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Research Article

First steps toward implementing Trojan-sex chromosomal control of the invasive jewel cichlid *Hemichromis guttatus*

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ABSTRACT. The jewel cichlid, *Hemichromis guttatus*, a fish native to Africa, was introduced into Cuatrociénegas, Mexico, a natural protected area and has negatively impacted native species. To manage its populations, the Trojan-sex chromosomes technique, a genetic method that skews sex ratios over time by releasing YY (trojan) individuals, was implemented. Screening for sexual genotypes while creating YY individuals is crucial to its success. In the current work, we tested two putative genetic markers for the sexual genotype of jewel cichlids after subjecting them to sex reversal treatments. From 15 to 60 days after hatching (DAH), jewel cichlid larvae were fed diets containing either 17β-estradiol (feminization) or fadrozole (masculinization). Using RT-qPCR, the relative gene expression of *cyp19a1a* and *foxl2* was measured at 15, 30, 45, and 60 DAH. Oral administration of 17β-estradiol resulted in 100% feminization; fadrozole achieved 50% jewel cichlid males. The treatments unaffected the organism's length, weight, or survival. Although *cyp19a1a* was expressed at 15 DAH, its expression was not found in subsequent sampling periods. At 30 DAH, *foxl2* levels in the feminized organisms were lower than in the control group. There were no variations in the *foxl2* levels between the masculinization and feminization treatments at 45 DAH. According to endpoint PCR, the expression levels started to decline at 60 DAH. The sex reversal period might be shortened if gene expression patterns are taken into account. Our findings support the use of *foxl2* for sexual genotype screening in later stages of the generation of YY males.

Keywords: *Hemichromis guttatus*; invasive species management; Trojan-sex chromosomes; sex differentiation; *cyp19a1a*; natural protected area; jewel cichlid

INTRODUCTION

Introducing invasive species is one of the leading causes of biodiversity loss (Pyšek et al. 2020), and freshwater ecosystems are especially vulnerable to this threat (Rahel 2002, Leprieur et al. 2008). The jewel cichlid *Hemichromis guttatus*, a fish native to Africa, was introduced into the Churince pool in Cuatrociénegas, Coahuila, Mexico, probably as an aquarium dumping (Contreras-Balderas & Ludlow 2003, APFFC 2008). Since its introduction, this species has quickly expanded to surrounding aquatic systems due to its strong reproductive capability (Cohen et al. 2005, Lozano-Vilano et al. 2006, Aguilar-Aguilar et al. 2014, Hernández et al. 2017).

Since Cuatrociénegas is a federal Natural Protected Area and a RAMSAR site, it is crucial to preserve its biodiversity (Carabias et al. 1999). Its native biota includes stromatolite-forming communities, which are notable since they are among the planet's oldest life forms (Carabias et al. 1999, Elser et al. 2005). The jewel cichlid in this area has already damaged local species (Lozano-Vilano et al. 2006, Marks et al. 2011, De la Maza-Benignos 2017). Due to dietary overlap and

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the potential inhibition of reproduction (Dugan 2014), the endangered cichlid *Herichthys minckleyi* populations have been significantly impacted (Marks et al. 2011). Additionally, modifications to the trophic networks and macroinvertebrate populations may affect the communities that create stromatolites, with potentially severe implications for local biodiversity (Elser et al. 2005, Hulsey et al. 2005, Dugan 2014).

To eradicate this exotic species, we have chosen the Trojan sex chromosomes (TSC) technique (Gutierrez & Teem 2006), which involves releasing individuals carrying two Y sex chromosomes (supermales) into the exotic population over several generations. The invasive population's sex ratio would shift over time since YY individuals only produce male offspring, which would result in the virtual extinction of the population once there are no more females left (Gutierrez & Teem 2006, Gutierrez et al. 2012).

Despite the theoretical interest, there have been few documented instances of the TSC approach being used to control exotic populations (e.g. Schill et al. 2016, Jiang et al. 2018). Production of YY individuals is routinely achieved in aquaculture by sex reversal treatments (Beardmore et al. 2001), where sex-reverted females (individuals with a female phenotype but an XY genotype) are obtained through hormone exposure in the early stages of development (Budd et al. 2015). Sex-reverted females are then crossed with normal XY males, giving rise to a progeny composed of XX females and XY and YY males (Fig. 1). For some species, YY females can be obtained with a second sex reversal step.

The availability of molecular markers for the detection of sexual genotypes is a crucial component of the implementation of the TSC method (Beardmore et al. 2001, Cotton & Wedekind 2007), given that it is usually not possible to differentiate XY from YY individuals morphologically (Schill et al. 2016, Hattori et al. 2018). When it comes to their function during sex differentiation events, the enzyme cytochrome P450 aromatase, family 19, subfamily A, polypeptide 1a (*cyp19a1a*), and its regulator forkhead box L2 (*foxl2*) play pivotal roles in the regulation of sex steroids biosynthesis and metabolism, thereby exerting crucial control over the intricate processes of sexual differentiation. Both are highly conserved genes within teleost fishes and may be used as molecular markers (Guiguen et al. 2010, Tao et al. 2013). Considering those mentioned above, it would be possible to determine the sex ratio of a population by sacrificing a small sample of individuals and quantifying the expression levels of these genes, even at the early stages

