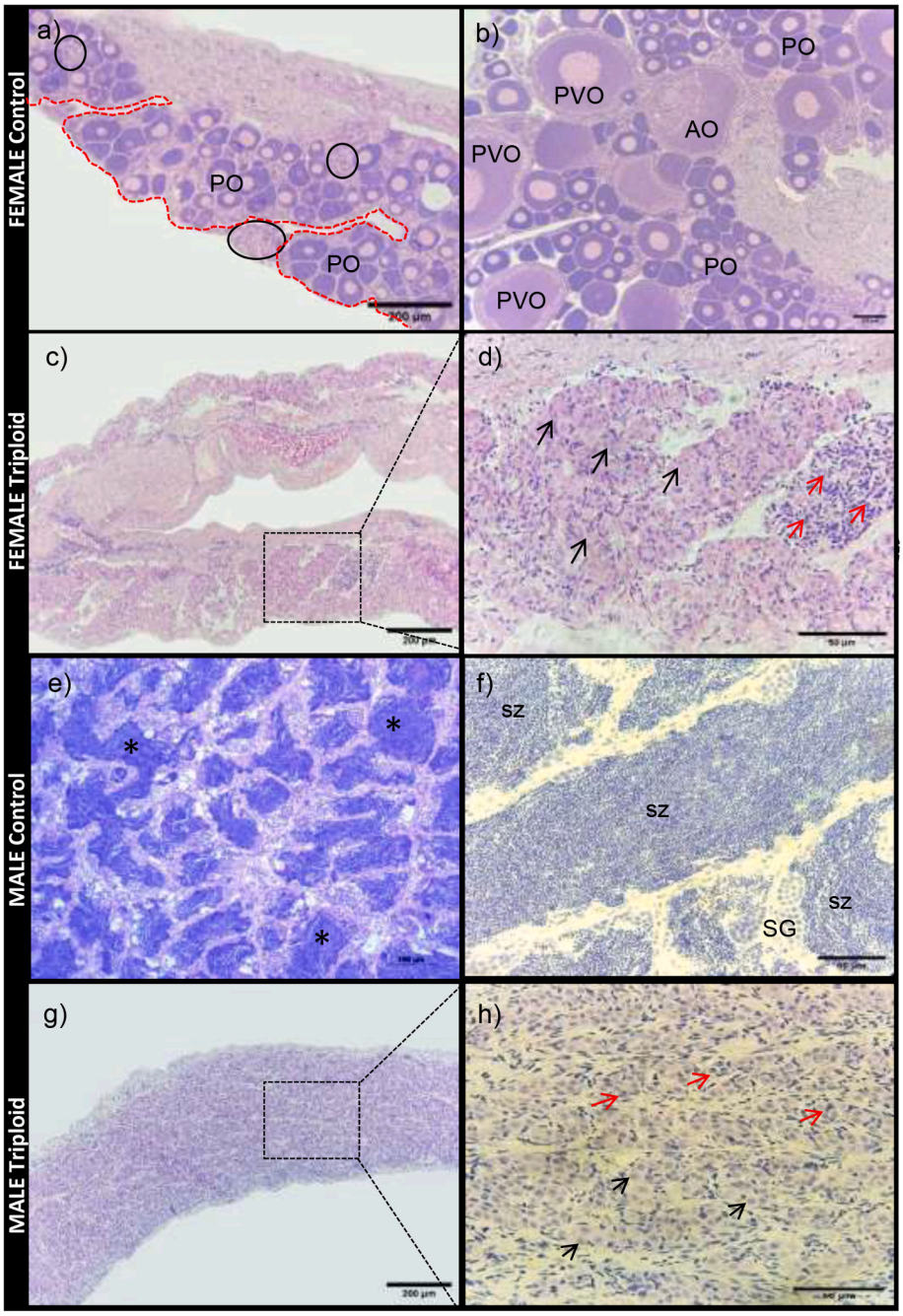

Fig. 7. Gonads of tambaqui *C. macropomum*. a) 2n immature ovary containing mainly primary oocytes (PO) and oogonia (circled in black) allocated in lamellae (circled in red); b) 2n primary growth ovary containing primary, previtellogenic (PVO) and atretic (AO) oocytes; c) 3n ovary without lamella structure, with germinal epithelium (circled in red) devoid of CG germ cells; d) 3n ovary without lamella structure, containing only oogonia (black arrows) in the germinal epithelium, which also presented large areas of apoptotic cells (red arrows); e) resting 2n testis containing residual spermatozoa (asterisks) and thick interstitium; f) mature 2n testis containing free spermatozoa (SZ) and few spermatognial cysts (SG); g) 3n testis presenting spermatogenic cyst in apoptosis (red arrows), and isolated type A spermatogonia (black arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diploids, demonstrating the advantages of triploidy were age-related for this species, probably concomitant with pubertal timing (Xu et al., 2008). On the contrary, in salmonids the growth is reduced in triploids, and the greatest advantage of producing such fish is the sterility aiming to preserve the genetic integrity of wild populations in case of any escape events of farmed fish (Madaro et al., 2022).

