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Effects of 17β-estradiol on early gonadal development and expression of genes implicated in sexual differentiation of a South American teleost, *Astyanax altiparanae*

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ABSTRACT

Gonadal sex differentiation in teleost fish shows greater plasticity as compared to other vertebrates, as it can be influenced by a variety of factors such as exogenous sex steroids. Exogenous estrogens, such as 17β-estradiol (E2), can induce feminization when administered during early embryonic development. However, the mechanisms underlying the E2-induced feminization are not fully understood, especially in Neotropical species. Therefore, the aim of this study was to evaluate the effects of E2 administration on the phenotypic sex characteristics, histological assessment of the gonads, and the expression of selected genes in *Astyanax altiparanae* exposed to dietary E2 prior to gonadal differentiation. At 4 days post-hatch (dph), groups of 30–40 undifferentiated larvae were fed with a diet containing varying amounts of E2 for 28 days, and fish were sampled at 90 dph. Previous studies revealed that ovary formation in *A. altiparanae* occurred at 58 dph, whereas the first sign of testis formation was found at 73 dph. In relation to the control, E2 exposure increased the proportion of phenotypic females in 120% and 148.4% for 4 and 6 mg E2/Kg, respectively. However, histological analysis revealed that treatments did not affect gonadal sex ratio between males and females, but induced intersex (testis-ova) in the group treated with 6 mg E2/Kg food. Treatment with E2 also altered gonadal transcript levels of a selected number of genes implicated in sexual differentiation. Males overexpressed *dmrt1*, *sox9* and *amh* following E2 treatment as compared to control. Females showed increased mRNA levels of *dmrt1* and *sox9*, which might be related to the down-regulation of *cyp19a1a* after E2 exposure. In summary, E2 exposure during early gonadal development affected male secondary characteristics without changing the gonadal sex ratio, and altered expression of genes implicated in sexual differentiation.

1. Introduction

The most critical stage in vertebrate sex determination is one in which the bipotential gonad anlage will become a testis or ovary. This process involves cell fate and differentiation, and both programs are regulated through cascades and networks of multiple genes (Graham et al., 2003; Herpin et al., 2013; Herpin and Schartl, 2015). Over the past decades, accumulated knowledge has shown that while the genetic machinery triggering the gonadal sex differentiation is diverse among vertebrates, the downstream components seem to be evolutionarily more conserved and appear to converge on the regulation of a few central common effectors (Graham et al., 2003; Herpin et al., 2013; Capel, 2017). In vertebrates, activation of male pathway initiates through up-regulation of Sox9 (Sry-related HMG box 9), followed by Sf1 (steroidogenic factor–1), Fgf9 (Fibroblast growth factor 9) and Dmrt1 (Doublesex and Mab-3 related transcription factor 1). Dmrt1 is

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not only important for maintaining the male pathway but also in suppressing the female networks. There are two female networks, one involving Foxl2 (Forkhead box transcription factor L2) and Esr1,2 (Estrogen receptor 1 and 2), while the other one comprises Rspo1 (R-spondin 1), Wnt4 (Wnt family member 4)/β-catenin and Fst (Follistatin) (Brennan and Capel, 2004; Yao et al., 2004; Herpin and Schartl, 2015; Biscotti et al., 2018).

Teleost fish exhibits the most diverse mechanisms of sex determination and differentiation among vertebrates (Nagahama, 1994; Devlin and Nagahama, 2002; Kobayashi et al., 2013). Additionally, multiple triggers can regulate the process of sexual differentiation including genetic or environmental factors (hormones, temperature, pH, oxygen, social condition and others) (Kobayashi et al., 2013; Godwin, 2010; Castañeda Cortés et al., 2019). Among these factors, sex steroid hormones are considered the major inducers of gonadal sex differentiation, and are also responsible for the maintenance of the differentiated gonad in fish as reviewed previously (Liu et al., 2017; Li et al., 2019).

Estrogens are produced by the conversion of androgens through cytochrome P450 aromatase, which is encoded by *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a - gonadal type) and *cyp19a1b* (cytochrome P450, family 19, subfamily A, polypeptide 1b - brain type) genes in most of teleost fish species (reviewed by Le Page et al., 2010; Li et al., 2019). The expression of *cyp19a1a* and the production of endogenous estrogens occur specifically in the female gonads during the critical period of molecular sexual differentiation (reviewed by Nagahama, 2002; Li et al., 2019). In this context, exposure to 17β-estradiol (E2) before the critical time window of gonadal differentiation has been shown to induce ovarian differentiation in genetic males of different families of fish including Salmonidae, Cichlidae, Anguillidae, Sparidae, Belontiidae, Poecilidae, Cyprinidae and Characidae (Pandian and Sheela, 1995; Piferrer, 2001; Chang et al., 1994; Bem et al., 2012; Díaz and Piferrer, 2017). Conversely, administration of aromatase inhibitors prior to sexual differentiation has been reported to induce masculinization of genotypic female fish, as reported in rainbow trout (*Oncorhynchus mykiss*) (Guiguen et al., 1999), tilapia (*Oreochromis niloticus*) (Guiguen et al., 1999), chinook salmon (*Oncorhynchus tshawytscha*) (Piferrer, 1994), protogynous orange-spotted grouper (*Epinephelus coioides*) (Tsai et al., 2011) and European sea bass (*Dicentrarchus labrax*) (Navarro-Martín et al., 2009). Altogether these data indicate that gonadal aromatase and E2 can act as inducers of ovarian differentiation in some teleost species (reviewed by Nagahama, 2002; Li et al., 2019). Treatments with E2 or aromatase inhibitors have been widely used for sex control in aquaculture, in particular for establishing monosex cultures when a species has a pronounced sexual dimorphism in weight and size or growth rate (Piferrer, 2001; Beardmore et al., 2001). However, the mechanisms underlying the E2-induced feminization in fish are not fully understood nor systematically compared, especially for Neotropical species (Fernandino and Hattori, 2018). For example, studies with South American pejerrey (*Odontesthes bonariensis*) have shown that E2 treatment during early gonadal development decreased the expression of genes related to testicular differentiation, such as *amh* (anti-Müllerian hormone) and *dmrt1* (doublesex and Mab-3 related transcription factor 1) (Fernandino et al., 2008a, 2008b). Simultaneously, E2 increased the transcript abundance of *cyp19a1a*, which is associated with ovarian differentiation (Fernandino et al., 2008a).

Astyanax altiparanae is a South American teleost fish, popularly known as lambari, native to the Upper Paraná basin in Brazil (Garutti and Britsky, 2000). *A. altiparanae* easily reproduce, have good survival rates for both larvae and juveniles, and display rapid growth rate and larger body size for females (Porto-Foresti et al., 2005). Therefore, the species is of substantial commercial importance (Porto-Foresti et al., 2005) and scientific relevance as a Neotropical experimental model (Gomes et al., 2013; Costa et al., 2014; Adolfi et al., 2015; De Paiva Camargo et al., 2017; Branco et al., 2019). Previous studies have induced meiotic gynogenesis in *A. altiparanae* and the sex ratio of gynogenetic progenies suggests a XX/XY chromosome system for this species (Do Nascimento et al., 2019). However, sex determining gene is still unknown and no genetic makers are available to genotype individuals regarding the sex.