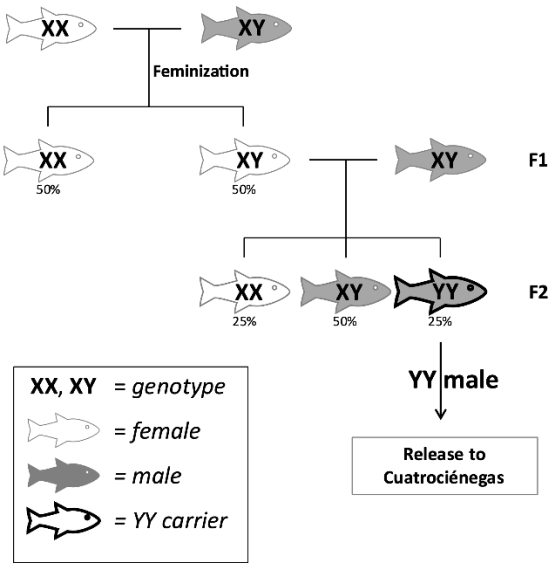

Figure 1. Diagram of reproductive events, feminization treatments, and introduction of YY carriers to control the exotic jewel cichlid *Hemichromis guttatus* in Cuatrociénegas through the TSC strategy by releasing YY males. Sexual genotypes and phenotypes are described in the box. The percentages show the proportion of individuals expected in the corresponding offspring (adapted from Gutierrez & Teem 2006).

(Piferrer & Guiguen 2008, Schill et al. 2016). As a result, it may be useful for identifying genotypes during the generation of YY male jewel cichlids.

The present study aimed to investigate the genetic expression of *cyp19a1a* and *foxl2* as potential molecular markers for sexual genotype screening and assess the efficacy of sex reversal treatments in jewel cichlid larvae, the first step to generating YY males.

MATERIALS AND METHODS

Collection and maintenance of jewel cichlids

Adult jewel cichlids (*Hemichromis guttatus*) were collected from the Churince pool (26°50'24.42"N, 102°8'2.31"W) in Cuatrociénegas, Mexico, using baited pots and creels (fishing permit PPF/DGOPA-188/14). Organisms were transferred and kept in captivity at the Laboratory of Ecophysiology, College of Biological Science of the Autonomous University of Nuevo León (UANL, by its Spanish acronym), where the bioassays were carried out.

Adults were kept in 20-gallon aquaria with artificial vegetation and a flowerpot as nesting material and spawning site. Each aquarium had five to six adults that

were given live food three times a day (earthworms, amphipods, larvae of chironomids, and mosquitoes) or commercial pellets (Nutripec, 32% protein) to apparent satiety. The rainy season was simulated to induce their reproductive behavior, with 30% water exchange twice a week. The rest of the fish were removed once a pair was formed, and the same conditions were maintained until spawning (Greenberg 1961). Because of the parental care of this species, larvae were kept with both parents until 14 days after hatching (DAH). During this period, larvae were fed *Paramecium* sp. three times a day from the start of the free-swimming stage (about 3-4 DAH) and *Artemia franciscana* nauplii from 12 DAH onwards. Larvae were placed at random in aquariums designed for sex reversal procedures at the age of 14 DAH.

Sex reversal

These experiments consisted of a feminization treatment in which larvae were orally exposed to 17β-estradiol (an estrogen, Sigma-Aldrich) and a masculinization treatment by the oral administration of fadrozole (an aromatase inhibitor, Sigma-Aldrich). Both compounds were dissolved in 70% ethanol at a concentration of 6.66 mg mL^{-1} and administered by exposing *Artemia* nauplii following previously published protocols (Martin-Robichaud et al. 1994, Stewart et al. 2001, Vidal-López et al. 2009). Daily, commercial *A. franciscana* cysts (Brine Shrimp Direct, Inc.) were hydrated for 1 h and decapsulated for 1 min in a 5% sodium hypochlorite solution. Chlorine was deactivated with sodium thiosulfate, and the cysts were filtered, rinsed with freshwater, and transferred to a conical container supplied with aeration from the bottom. Decapsulated cysts were incubated at 25°C in a saline medium of 30 for 24 h. After hatching, nauplii density was calculated (as the average number of nauplii in 10 samples of 1 mL) and moved to three incubation media in accordance with sex reversal treatments. For the feminization treatment, nauplii were enriched in a nutritive medium (according to Lozano-Peña 2017) given a weight of 10 µg per nauplii and a concentration of 17 beta of 200 mg kg^{-1}, the incorporation of estradiol would be 461 ng per mg of nauplii biomass (Stewart et al. 2001). Despite the lack of prior reports on the enrichment of nauplii using this chemical, the identical enrichment technique was carried out for the masculinization treatment employing fadrozole at 100 mg kg^{-1} of nauplii; this concentration has been successfully employed in several fish species using formulated diets (Kitano et al. 2000, Afonso et al. 2001, Uchida et al. 2004). The same amount of ethanol without any hormone content was given to the nauplii utilized in the control treatment. The maximum ethanol concentration at which organisms were exposed was 0.21% for nauplii and 0.01% for jewel cichlid larvae.