Our analysis comparing the erythrocyte sizes of diploid and triploid fish showed that triploid fish (confirmed by the observation of more than 70 chromosomes in the metaphase of such fish) showed larger erythrocytes compared to the diploid fish, as evidenced by the average size increase from 12.7 μm to 15.8 μm. and describing some key observations. The nuclear size of the erythrocytes in triploid fish being larger than 15 μm further supports the genetic differences, possibly related to the increased number of chromosomes and the additional genetic material. This finding is in line with observations in other teleost's such as salmonids (Dorafshan et al., 2008; Saranyan et al., 2017), commom carp (Zhu et al., 2021), jundia (Fukushima et al., 2012) and zebrafish (Small et al., 2024) ((Benfey, 1999), among others. Considering that erythrocytes are key mediators of physiological adjustments in oxygen transport efficiency, ongoing research by our group is focused on assessing the

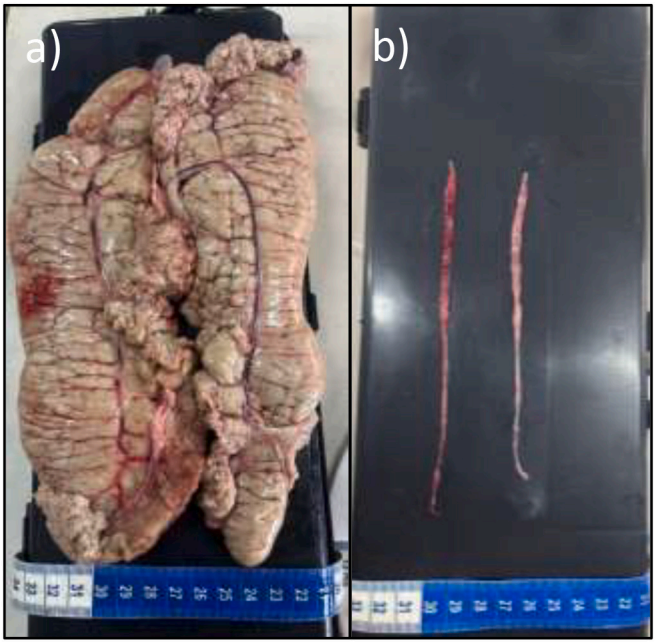

Fig. 8. Ovary of three-year-old (a) diploid and (b) triploid tambaqui *C. macropomum*.

hematological condition and health status of triploid tambaqui to better understand the potential implications of triploidy on the metabolism and physiology of the fish.