Considering that *A. altiparanae* females are more economically attractive than males due to their faster growth rate and larger size (Porto-Foresti et al., 2005), studies to induce feminization in this species are of great interest. Nevertheless, this topic remains poorly investigated in *A. altiparanae* and the molecular mechanisms underlying the E2-induced feminization are unknown. Therefore, the aim of the present study was to evaluate the effects of dietary E2 on phenotypic sex characteristics, gonadal histology, and gonadal gene expression in *A. altiparanae* exposed to dietary E2 prior to gonadal differentiation.

2. Material and methods

2.1. Animals and experimental design

The specimens used in this study were obtained from the Center of Aquaculture of São Paulo State University (CAUNESP), Jaboticabal (São Paulo State, Brazil). All experimental procedures were performed according to the São Paulo State University Animal Care and Use Committee Protocol (CEUA - 9496). Throughout the experiment, water quality parameters were monitored daily. The average monitored conditions were pH 6.6 ± 0.7 (pH meter YSI, model PH100), dissolved oxygen 6.2 ± 0.6 mg/L (oximeter YSI model 55), ammonia < 0.1 mg/L, and nitrite 0.11 mg/L. Artificial reproduction was performed using *A. altiparanae* broodstocks (1 female: 2 males) that were kept in 100 L tanks under 14 h light and 10 h dark, and constant temperature (28 °C). Spawning was induced by a single dose of 3–6 mg carp pituitary extract/Kg of fish. Fertilized eggs were collected and incubated in a controlled incubation system until the larva-mouth opening period, which is approximately 4 days post-hatching (dph) under these conditions. At this stage of development, *A. altiparanae* larvae are considered undifferentiated, as described previously (Adolfi et al., 2015). Three groups of 30–40 larvae each were distributed into 60 L tanks and fed with diet containing either 0, 4, or 6 mg of E2/kg food (Fig. 1). Each treatment was performed in 4 replicate tanks, for a total of 130 larvae (Fig. 1). Fish were fed with E2-containing feed (see methods described below) for 28 days, from 4 to 32 dph (Fig. 1). This period has been chosen as it precedes the critical time window of sexual differentiation for this species (Adolfi et al., 2015). In this species, the first sign of morphological ovarian differentiation was observed at 58 dph, whereas the first sign of testis formation was found at 73 dph (Adolfi et al., 2015). After 28 days of treatment, juvenile fish were fed *ad libitum* twice daily with commercial powdered food without any hormonal treatment (Fig. 1). Fish were sampled at 90 dph (Fig. 1); length and weight were recorded (Supplemental Table 1), and gonads dissected and collected for histology and gene expression analysis. According to previous study (Ferreira do Nascimento et al., 2017), gonads are already differentiated in *A. altiparanae* with 83 dph; ovaries display oogonia, oocytes at primary and secondary growth, and vitellogenic oocytes, while testes present spermatogonia, spermatocytes, spermatids and spermatozoa. Initial phenotypic sex ratio was determined in all individuals at 90 dph by the presence of spinelets in the anal fin, a secondary sex characteristic exclusive to *A. altiparanae* males (Supplemental Table 1). Sex ratio was further examined by gonadal histology.

2.2. Steroid diets

Treatment with estrogens prior to sexual differentiation have been extensively used to induce feminization in fish (reviewed by Piferrer, 2001). In the case of *A. altiparanae*, de Bem et al. (2012) used a synthetic estrogen (estradiol valerate) at elevated concentrations (20, 40 and 80 mg/Kg) to induce 70% feminization in this species. Conversely, there is a study showing that estradiol valerate is a reproductive

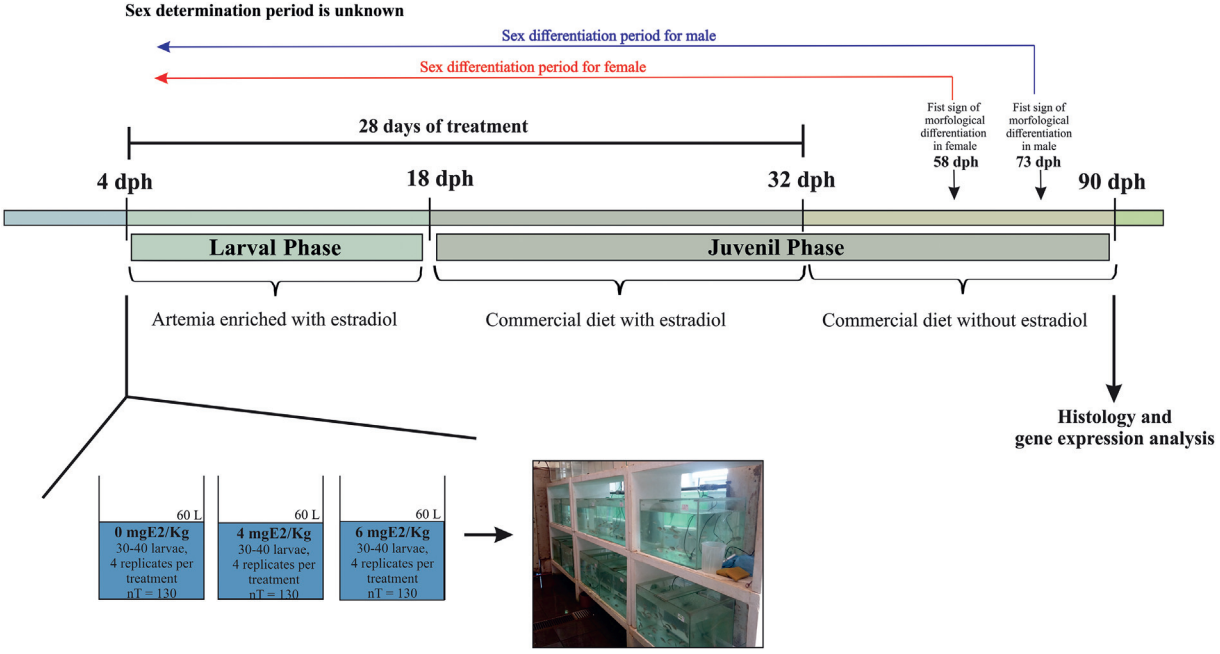

Fig. 1. Experimental design. Three groups containing approximately 30–40 undifferentiated larvae each were fed with diet containing increasing concentrations of E2 (0, 4 or 6 mg/Kg). Each treatment group was performed in 4 replicate tanks (n_T = 130 larvae per treatment). Hormonal treatment occurred for 28 days, from 4 until 32 days post-hatching (dph). Fish were sampled at 90 dph for phenotypic, histological and gene expression analysis.

toxicant for fish (Lei et al., 2013). Therefore, to avoid toxicological adverse effects, the present study used a natural estrogen (E2) but at lower concentrations due to the following reasons: 1) There are evidences that greater concentrations of E2 (10, 20 or > 40 ppm or mg/L or mg/Kg) increased fish mortality and induced toxicological effects on the brain-pituitary-gonadal axis (reviwed by Piferrer and Donaldson, 1992; Kim et al., 1997; Park et al., 2004); 2) E2 has been shown to affect growth during the post-treatment period in some fish species (reviewed by Donaldson et al., 1979); 3) There is an environmental concern when using increased concentrations of E2 due to difficulties of water clearance and steroid incorporation into the fish after treatment (Specker and Chandlee, 2003); and 4) There is a study showing effective feminization in the bagrid catfish *Pseudobagrus fulvidraco* by using low concentration of E2 (5 mg/L or mg/Kg) for 20 days (Park et al., 2004). Based on these evidences, we decided to use E2 at 4 and 6 mg/L or mg/Kg to investigate a minimum dose able to induce feminization, low mortality and no toxicological adverse effects.