Treatments for sex reversal were performed in triplicate in 10-gallon aquariums with a 20 L water volume, continual aeration, and twice-weekly 50% water changes to preserve water quality. Each aquarium received 50 larvae. Larvae and treatments were distributed at random. Larvae were fed the corresponding enriched nauplii to apparent satiation twice a day after 2 and 5 h of the enrichment of nauplii. Sex reversal treatments lasted 45 days, from 15 to 60 DAH.

The temperature was measured thrice daily, the pH twice weekly, and the levels of ammonia, nitrites, and nitrates once weekly using a colorimetric kit (Freshwater Master test kit, API). Throughout the trial, water quality was maintained within the parameters advised for this species. For the control, feminization, and masculinization treatments, the temperatures were kept at 27.7 ± 2.2, 27.6 ± 2.2, and 27.6 ± 2.1°C, respectively. The recorded lowest and highest temperatures were 22 and 33.2°C. pH values for the control, feminization, and masculinization treatments were 8.2 ± 0.25, 8.4 ± 0.22, and 8.4 ± 0.31, respectively. It was not possible to measure oxygen levels. However, given the low organism density (2.5 larvae L^{-1}), low oxygen levels were probably not a problem. Nitrate, nitrite, and ammonia concentrations were always below the minimum detection level.

Four larvae per replicate (12 per treatment) were sacrificed for the gene expression analysis at 15, 30, 45, and 60 DAH. After swiftly measuring the larvae's wet weight, they were immediately placed in RNAlater (Qiagen), where they were maintained at -20°C until the RNA was extracted. At the end of the sex reversal treatments (60 DAH), total length (TL) and survival rate were determined. The remaining organisms were then kept alive with a commercial feed.

The sex ratio was established by sacrificing 36 individuals for each treatment between the ages of 199 and 235 DAH to assess the effectiveness of sex reversal treatments. Sex was determined by dissection and visual inspection of the gonad after aceto-carmine staining (Wassermann & Bertolla-Afonso 2002). An aceto-carmine solution (0.5% indigo-carmine, 45% acetic acid), heated to boiling for 5 min and filtered, was made for the gonad staining. Once the gonad was removed, the tissue sample was placed on a glass slide with a few drops of aceto-carmine and pressed with a coverslip (gonadal squash). Preparations were examined under a 100 and 200x optical microscope to

identify the gender of the organisms based on the morphological structures present (Guerrero & Shelton 1974, Wassermann & Bertolla-Afonso 2002).

Genetic expression

To quantify *cyp19a1a*, also known as aromatase, gene expression, total RNA from each larva was extracted in Trizol (Ambion) according to the manufacturer's protocol. Ten volumes of Trizol were used during homogenization to reduce contamination with genomic DNA (Heidary & Pahlevan 2014). Briefly, each larva was homogenized in Trizol and centrifuged at 12,000 *g* (10 min, 4°C). The supernatant was incubated at room temperature (5 min), mixed with chloroform, and centrifuged at 12,000 *g* (15 min, 4°C). The upper aqueous phase was recovered with 90% isopropanol and incubated overnight at -20°C. Subsequently, it was centrifuged at 12,000 *g* (10 min, 4°C). The precipitate (RNA) was washed with 70% ethanol, centrifuged again at 7500 *g* (5 min, 4°C), and the pellet was allowed to dry for 5-10 min in a laminar flow hood and immediately dissolved in sterile water. Because of their small size, larval samples of 15 DAH were processed in of three pools. RNA concentration was quantified by spectrophotometry (Nanodrop, Thermoscientific), and its quality was determined by its 260/280 optical density. The integrity of the 18s and 28s ribosomal RNA subunits was evaluated by electrophoresis of 1 µg of total RNA on 1% agarose gel, including 1% commercial chlorine as a denaturing agent and ribonuclease inhibitor (Gayral et al. 2011, Aranda et al. 2012). Ethidium bromide was used for staining, and the gels were analyzed in a Gel Doc EZ + photodocumenter using Image Lab 5.2.1 software (Bio-Rad).

The complementary DNA (cDNA) was constructed with an Omniscript reverse transcription kit (Qiagen) using random hexamers according to the manufacturer's protocol. Briefly, 125 ng of total RNA were incubated at 65°C for 5 min and then at room temperature for another 5 min. Reverse transcription was performed in a reaction mix of 10X RT buffer (0.125 µL), 5 mM dNTP (0.5 µL), 2.5 ng of random hexamers, reverse transcriptase (0.125 µL), RNAse inhibitor (0.01 µL) and water to make up 10 µL. The mixture was incubated in a thermal cycler for 10 min at 25°C, 60 min at 37°C, and 15 min at 70°C. The cDNA was immediately used or stored at -20°C until amplification.

DNA was isolated from adult muscle tissue using the phenol:chloroform procedure to confirm the size of the amplified region of aromatase (because of the presence of introns). The tissue was incubated at 48°C for 90 min in a lysis buffer (0.5% SDS, 100 mM EDTA pH 8, 200 mM Tris-HCl pH 8, 250 mM NaCl, and 0.3 mg mL^{-1} proteinase *K*) and then homogenized in phenol:chloroform:isoamyl alcohol solution (25:24:1) and centrifuged at 5000 *g* (10 min, 25°C). The aqueous phase was recovered, and DNA was precipitated with isopropanol at -20°C overnight. Then, it was centrifuged again at 5000 *g* (10 min, 25°C), and the pellet was allowed to dry in a laminar flow hood, dissolved in sterile water, quantified in Nanodrop, and stored at -20°C until use.