Histological analysis showed that triploidy induction impaired the development of the gonad germline in both female and male tambaqui and more evident by the presence of increased cellularity in the interstitium in triploid tambaqui. Typically, sterility is more evident in triploid females as observed for Atlantic cod (*Gadus morhua*; Feindel et al., 2011), pufferfish (*Takifugu nifobicas*; Hamasaki et al., 2013) and rainbow trout (*Oncorhynchus mykiss*; Han et al., 2010). In tambaqui, the gonads of triploid females were immature, with great proliferation of oogonia by mitosis and no signs of meiotic oocytes. Moreover, triploid ovaries presented a dense structure of connective tissue with high cellularity, most likely these cells are responsible for the cleaning of apoptotic oogonia and/or early oocytes (Golpour et al., 2016).

In triploid males only spermatogonium were observed, while all diploid males presented post-pubertal testis (in final maturation, mature or resting). Similar results were observed in *Heteropneustes fossilis* (Tiwary et al., 2000) and in the sea bass *Dicentrarchus labrax* (Felip et al., 2001). Moreover, in male sea bass and sea bream *Sparus aurata* (Haffray et al., 2005), triploidy severely affects meiosis II, when secondary spermatocytes differentiate into spermatids. The sterility opens an opportunity for these males to become recipients for surrogate experiments (Siqueira-Silva et al., 2018). However, with the recent discovery that triploid brown trout develops fertility with aging (Lahnsteiner and Dünser, 2024), it is important that gonad status of older triploid tambaqui is evaluated to confirm if the sterility observed in the present study is permanent or transitory. Although only one fish was sampled, the sterility status of triploid female was further confirmed at three years with an undeveloped ovary compared to the fully developed ovary in 4 control diploid fish.

An important implication of the present study, beyond its potential to improve the aquaculture performance of triploid *C. macropomum*, lies in the application of triploidization as a biosafety measure to prevent the establishment of fertile hybrid escapees from aquaculture facilities. Hybrid fish production is a well-documented practice in South America, and hybrid individuals have already been reported in wild populations (Hashimoto et al., 2012). Such occurrences contribute to genetic introgression, posing a significant threat to the integrity of wild genetic resources that must be conserved (Hashimoto et al., 2012). Similar regulatory measures have been implemented in other regions, such as the United States, where triploid grass carp are used to mitigate ecological risks associated with fertile escapes (Zajicek et al., 2011).

In conclusion, our results demonstrated that pressure shock at an appropriate time post fertilization (95 spf) for a duration of 90 s would result in 100% triploidization and higher FGS (97%) and ES (97%). Our results also demonstrated that pressure shock treatment of the newly fertilized tambaqui eggs is a better way to produce triploidy than the previously used thermal shock methods. Our study presents an efficient and reproducible protocol for the production of triploid tambaqui, a significant advancement for the tambaqui aquaculture industry. Triploid tambaqui demonstrated sterility, a critical attribute that can mitigate the risk of genetic contamination in natural populations and improve farmed stock management. This sterility also provides an opportunity to optimize energy allocation for somatic growth, potentially enhancing productivity. The findings underline the practical benefits of triploidy as a sustainable solution for the tambaqui farming sector, paving the way for further research and broader application of this technique in aquaculture.

Authors statement

1. We confirm that all listed authors in the article have contributed significantly and agree with the final manuscript content.
2. All funding sources, including grants and their numbers, that supported the research have been declared at the end of the manuscript.
3. No potential conflict of interest was reported by the author (s).
4. The ethical statement has been included in the manuscript.

CRediT authorship contribution statement

Aldessandro da C. Amaral: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Lucas S. Torati:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Luciana N. Ganeco-Kirschnik:** Methodology, Investigation. **Luciana C.V. Villela:** Writing – review & editing, Investigation. **André Silverio Pereira:** Writing – review & editing, Investigation. **Julia T. Lopes:** Investigation. **Yury O. Chaves:** Methodology, Investigation. **Diogo T. Hashimoto:** Investigation. **Velmurugu Puvanendran:** Writing – review & editing, Visualization, Validation, Supervision, Investigation, Funding acquisition, Conceptualization. **Fernanda L. Almeida O'Sullivan:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

Data will be made available on request.

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