In this study, E2-enriched live diets (*Artemia*) were used to feed *A. altiparanae* larvae, as hormone-treated commercial diets are unsuitable for early stages of development. For this purpose, steroids were added to *Artemia* at the first feeding instar II stage of development according to a previously described protocol (Martin-Robichaud et al., 1994; Stewart et al., 2001). Decapsulated *Artemia* cysts were incubated at 3 g of cysts per L of hatching medium. Hatching medium was prepared fresh daily by dissolving E2 (stock solution) into 1 L seawater. Hormone–alcohol mixture was prepared as a stock solution of 500 mg of E2 (Cayman Chemical, Ann Arbor, MI, EUA) in 75 mL of 70% ethanol. Subsequently, 600 and 900 μLof stock solution was added to 1 L hatching medium to prepare 4 and 6 mg E2 per L as final concentration, respectively. Control medium (without hormone) was prepared by adding 900 μL of 70% ethanol alone into 1 L hatching medium. Then, *Artemia* cysts were cultivated in control (0 mg E2/L) or hatching medium containing different concentrations of E2 (4 or 6 mg E2/L) at 18–20 °C for 24 h. Nauplii were then collected by siphoning, filtered through brine shrimp netting, and rinsed in 1 L tap water. In this study, concentration of E2 incorporated into the *Artemia* nauplii was not quantified. A previous study showed that *Artemia* nauplii cultivated in a lipid-enriched media containing 0, 5, 10 or 20 mg E2/L for 24 h incorporated 0, 140, 90 and 231–407 ng E2/mg dry wt., respectively (Martin-Robichaud et al., 1994). Similar, Stewart and collaborators (2001) have detected approximately 461 ng E2/mg dry wt. when *Artemia* were cultivated in hatching medium containing 20 mg E2/L for 24 h. Moreover, Martin-Robichaud and collaborators (1994) showed 99% feminization when larvae of lumpfish (*Cyclopterus lumpus*) were fed with *Artemia* containing 231–407 ng E2/mg dry wt. at first feeding for 20 days. Altogether, these data indicate that enrichment of *Artemia* with steroids is a feasible procedure for introducing steroids to first feeding fish larvae.

As previously described, the three groups of *A. altiparanae* larvae were fed for 14 days, from 4 to 18 dph, with *Artemia* nauplii cultivated in media containing increasing concentrations of E2 (0, 4 or 6 mg E2/L) (Fig. 1). After this period, specimens were fed with commercial powdered food containing 0, 4 or 6 mg E2 per Kg of food for 14 days, from 18 to 32 dph (Fig. 1). In this period, the hormone-treated diet was prepared following a previously described method (Popma and Green, 1990). Briefly, a hormone–alcohol (100% ethanol) mixture of 4 or 6 mg E2/L was sprayed onto 1 Kg of powdered food. The food was stored in the absence of light at room temperature for 12 h to volatilize the solvent, and subsequently stored at 4 °C. The control diet (0 mg E2/Kg food) was impregnated with 100% ethanol alone.

2.3. Histology

Specimens at 90 dph from each experimental group [0 (n = 6 per replicate, n_T = 24), 4 (n = 6–7 per replicate, n_T = 29) and 6 mg E2/Kg (n = 10–11 per replicate, n_T = 41)] were collected randomly for histological assessment of the gonads . For this purpose, gonads were then dissected, fixed in modified Karnovsky solution (2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2]) for at least 24 h at room temperature, and subsequently dehydrated, embedded in resin (Leica HistoResin) and sectioned at 3 μm thickness for staining with hematoxylin and eosin. Additional samples were also fixed in 4% paraformaldehyde solution in phosphate buffered saline (PBS) for at least 24 h at room temperature, dehydrated, embedded in Paraplast High Melt (Leica, Wetzlar, HE, Germany) and sectioned with 5 μm thickness for staining with hematoxylin and eosin. Histological sections

were examined and classified as ovary, testis, or testis-ova (occurrence of oocytes in the testis) using a Leica DMI6000 microscope.

2.4. Gonadal gene expression

Transcript abundance for a selected number of genes was measured in the gonads (testes and ovaries) from individuals at 90 dph that received diets containing 0 mg E2/Kg (control) (n = 4 males per replicate, n_T = 16 males; n= 4 females per replicate, n_T = 16 females; n_T = 32 animals/group) and 6 mg E2/Kg (n = 4 males per replicate, n_T = 16 males; n = 4 females per replicate, n_T = 16 females; n_T = 32 animals/group). Testes and ovaries were selected from phenotypic males and females, respectively. Furthermore, to determine if the selected genes display a sex-biased expression, quantitative polymerase chain reactions (qRT-PCR) were also performed using adult testes and ovaries (n = 6 males; n = 6 females), as their gonads are fully differentiated. For the analysis of gonadal gene expression, total RNA was extracted from samples of testicular and ovarian tissue, previously identified as such by histology, using TRIzol™ (Invitrogen, Carlsbad, CA, EUA) following manufacturer's instructions. After verifying RNA integrity and quality, cDNA synthesis was performed using 1 μg total RNA and 4 μL 5× iScript reaction mix for 5 min at 25 °C. Reverse transcription was performed at 46 °C for 20 min using iScript Reverse Transcriptase (Bio-Rad Laboratories, Hercules, CA, EUA), as described by the manufacturer. Reactions were performed in a Step One Plus Real-Time PCR Systems (Life Technologies, Carlsbad, CA, EUA), where each reaction contained 2.5 μL each primer (1.125 nM), 5.0 μL SYBR® Green Master Mix (Bio-Rad Laboratories, Hercules, CA, EUA) and 2.5 μL total cDNA. All reactions were subjected to 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min with a final denaturation of 95 °C for 15 s. Species-specific primers of *dmrt1, elf1a* (elongation factor 1-alpha) and *sox9* (SRY-box transcription factor 9) (Table 1) were obtained from Adolfi et al. (2015). Primers for *amh, ar* (androgen receptor), *cyp19a1a*, *foxl2* (forkhead box L2a), *esr* (estrogen receptor) and *17β-hsd* (hydroxysteroid 17-beta dehydrogenase) were designed based on highly conserved nucleotide regions of *A. mexicanus*, a related species of *A. altiparanae* (Table 1). Sequences to design the primers for *rspo1* (R-spondin 1) and *esr* were based on the transcript variant X1 of the available genome of *A. mexicanus*. These sequences correspond to *rspo1*, *esr1* or *esrα* in other species. Based on the genome of *A. mexicanus*, only one isoform was found for *ar*, *esr* and the rest of the evaluated genes. Expression of the endogenous control *elf1a* remained stably expressed under the different experimental conditions. The validity of the qRT-PCR reactions was confirmed by melting curve analysis (Supplemental Fig. 1) and visualization of amplified fragments by gel electrophoresis. Relative mRNA levels were determined using the $2^{-(\Delta\Delta CT)}$ method, where the expression of the target gene was first normalized to the reference gene and subsequently calibrated to its average expression level detected among all experimental groups.