For the amplification of aromatase *cyp19a1a*, primers were designed from conserved regions of the sequences available for other cichlid species at the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov) using MEGA for sequence alignment. Additionally, the genetic expression of *foxl2* was evaluated (Böhne et al. 2013, Flores-Salinas 2017). As a reference gene, *18s* rRNA was used (Yang et al. 2013, Aguilar 2017). All oligonucleotides were analyzed using Snapgene viewer and PrimerBlast software. It was confirmed using Oligoanalyzer (IDT technologies) that the ΔG values between the designed oligonucleotides were greater than -7 for homodimerization, greater than -6 for heterodimerization, and with a temperature not exceeding 40°C for hairpin formation. Primer sequences, alignment temperatures, and expected fragment sizes for each gene are shown (Table 1).

Expression levels for each gene were determined by reverse transcription quantitative PCR (RT-qPCR) using fluorescence accumulation values during amplification with SYBRgreen (SsoAdvanced Universal SYBR Green Supermix, Bio-Rad). Each sample was run in duplicate in a 10 µL reaction volume of 1 µL cDNA, 1 µL primers (5 µM each), 3 µL sterile H_2O, and 5 µL SYBR Green 2X supermix. Amplification was performed using 96-well microplates in a CFX96 RealTime System thermal cycler (Bio-Rad) under the following conditions: 95°C hot start (3 min), 40 cycles at 95°C (30 s), alignment temperatures according to Table 1, 72°C (30 s), and final extension at 72°C (1 min). Fluorescence readings were done at the end of each extension cycle to analyze the purity of the product. A melting curve was performed at the end of the final extension with a temperature gradient ranging from 65 to 95°C in 0.5°C/5 s increments. No template controls (NTC) were included at each run.

Fluorescence amplification values were retrieved using the CFX manager software (Bio-Rad). Quantification was performed for each curve using the Cy0 method (Guescini et al. 2008) and the qpcR package (Ritz & Spiess 2008) to account for varying

Table 1. Primers used for the amplification of jewel cichlid *Hemichromis guttatus* genes during this study. [1]Aguilar (2017). [2]Flores-Salinas (2017).

Gene	Primer	Sequence	Fragment size	Alignment temperature (°C)
cyp19a1a[1]	Forward	5' CAT GAA CGA GAG AGG CAT CA 3'	206 pb	56.1
	Reverse	5' AGA TGT CCA CCA CAG TGC AG 3'		
foxl2[2]	Forward	' CCG GAT CCG TCC CAG AA '	59 pb	56.9
	Reverse	5' CGG ATA GCC ATG GCA ATGA 3'		
18s[1]	Forward	5' GGA CAC GGA AAG GAT TGA CAG 3'	111 pb	54.5
	Reverse	5' GTT CGT TAT CGG AAT TAA CCA GAC 3'		

PCR efficiencies. The relative expression of *cyp19a1a* or *foxl2* (target gene) was calculated by normalizing their values with those of the *18s* gene using the $2^{-\Delta Cy0}$ method, where $\Delta Cy0 = Cy0_{target\ gene} - Cy0_{18s}$.

Statistical analysis

Data normality and homoscedasticity assumptions were evaluated with the Shapiro-Wilk and Levene tests, respectively. A one-way ANOVA was performed to evaluate the effect of treatments on weight at 30, 45, and 60 DAH and total length at 60 DAH. After arcsine transformation of percentages, survival was assessed using a one-way ANOVA. Relative expression values did not meet ANOVA assumptions. They were evaluated with a randomization test by randomly reallocating (n = 49,999) expression values between each treatment group and the control while maintaining the sample size and characteristics of the original data, allowing us to evaluate the observed expression ratio against the expected ratio distribution. The results were then used to assess lower and higher expressions based on their relative positions within the distribution (Manly 2006, Pfaffl 2006). The sex ratio was evaluated against the expected 1:1 ratio using the X^2 test (Mair et al. 2011). All statistical analyses were performed in R v4.0.2 (R Core Team 2020).

RESULTS

Sex reversal

Two breeding pairs produced the jewel cichlid (*H. guttatus*) larvae used in the sex reversal treatments, with a 4-h gap between spawnings. Because of this species parental care, the number of eggs and the hatching rate were not determined (females usually protect their eggs in their mouths during the first few days). At 14 DAH, 424 larvae from one pair and 26 larvae from another pair were used, with an average weight of 2.42 ± 0.46 mg and a total length of 6.31 ± 0.42 mm. Individuals at 60 DAH did not differ in weight ($P = 0.48$) or total length ($P = 0.37$) (Table 2).

The survival rate was not affected by treatments ($P = 0.95$). At 60 DAH, there was a mean survival of 75.3 ± 6.1% for the control, 70.6 ± 16% for the feminization, and 72 ± 16.4% for the masculinization treatment. Mortality mostly occurred in the first week of the experiment, presumably due to handling the larvae during distribution into the experimental aquaria.

By sacrificing 36 individuals per treatment and determining sex using gonadal examination around 200 DAH, the effectiveness of reversing sex was assessed. Because the gonads were fully grown and the oocytes could be easily identified, suggesting that the organisms were going through sexual maturity, it was quite simple to identify females. Under microscopic observation, the ovarian tissue was characterized by oocytes at the perinucleolar stage. The testicular structure showed the characteristic morphology of spermatogonia. The sex ratio in the control group was 1:1.4 (Table 2) and thus was not different from the expected 1:1 ratio ($X^2 = 1$; $P = 0.31$). The feminization treatment was highly effective, resulting in 100% of females ($X^2 = 32$; $P = 0.001$). The masculinization treatment resulted in a sex ratio of 1:1.

Genetic expression

The amplified *cyp19a1a* fragment corresponded to the expected size of 206 pb for cDNA and 320 pb for DNA (Ye et al. 2012). Sequence alignment of the primers in several cichlid species (*Oreochromis niloticus*, NCBI GenBank Accession No: U72071; *Oreochromis aureus*, DQ279891.1; *Astronotus ocellatus*, KT337402.1; *Oreochromis mossambicus*, AF135851.1; *Neolamprologus pulcher*, KC684565.1; and *Cichlasoma dimerus*, KX260955.1) showed that the fragment corresponded to a partial region of the aromatase cDNA spanning exons 3 and 4 (nucleotides 453-658 of the cDNA), including intron 3 in DNA (Chang et al. 1997, 2005). This difference in fragment size allowed us to distinguish contamination by genomic DNA during the RNA extraction and subsequent amplification proto-

Table 2. Sex ratio of jewel cichlid *Hemichromis guttatus* exposed to sex reversal treatments. *Indicates differences to the expected 1:1 ratio (X^2, $P < 0.001$). SD: standard deviation.

Treatment	Weight (mg ± SD)	Length (mm ± SD)	Number of males	Number of females	% of females
Control	160.2 (34.6)	21.35 (1.74)	15	21	58
Feminization	152.2 (42)	20.33 (1.79)	0	36	100*
Masculinization	157.6 (47.6)	20.62 (1.89)	18	18	50

cols. RNA quality was verified utilizing $A_{260/280}$ absorbance values.

Quantifying aromatase genetic expression at 15 DAH was impossible due to non-specific bands (Fig. 2); however, the anticipated presence of cDNA amplicon (206 bp) indicates that aromatase is expressed at this age. Semiquantitative imaging analysis for these fragments showed a 10.73 times difference in the relative intensity among bands, suggesting a difference in expression levels among organisms because these larvae were sampled before sex reversal treatments.

Aromatase could not be detected at 30, 45, or 60 DAH, as indicated by the fluorescence levels during amplification, which were close to the NTC control. Different modifications were made to the RNA extraction process and reverse transcription protocol to rule out a potential influence owing to the size of the amplified product (>150 bp); however, aromatase expression could not still be found at this age (Fig. 2). On the other hand, the 18s ribosomal gene and *foxl2*, one of the key regulators of aromatase transcription, were amplified.

We chose to quantify *foxl2* by RT-qPCR because this gene is a transcriptional factor that controls the transcription of the aromatase and because RT-PCR at 30 and 45 DAH confirmed the expression levels of this gene. As a result, differences were found in the relative expression levels at 30 DAH. Specifically, and unexpectedly, the expression levels in the feminization treatment were lower than the control group (fold change = 0.735; $P = 0.009$, Fig. 3). The expression levels in the masculinization treatment were not different from those of the control group (fold change = 0.99; $P = 0.49$).

All treatments displayed increased variation at 45 DAH, but no marked differences between treatments were observed; expression levels in the feminization treatment were not different from the control group (fold change = 0.976; $P = 0.469$), while expression levels in the masculinization treatment were slightly higher (fold change = 1.365; $P = 0.128$).

From 30 to 45 DAH expression levels in the control group decreased slightly (fold change = 0.751; $P = 0.105$) but remained unchanged in the feminization (fold ratio = 0.997; $P = 0.49$) and masculinization treatment (fold change = 1.031; $P = 0.55$). At 60 DAH, the expression levels of *foxl2* were lower according to endpoint RT-PCR, and they were not quantified by qPCR.

DISCUSSION

Negative impacts on populations of endemic species have already been reported in Cuatrociénegas due to the introduction of the jewel cichlid (*H. guttatus*) (Lozano-Vilano et al. 2006, Marks et al. 2011, Dugan 2014). As a control strategy, we propose introducing YY individuals to skew the population sex ratio over time (Gutierrez & Teem 2006). The sex reversal technique and the sexual characterization of the offspring are two essential phases to implement the TSC strategy. The present study addresses the first approximation of the production of sex-reverted females and the identification of a sex molecular marker for this species.