2.5. Statistical analysis

Results were expressed as mean values ± SEM. Significant differences between two groups were identified using unpaired Student *t*-test ($p < .05$). Comparisons of more than two groups were performed with one-way ANOVA followed by Student-Newman-Keuls test ($p < .05$). A χ^2 contingency test was used to compare the sex ratios of each treatment against control ($p < .05$). Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, (http://www.graphpad.com) was used for all statistical analysis.

3. Results

3.1. Early exposure to E2 affected the male secondary sex characteristics of A. altiparanae

Analysis of secondary sex characteristics revealed that E2 exposure affected the phenotypic sex of *A. altiparanae* when compared to control (Table 2; Supplemental Table 1). In the control group, the phenotypic sex ratio (male:female) was 0.88 (51:58), and after treatment, phenotypic sex ratios changed significantly to 0.56 (45:80) and 0.26 (21:80) in the groups that received 4 mg and 6 mg E2/Kg, respectively (Table 2). Our results showed that treatment with E2, in a concentration-dependent manner, increased significantly the proportion of individuals with female phenotype (Table 2). In relation to the control, there is an increase of 120% and 148.4% in the proportion of phenotypic females for the groups of 4 and 6 mg E2/Kg, respectively (Table 2).

In this species, females are phenotypically characterized as being larger than males, usually with a protuberant ventral region, and no spinelets are present in their anal fin (Fig. 2A). On the other hand, males have an elongated body and display spinelets in their anal fin (only perceptible by touching) (Fig. 2B). The presence of spinelets is the principal characteristic to identify phenotypic males in this species (Malabarba and Weitzman, 2003). When analyzing the total length and weight, there was a tendency for an increase in the group treated with

Table 1
Specifications of the primers used in all qRT-PCR reactions.

Gene	Primer sequence (5′-3′)	Accession no.	Species	Amplify length (bp)	Annealing temp. (°C)	PCR efficiency (%)
amh	**Forward:** AGGCTTTGCAAGCTGTGTTG **Reverse:** GCTTTGTTGTGCCATCCTGG	XM_022669774.1	*Astyanax mexicanus*	84	58	100
ar	**Forward:** GCTAAGGCAGGGTTAAGGCA **Reverse:** ACACCTAAGCCTGTGTCTGC	XM_022685081.1	*Astyanax mexicanus*	135	58	104
cyp19a1a	**Forward:** TGCAGTTCCTGAGCAGCAG **Reverse:** TTCGACACTTGGCAGACAGT	XM_022687021.1	*Astyanax mexicanus*	93	60	102
dmrt1	**Forward:** CAGCCTACTACAGCAACCTCTACAAT **Reverse:** TGGCTGGACAGACGGCTATC	KM502983.1	*Astyanax altiparanae*	76	60	100
esr	**Forward:** GTCCACTTCCTGGATGGAGC **Reverse:** AAGAGTTTCTTCGGCCAGGG	XM_007253897.3	*Astyanax mexicanus*	120	60	107
elf1a	**Forward:** CTTCTCAGGCTGACTGTGC **Reverse:** CCGCTAGCATTACCCTCC	XM_007253897.3	*Astyanax mexicanus*	112	60	110
foxl2	**Forward:** ACCTGAGCCTTAACGAGTGC **Reverse:** ATGTCTTCACACGTCGGGTC	XM_007232295.3	*Astyanax mexicanus*	97	58	100
hsd17b1	**Forward:** AGAGCAACATCACTGAGGGC **Reverse:** GCTTCCAGTGGTCCCATCAA	XM_007234470.2	*Astyanax mexicanus*	76	58	98
rspo1	**Forward:** ACATGAGAAGAAGATGAGTGGG **Reverse:** CTGGAATCCTACGCGTGACA	XM_007251793.3	*Astyanax mexicanus*	81	58	101
sox9	**Forward:** CCAGCATGGGCGAAGTG **Reverse:** CGTCGGTGGCGTTGGA	KM502984.1	*Astyanax altiparanae*	71	58	100

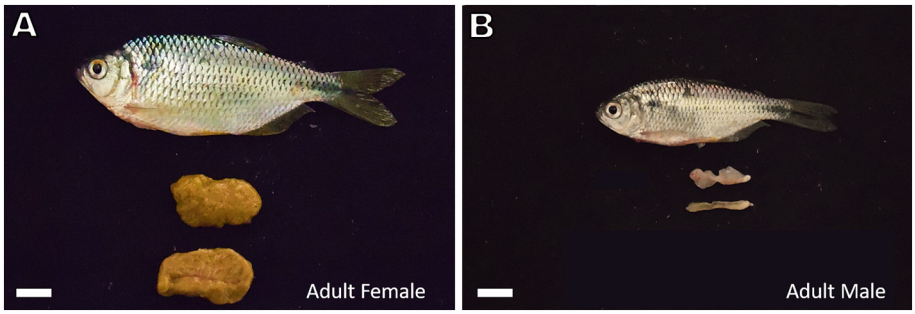

Fig. 2. Phenotypic differences between *A. altiparanae* adult females (A) and males (B). Females are usually larger than males. Males display an elongated body and have spinelets in their anal fin (only perceptible by touching). Mature gonads are also shown in the figure. Ovaries are bigger, yellowish in color, and some oocytes can be seen with the naked eye. Testes are more elongated and white in color. Scale bar: 1,0 cm.

Table 2
Sexual proportion and sex ratio observed in each treatment (0[Control], 4 and 6 mg E2/Kg) at 90 dph according to phenotypic sexual characteristics. * (p = .01); **** (p < .0001); E2 = estradiol.

Treatment	Female phenotype (%)	Male phenotype (%)	Sex ratio (♂:♀)	n	χ^2
Control	53.2	46.8	0.88 (51:58)	109	
4 mg E2/Kg	64	36	0.56 (45:80)	125	*
6 mg E2/Kg	79	21	0.26 (21:80)	101	****

the greatest dietary concentration of E2, however it was without significance (p > .05; Table 3, Supplemental Table 1).