In this research, the sex reversal of jewel cichlid larvae was done using hormone-enriched *Artemia* nauplii. The sex ratio in the control treatment did not differ from the expected 1:1 proportion, confirming no marked influence of temperature on sex reversal. The temperature ranged between 22 and 33.3°C during the experiment. Temperature is one of the most crucial factors affecting sex differentiation in teleost fishes and usually influences the sex ratio (Tenugu et al. 2022). Although several studies have demonstrated that temperature affects the sex ratio of some cichlid species (Bezault et al. 2007, Azaza et al. 2008), either increasing female or male rates, to our knowledge, no studies have reported the influence of temperature on the sex ratio in *Hemichromis* spp. By supplying 17β-estradiol-enriched nauplii at a concentration of 200 mg kg^{-1}, 100% of feminization of the jewel cichlid could be achieved without impairing growth and survival. We assumed that *Artemia* nauplii assimilated 100% of

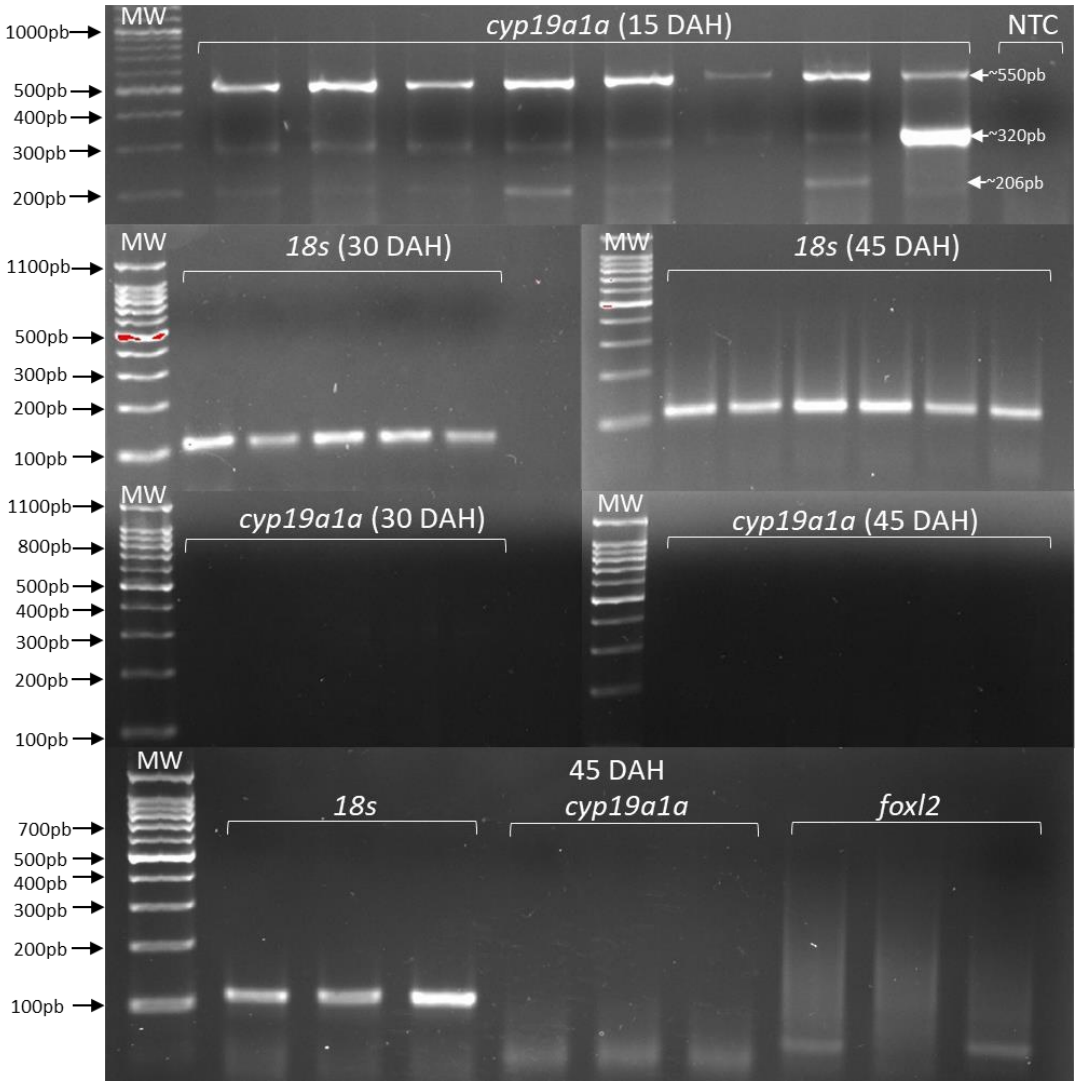

Figure 2. Amplification of *cyp19a1a*, *foxl2*, and *18s* by endpoint RT-PCR in jewel cichlid *Hemichromis guttatus* at 15, 30, and 45 days after hatching (DAH). Each band corresponds to the expression levels of the indicated gene in different individuals. Two percent agarose gel electrophoresis stained with bromide ethidium. MW: molecular weight marker, NTC: no template control.

estradiol; however, it has been noted that 4 h after enrichment, estradiol-enriched nauplii can lose up to 13% of the assimilated hormone (Martin-Robichaud et al. 1994). Consequently, the real amount of estradiol consumed by the larvae was probably smaller. In this regard, the use of *Artemia* nauplii as a vehicle for hormone administration has been successfully used for the feminization of other fish species. For example, 100% feminization was achieved in *Micropterus salmoides* larvae and 98% in *Cyclopterus lumpus* using estradiol-enriched *Artemia* nauplii at concentrations of 5 and 20 mg L^{-1}, respectively (Garrett 1989, Martin-Robichaud et al. 1994).