3.2. Early exposure to E2 did not change gonadal sex ratio but induced intersex (testis-ova)

Histological assessment of the gonads revealed that despite of having a female phenotype, some individuals treated with dietary E2 displayed testes . When analyzing the number of individuals with testes or ovaries, results showed that E2 exposure did not affect significantly the gonadal sex ratio as compared to the control (Table 4). In the control, the gonadal sex ratio (testes:ovaries) was 1 (12:12), while in the greatest concentration of E2 (6 mg/Kg food), the ratio was about of 0.78 (18:23) (Table 4). In the group that received 4 mg E2/Kg food, the gonadal sex ratio was 1.9 (19:10) (Table 4). Although in this group (4 mg E2/Kg food), there is an increase of individuals with testes, statistical analysis showed no significant differences when compared to the control. This result is more likely associated with a sample size problem rather than E2-related effects, since no changes on gonadal sex ratio were reported in the greatest concentration of E2 (6 mg/Kg food). With respect to proportion, the control group (0 mg E2/Kg) exhibited 50% ovaries and 50% testes (Table 4). The group that received 4 mg E2/Kg food was comprised of 34.5% ovaries and 65.5% testes (Table 4), while the 6 mg E2/Kg food displayed 52.3% ovaries and 40.9% testes (Table 4). Additionally, histological analysis showed that about of 6.8% of individuals in the greatest concentration (6 mg/Kg food) were intersex (Table 4). These intersex individuals displayed a female phenotype and their gonads were classified as testis-ova (Fig. 3E). This type of gonad is characterized as having groups of oocytes inside the testis (Fig. 3E). No intersex individuals were found in the control and 4 mg E2/Kg food group (Table 4).

Table 3
Total length (cm) and weight (g) observed in each treatment (0[Control], 4 and 6 mg E2/Kg) at 90 dph. Values are indicated as mean ± standard deviation. No significant differences were observed between the control and treatment groups (4 and 6 mg E2/Kg); p > .05; E2 = estradiol. Means not significantly different share the same letter (a).

Treatment	Total length (cm)	Weight (g)
Control	6.95 ± 0.79 [a]	5.29 ± 1.90 [a]
4 mg E2/Kg	6.86 ± 0.86 [a] (p = .32)	4.96 ± 1.81 [a] (p = .175)
6 mg E2/Kg	7.08 ± 0.84 [a] (p = .34)	5.50 ± 1.90 [a] (p = .467)

Moreover, histological analysis showed no differences between gonads of control and E2 treatment groups (Fig. 3). The ovaries presented numerous lamellae that project into the ovarian lumen and oocytes at different stages of development, many at primary and secondary growth phases (Fig. 3A,C). Testes exhibited an anastomosing tubular structure and germinal epithelium composed of cysts at different stages of germ cell development (Fig. 3B,D). The testicular lumen was filled with spermatozoa and secretion (Fig. 3B,D). In testis-ova, we observed some previtellogenic oocytes (single or in clusters) distributed among the male germ cell cysts (Fig. 3E).

3.3. Early exposure to E2 altered the expression of genes implicated in sexual differentiation

Early exposure to E2 altered the transcript abundance of several genes implicated in sexual differentiation, as demonstrated through their average expression (Fig. 4), or relative expression ratio (R) for each individual from control or treatment group (Supplemental Fig. 2). For gonadal gene expression analysis, only individuals that received 6 mg E2/Kg were selected because this treatment induced more phenotypic females and intersex testis-ova. In males, treatment (6 mg E2/Kg) significantly increased the transcript levels of *dmrt1*, *amh* and *sox9* when compared to the control (Fig. 4A,B,D; Supplemental Fig. 2A). In addition, the expression of testicular *17β-hsd*, which is required for the conversion of dihydrotestosterone into androstenediol, androstenedione into testosterone and estrone into estradiol, was decreased following E2 exposure (Fig. 4E; Supplemental Fig. 2A). On the other hand, genes related to ovarian differentiation, such as *esr* and *rspo,* were up-regulated in males due to treatment (Fig. 4G,I; Supplemental Fig. 2A). Interestingly, *cyp19a1a* mRNA levels showed a significant reduction following E2 treatment when compared to control males (Fig. 4F; Supplemental Fig. 2B).

Strikingly, the relative mRNA levels of *dmrt1* and *sox9* were significantly increased in females that received E2 diet when compared to control females (Fig. 4A,D; Supplemental Fig. 2B). On the other hand, *amh* was without significant change in treated females when compared to control (Fig. 4B; Supplemental Fig. 2B). For the genes related to ovarian differentiation (*esr*, *rspo* and *foxl2*), no significant changes were observed after E2 exposure (Fig. 4G,H,I; Supplemental Fig. 2B), while the gene encoding the gonadal aromatase (*cyp19a1a)* was significantly down-regulated in E2-treated females (Fig. 4F; Supplemental Fig. 2B).

3.4. Sex-biased genes

Further analysis compared the expression of the selected genes between the adult testes and ovaries of *A. altiparanae* in order to identify sex-biased genes for this species (Fig. 5), and to help elucidate E2-induced changes in gene expression. Quantitative RT-PCR analysis revealed a dimorphic expression for *dmrt1, amh* and *sox9*, showing that these transcripts are highly expressed in the testes than in ovaries (Fig. 5). Sexually dimorphic expression in *ar*, *17β-hsd, esr*, *rspo*, *cyp19a1a* and *foxl2* did not occur (Fig. 5).

Table 4
Proportion and sex ratio observed in each treatment (0[Control], 4 and 6 mg E2/Kg) at 90 dph according to histological assessment of the gonads. No significant differences were observed between the control and treatment groups (4 and 6 mg E2/Kg); p > .05; E2 = estradiol; NS = not significant.

Treatment	Female (Ovaries) (%)	Male (Testes) (%)	Intersex (Testes-ova) (%)	Sex ratio (♂:♀)	n	χ^2
Control	50	50	0	1 (12:12)	24	
4 mg E2/Kg	34.5	65.5	0	1.9 (19:10)	29	NS $^{p=.2538}$
6 mg E2/Kg	56.1	43.9	6.8	0.78 (18:23)	41	NS $^{p=.6341}$

4. Discussion

The present study examined the effects of E2 on the phenotypic characteristics, histological assessment of the gonads, and the expression of selected genes in the gonads of a South American fish, *A. altiparanae,* exposed to dietary E2 for 28 days during the period that precedes the gonadal differentiation. The results showed a concentration-dependent effect of E2 on the phenotypic sex of *A. altiparanae*. Early exposure to E2 (4 and 6 mg/Kg) significantly affected the establishment of male secondary characteristics (spinelets in the anal fin) in the majority of fish. In fish, the development and manifestation of secondary sexual characteristics, such as intromittent organs, size, coloration, caudal finnage, are under control of sex steroid hormones (Wootton and Smith, 2014; Wheeler et al., 2020). A recent study in zebrafish (*Danio rerio*) showed that genetic loss of the C17–20 lyase enzyme, required for androgen production, compromised the male secondary sex characters, including breeding tubercles, body pigmentation, and anal fin coloration, but did not affect testis development and spermatogenesis (Zhai et al., 2018). In our study, gene expression analysis showed that following dietary E2 (6 mg/Kg), *17β-hsd*, an enzyme required for androstenediol, testosterone and estradiol production, and *cyp19a1a* were down-regulated in treated males when compared to control. Altogether these findings indicate that E2 may have affected male secondary characteristics of *A. altiparanae* by suppressing or decreasing androgen/steroid production in the treated males as compared to the control.

Although dietary E2 affected the phenotypic sex, gonadal histology was either unchanged or resulted in an intersex condition as shown in the greatest concentration of E2 (6 mg/Kg). Intersex is a condition where there is a simultaneous presence of male and female gonadal tissue in a gonochoristic species (Adolfi et al., 2019a), and in fish, is often viewed as the direct effect of exposure to endocrine disrupting chemicals (Bahamonde et al., 2013). The occurrence of intersex as result of E2 exposure has been reported in several teleosts. For example, 100 ng and 1 μg/L E2 caused 10% intersex in chinook salmon (*Oncorhynchus tshawytscha*) (Bjerregaard et al., 2008). In Japanese medaka (*Oryzias latipes*), following treatment with 10 ng/L E2 for 100 dph, 10% of individuals presented intersex gonads without affecting the sex ratio (Bjerregaard et al., 2008). In the present study, the percentage of intersex individuals was 6.8%, indicating that these concentrations of E2 were insufficient to induce complete male-to-female sex reversal.

To further examine the molecular mechanisms, the current study also examined the effects of E2 on transcript abundance for a selected number of genes involved in gonadal sex differentiation. For this purpose, testes and ovaries were first evaluated for sexually dimorphic expression in mature adults. Data revealed a male-biased expression for *dmrt1* and *sox9*, which is in accordance with previous studies showing

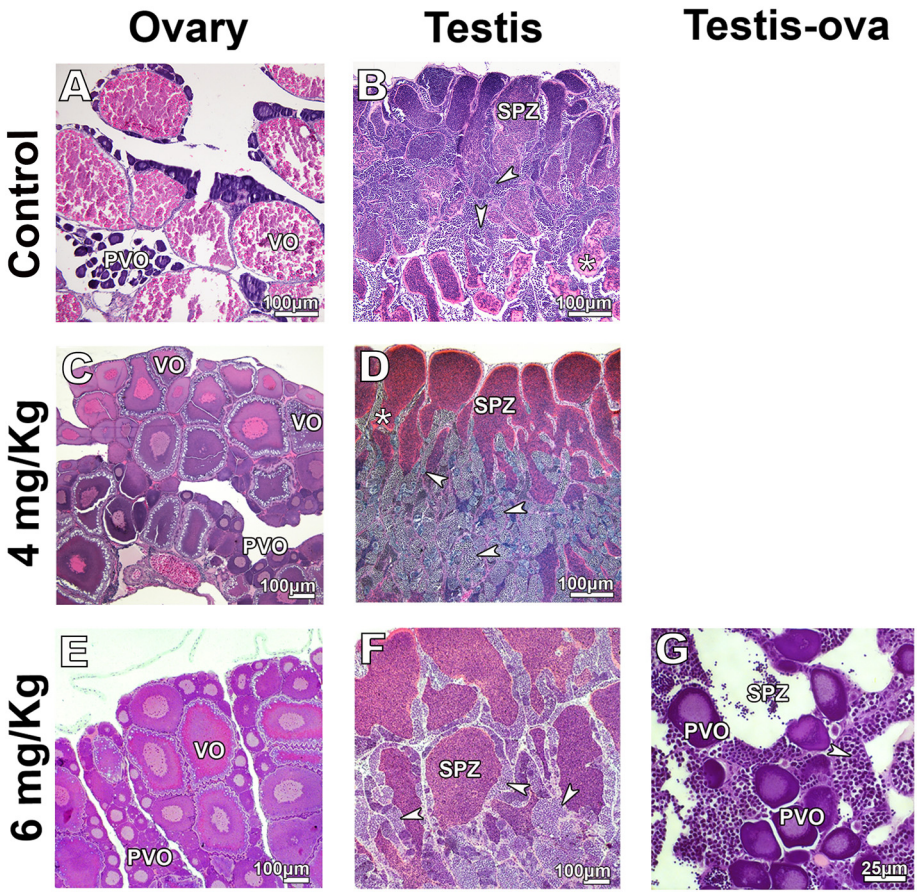

Fig. 3. Gonadal histology of *Astyanax altiparanae* that received diets containing 0 (control), 4 and 6 mg E2/Kg food. The gonadal morphology among the groups were similar, no structural differences were observed. Females (A,C,E) showed similar morphology; numerous lamellae with oocytes at different stages of development, especially at primary and secondary growth phases, including previtellogenic oocytes (perinucleolar) (PVO) and vitellogenic oocytes (VO). Males (B,D,F) exhibited testes with anastomosing tubular structure and germinal epithelium composed of cysts (arrowheads) at different stages of spermatogenesis. Spermatozoa (SZ) and secretion (asterisks) are also seen in the testicular lumen. The intersex individuals (G) presented a testis-ova gonad with oocytes (PVO) intermixed with male germ cell cysts (arrowheads). Staining: Hematoxylin and eosin (H&E).