On the other hand, fadrozole could not masculinize every jewel cichlid larva at the employed concentration. Even though the YY male production does not require a masculinization phase, this treatment was carried out to serve as a negative control for aromatase expression analysis since both male and female gene expression patterns were anticipated for the control group. This finding indicates that the masculinization of the jewel cichlid larvae would have required a greater fadrozole concentration. However, 100% reversion has been accomplished in other fish species using comparable fadrozole doses. For example, 100 mg kg^{-1} of food completely masculinized

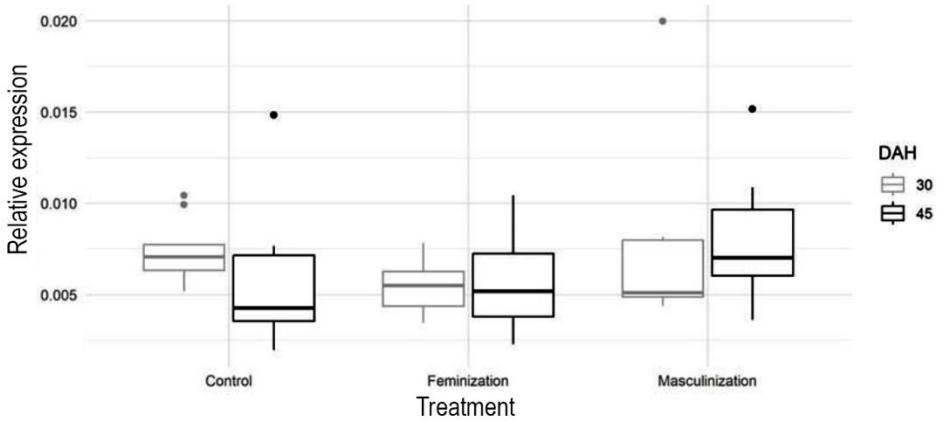

Figure 3. Relative expression of *foxl2* in jewel cichlids *Hemichromis guttatus* exposed to sex reversal treatments at 30 and 45 days after hatching (DAH).

Danio rerio females, while a dose of 10 mg kg^{-1} masculinized 62.5% (Uchida et al. 2004).

Sensitivity to fadrozole seems species-specific, as 100% sex reversal was achieved with *Paralichthys olivaceus* larvae using 100 mg kg^{-1}. A dose of as little as 1 mg kg^{-1} was enough to masculinize 25% of females (Kitano et al. 2000). However, conflicting results have been noted in other cichlid species, including *Oreochromis niloticus*, where 100% masculinization has been reported after exposure of larvae to 75 mg kg^{-1} for 30 days (Afonso et al. 2001). However, a dose of 500 mg kg^{-1} was insufficient to produce 100% males in a different study with the same species (Kwon et al. 2000). It should be emphasized that fadrozole was given through a commercial diet in the research mentioned above, so a potential impact from the method of delivery cannot be completely ruled out. Additionally, there may be losses in the retention by nauplii after enrichment, as was previously indicated. To our knowledge, no reports of enrichment of nauplii with fadrozole exist.

Despite theoretical interest, there have been few reported attempts to control alien populations using the TSC technique. So far, YY male production of the brook trout *Salvelinus fontinalis* and the common carp *Cyprinus carpio* have been documented. In the case of *S. fontinalis*, the organisms were feminized to a 99.6% feminization rate by being fed a commercial diet containing estradiol at a dose of 20 mg kg^{-1} (Schill et al. 2016), whereas *C. carpio* YY males were produced by androgenesis (Jiang et al. 2018).

Given that traditional test crossing is time-consuming (Jiang et al. 2018), the availability of molecular markers for the identification of sexual genotypes is essential for the execution of the TSC method (Cotton & Wedekind 2007). Because the sex-determining master gene is unknown for this species and no sex-linked markers have been reported for any jewel cichlid species, aromatase was the best option (Piferrer & Guiguen 2008). One of our primary goals was to measure the expression levels of the gene *cyp19a1a*, which encodes the aromatase enzyme. This gene's expression is highly conserved throughout vertebrates. At early developmental stages, the levels of this gene typically differ between the sexes (Böhne et al. 2013) and even among normal and sex-reverted organisms in some species (Kotula-Balak et al. 2008). Even in larvae or juveniles, the sex ratio in a population can be determined by sacrificing a small sample of animals and analyzing aromatase expression levels (Piferrer & Guiguen 2008), which shortens the time required to get YY males (Schill et al. 2016).

The present research is the first report on the aromatase gene being amplified in any *Hemichromis* species. The 320 bp DNA amplified fragment corresponds to a partial region of the aromatase gene according to sequences reported from other cichlid species. The *cyp19a1a* gene comprises 10 exons and 9 introns in all teleost species, except for some groups that have lost introns during their evolution (Wang et al. 2014). Alignment of primers with the sequences reported showed that the 206 bp amplified fragment by RT-PCR corresponds to the expected size in cDNA, spanning exons 3 and 4, and the 320 bp amplified fragment by PCR corresponds to the expected size in DNA, which includes intron 3.