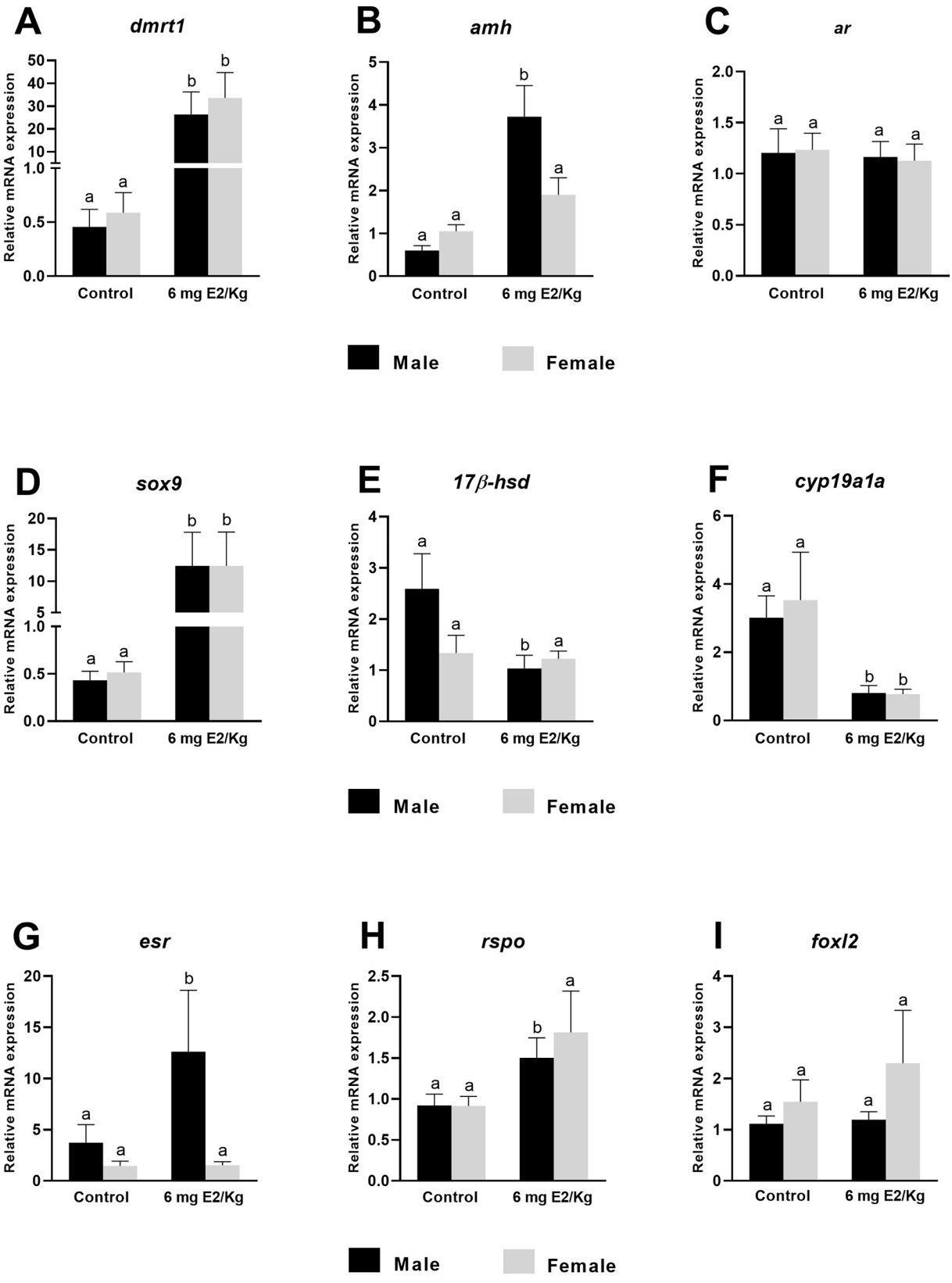

Fig. 4. The transcript abundance for the selected genes following E2 (6 mg/Kg) exposure at 90 days post-hatching (dph). Control males (n = 4 per replicate, n_T = 16); control females (n = 4 per replicate, n_T = 16), 6 mg E2/Kg males (n = 4 per replicate, n_T = 16) and 6 mg E2/Kg females (n = 4 per replicate, n_T = 16). A: *dmrt1* [doublesex and mab-3-related transcription factor 1]. B: *amh* [Anti-Müllerian Hormone]. C: *ar* [androgen receptor]. D: *sox9* [SRY-box transcription factor 9]. E: *17β-hsd* [17-beta hydroxysteroid dehydrogenase]. F: *cyp19a1a* [aromatase]. G: *esr* [estrogen receptor]. H: *rspo* [R-spondin 1]. I: *foxl2a* [forkhead box L2 a]). The expression level for each gene was normalized with *elf1a* and expressed as relative value to its average expression in both experimental groups. Each bar represents the mean ± SEM. Different letters denote significant differences ($p < .05$) between control and treatment group for the same gonad.

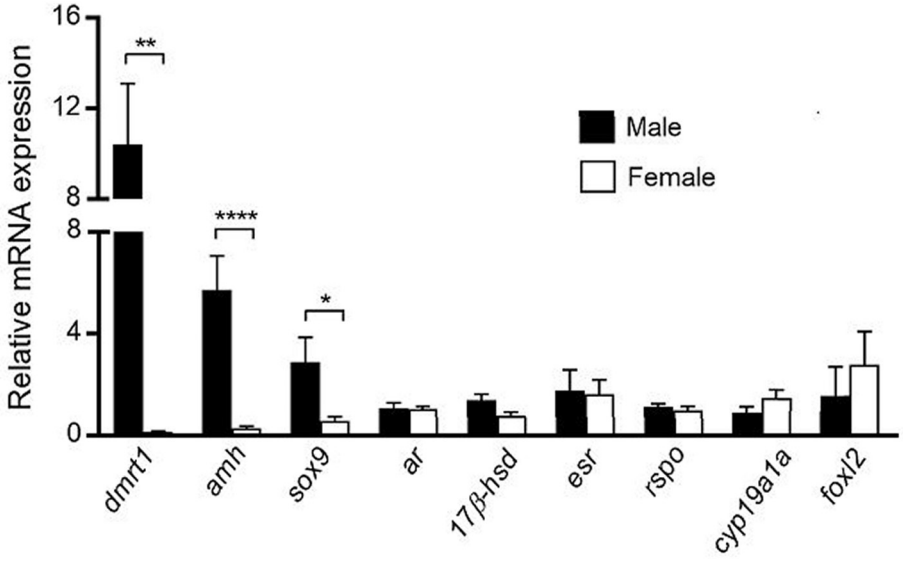

Fig. 5. Sex-biased gene expression analysis. The relative expression of *dmrt1* [doublesex and mab-3-related transcription factor 1], *amh* [Anti-Müllerian Hormone], *sox9* [SRY-box transcription factor 9], *ar* [androgen receptor], *17β-hsd* [17-beta hydroxysteroid dehydrogenase], *esr* [estrogen receptor], *rspo* [R-spondin 1], *cyp19a1a* [aromatase] and *foxl2a* [forkhead box L2 a] in adult gonads of *A. altiparanae*; testes ($n = 6$) and ovaries ($n = 6$). The expression level of each gene was normalized to *elf1a* and expressed as relative values to its average expression in both gonads. Data are expressed as mean ± SEM. Asterisks denote significant differences between testes and ovaries: * ($p < .05$); ** ($p < .01$); **** ($p < .0001$).

the role of these genes during testicular differentiation of *A. altiparanae* (Adolfi et al., 2015). Additionally, gene expression analysis showed that *amh* is highly expressed in the adult testes of *A. altiparanae* when compared to the ovaries. Amh is a member of transforming growth factor β, and is expressed in the undifferentiated gonads of both sexes at relatively low and equal levels of several teleosts as reviewed previously (Pfennig et al., 2015; Adolfi et al., 2019b). During testis differentiation, *amh* expression increases and remains increased during and after puberty in adult fish males (Pfennig et al., 2015; Adolfi et al., 2019b). Several studies in medaka have shown that *amh* plays a role in testis differentiation, *amhr2* (anti-Müllerian hormone receptor type 2) loss-of-function mutant *hotei* leads to an excessive germ cell proliferation and male-to-female sex reversal in 50% of the XY animals (Morinaga et al., 2007; Nakamura et al., 2012). Considering the role of Amh in other teleost fish, our results indicate that *amh,* a male-biased gene in *A. altiparanae*, may have a potential role during testicular differentiation of this species.

When analyzing the transcript abundance for *esr*, *rspo*, *foxl2* and *cyp19a1a,* none of them exhibited a dimorphic expression in the adult gonads of *A. altiparanae*. These results suggest either that other effectors could be involved in the female pathway program, or a specific expression of these genes in the female gonads during the critical period of molecular sexual differentiation. With respect to *cyp19a1a,* although not sexually dimorphic, it is likely that gonadal aromatase and consequently estrogens can act as inducers of ovarian differentiation in *A. altiparanae*. This suggestion is supported by previous work that showed 70–76% feminization when undifferentiated larvae of *A. altiparanae* were exposed to estradiol valerate (Bem et al., 2012). However, more studies are necessary to address the role of gonadal aromatase during gonadal sexual differentiation of this species.