Due to the presence of two non-specific bands of 320 and 550 bp, aromatase could not be quantified des-

pite expression levels suggesting that it is expressed at 15 DAH. The nature of the 550 bp band is unknown, and no band larger than 400 bp was found during standardization with the alignment temperatures used. The band may be associated with a specific transcript of early development, given that cDNA was obtained for standardization from juveniles or adults (>30 DAH). The 320 bp band corresponds to the expected size of aromatase in genomic DNA, so it could result from DNA contamination during RNA extraction, which is a common issue when RNA is extracted from tiny samples using Trizol (Gayral et al. 2011).

Despite these technical difficulties, observing a 206 bp product, the expected mRNA size for aromatase, was possible, suggesting that it is expressed at 15 DAH. Moreover, semiquantitative imaging analysis showed variations in the relative intensity of this fragment among samples. Specifically, the most intense band was 10.73 times more intense than the weakest one, which might indicate differences in aromatase expression between males and females at this age. The expected fragment was not observed at 30, 45, and 60 DAH. Thus, this gene may be expressed only early in this species. In this regard, expression levels of aromatase in *O. niloticus*, another cichlid, were shown to be different between males and females at 8 DAH (Li et al. 2013) and have even been found to reach significant increases in females as early as 5 DAH (Tao et al. 2013). As a result, the jewel cichlid's patterns of aromatase expression may resemble those of tilapia.

Aromatase expression was detected in adult gonadal samples (not shown), so the expression will likely remain at baseline levels in juveniles until the period of gonadal maturation and subsequent recrudescence events (Rasheeda et al. 2010, Johnsen et al. 2013), which suggests that future research should assess aromatase levels in jewel cichlids at very early stages, between 5 and 20 DAH.

We were able to quantify the expression of *foxl2*, a transcription factor that suppresses the action of male-related genes, such as *dmrt1* (doublesex and mab-3 related transcription factor 1), while promoting aromatase expression in females (Li et al. 2013, Tao et al. 2013, Wang et al. 2015). Specifically, it binds to the promoter region of the *cyp19a1a* gene through a forkhead domain, activating its transcription (Wang et al. 2007). Expression of *foxl2* has been found in other tissues such as the brain, gills, and spleen. Still, the highest levels are always found in the ovary during sex differentiation (Wang et al. 2004, 2012, Yamaguchi & Kitano 2008). However, some fish species express it in testis at basal levels (Wang et al. 2015).

It was possible to detect *foxl2* at 30, 45, and 60 DAH. At 30 DAH, the relative expression in the masculinization treatment was not different from the control group, which was expected given the similar sex ratio. Unexpectedly, at 30 DAH, the expression of *foxl2* was lower in the feminization treatment than in the control group.

Exogenous estrogen has been found to either up-regulate or not repress *foxl2* expression in a variety of fish species, even though it may downregulate the expression of other genes, such as aromatase (Jiang et al. 2011, Wang et al. 2012, Fan et al. 2019, Zou et al. 2020). It is conceivable that exogenous estradiol administration, which was sufficient to cause female sex differentiation, might have suppressed *foxl2* expression through negative feedback. In this regard, paradoxical masculinization following exogenous estrogen treatment has been observed in *Gambusia holbrooki* and *O. niloticus* (Alcántar-Vázquez 2018, Patil et al. 2020). Exogenous estrogens have been hypothesized to suppress the expression of aromatase in some species, preventing the conversion of androgens to estrogens and causing an accumulation of testosterone in blood plasma (Warner et al. 2014, Patil et al. 2020), which may have caused *foxl2* to be downregulated in our study.

Regarding the sex ratios, the *foxl2* expression pattern in feminized organisms could be associated with the phenotypic sex because the other treatments showed a sex ratio close to 1:1. *foxl2* levels in females from the control group, however, will not necessarily match those seen in feminized organisms due to the potential influence of exogenous estrogen.

At 45 DAH, there was greater variation in *foxl2* expression levels in the control group, indicating higher disparities in expression levels between sexes. However, no changes in *foxl2* expression were observed among treatments at this sampling period. It could imply that at this point of sex differentiation, the effects of hormone therapies have no impact on gene expression. At 60 DAH, RT-PCR showed that the expression levels fell for all treatments; hence, qPCR was not performed. These findings imply that *foxl2* expression levels in jewel cichlids may peak between 30 and 60 DAH and then decline to basal levels.

Since expression levels in the control group decreased slightly between 30 and 45 DAH and dropped approaching 60 DAH, the exposure duration to estradiol could be shortened (Piferrer & Lim 1997). *O. niloticus*, a species phylogenetically near *Hemichromis* sp., has a sensitive phase for sex reversal between 5 and 30 DAH (Farias et al. 1999, Mair et al. 2011, Zhang et

al. 2017). The exposure period could likely be shortened to 5-20 days. Given that the TSC technique requires introducing YY individuals over multiple generations, this adjustment merits consideration.

The current study discusses a first approach for using the Trojan-sex chromosome concept in controlling invasive *H. guttatus* populations. 100% feminization was accomplished using enriched artemia nauplii with 17 beta. It has been shown that this species' genome contains the aromatase gene *cyp19a1a*, which is expressed at very early stages of the period of sex differentiation. Furthermore, increased *foxl2* expression levels were linked to feminized organisms at 30 DAH, making it a trustworthy option to assess the sex ratio in later stages of the YY male production by sacrificing a small progeny sample.

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