Analysis of transcript abundance revealed that males expressed more *dmrt1*, *sox9* and *amh* (testicular-biased genes) after E2 treatment (6 mg/Kg) when compared to control males (Fig. 6). This altered expression could be considered a compensatory mechanism triggered by a genetic factor, or a mechanism of resilience, where males resisted to feminization by enhancing the expression of genes related to testicular differentiation. In addition, gene expression analysis showed that relative mRNA levels of *esr* and *rspo* were significantly up-regulated in the males after E2 exposure. Overall, although androgen pathway was likely affected and *esr* and *rspo* were highly expressed, this genetic profile was insufficient to transform *A. altiparanae* testes into ovaries (Fig. 6). In this context, it is suggested that E2 treatment (6 mg/Kg) was not able to transform *A. altiparanae* testes into ovaries due to the increased levels of *amh* in the testes. Schulz et al. (2007) have

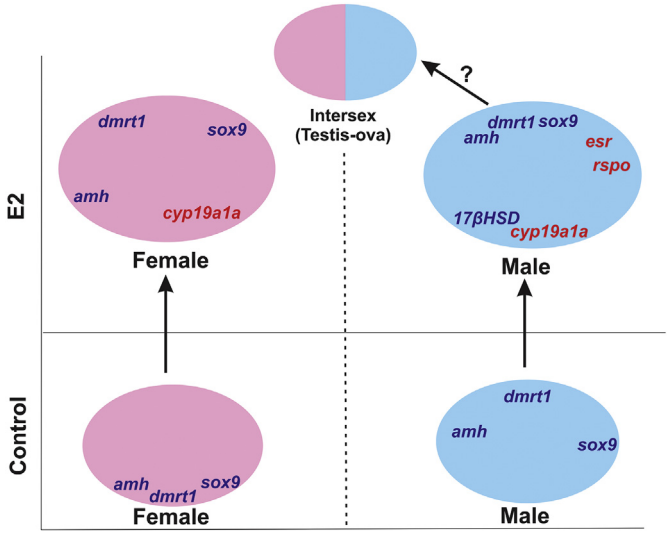

Fig. 6. Gonadal plasticity of *A. altiparanae* following E2 exposure in the diet (6 mg/Kg). The existing dichotomy between male and female originates a complex gonadal plasticity when undifferentiated larvae were treated with 6 mg E2/Kg for 28 days. The "E2 resistant males" comprises males that were resilient to E2-induced feminization. These males overexpressed genes related to testicular differentiation, such as *dmrt1*, *sox9* and *amh*. *17β-hsd* and *cyp19a1a* were down-regulated, while *esr* and *rspo* were up-regulated in these males. The question mark (?) speculates whether intersex individuals came from the E2 resistant males that somehow lost their resistance and underwent incomplete sex reversal (male-to-female). The females exhibited a normal ovarian morphology but showed a "male-like" gene expression. *dmrt1* and *sox9* were up-regulated, while *cyp19a1a* was down-regulated in the ovaries. Genes in the upper part are up-regulated, while the ones in the lower are down-regulated.

demonstrated that 17a-ethinylestradiol (EE2) exposure in early development of zebrafish down-regulated *amh* and consequently disrupted male gonadal development. Similarly, E2 treatment during early gonadal development of South American pejerrey (*O. bonariensis*) decreased the expression of *amh* and induced male-to-female sex reversal (Fernandino et al., 2008b). Therefore, further studies are needed to address the role of *amh* during *A. altiparanae* sex differentiation and whether *amh* is involved in gonadal sex change (male-to-female) in this species.

Moreover, in this study, we found that females strikingly expressed testicular-biased genes (*dmrt1* and *sox9*) following E2 treatment, while

gonadal aromatase (*cyp19a1a*) was significantly down-regulated when compared to control (Fig. 6). This expression profile can be explained as a consequence of the complex feedback loop between the transcriptional regulation of *dmrt1* and *cyp19a1a* with the steroid hormonal activity (Herpin and Schartl, 2011). In this loop, it has been shown that Dmrt1 regulates gonadal aromatase expression and thereby affects the estrogen balance that would feedback (negatively or positively) on *dmrt1* expression (reviewed by Herpin and Schartl, 2011). In our study, since E2 decreased the mRNA levels of *cyp19a1a*, it is likely expected a decreased activity for gonadal aromatase, and consequently lower E2 endogenous levels, which in turn, would positively regulate *dmrt1* expression in treated females of *A. altiparanae*. To support this hypothesis, previous studies in tilapia, zebrafish and rainbow trout have shown that *dmrt1* down-regulates *cyp19a1a* transcription, resulting in a decreased activity of gonadal aromatase and decreased plasma E2 levels (Guiguen et al., 2010; Wang et al., 2010; Pradhan and Olsson, 2016). On the other hand, the increased expression of *sox9* after E2 treatment could be explained due to a positive regulation by *dmrt1*. This is supported by a recent study showing that Dmrt1 directly stimulates the transcription of *sox9b* in tilapia (Wei et al., 2019).

Although females expressed male-specific genes (*dmrt1* and *sox9*), the ovarian morphology remained unaffected following E2 exposure. This finding demonstrates the extraordinary plasticity of gonadal sex differentiation in fish as compared to other vertebrates. Similar complexity has been reported in zebrafish by Ribas et al. (2017), who found females that were able to resist heat-induced masculinization, maintaining ovarian morphology despite changing their gonadal transcriptome to a testis-like one. These females displayed altered gene expression; *cyp19a1a* and *vtg5* (vitellogenin 5 gene) were down-regulated, while genes related to testicular differentiation were up-regulated (Ribas et al., 2017), similarly to our findings for *A. altiparanae* females.

In summary, our results showed that E2 exposure in the diet (4 and 6 mg E2/Kg) during early gonadal development of *A. altiparanae* was able to affect, in a concentration-dependent manner, the male secondary characteristics in the majority of fish. Interestingly, histological analysis revealed that gonadal sex ratio remained similar to that of controls. However, E2 was able to induce an intersex testis-ova in the greatest concentration. Finally, the present study showed a complex gonadal plasticity in terms of genetic expression profiles after E2 treatment (Fig. 6): 1 - males overexpressed the male-biased genes *dmrt1*, *sox9* and *amh* as a possible mechanism to resist the E2-induced feminization; and 2 - females showed increased levels of *dmrt1* and *sox9*, while gonadal aromatase (*cyp19a1a*) was down-regulated as compared to non-treated females. This study raises interesting questions, such as required concentration of E2 and alteration of genes that would be needed to induce complete sex inversion, and whether the gonadal physiology and fecundity are affected after E2 exposure. In total, this model provides new possibilities for investigating the mechanisms of gonadal sex differentiation, as well as the consequences of environmental exposures to xenoestrogens for Neotropical fish.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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References

Adolfi, M.C., Carreira, A.C., Jesus, L.W., Bogerd, J., Funes, R.M., Schartl, M., Borella, M.I., 2015. Molecular cloning and expression analysis of *dmrt1* and *sox9* during gonad development and male reproductive cycle in the lambari fish, *Astyanax altiparanae*. Reprod. Biol. Endocrinol. 13 (1), 2. https://doi.org/10.1186/1477-7827-13-2.

Adolfi, M.C., Fischer, P., Herpin, A., Regensburger, M., Kikuchi, M., Tanaka, M., Schartl, M., 2019a. Increase of cortisol levels after temperature stress activates *dmrt1a* causing female-to-male sex reversal and reduced germ cell number in medaka. Mol. Reprod. Dev. https://doi.org/10.1002/mrd.23177. mrd.23177.

Adolfi, M.C., Nakajima, R.T., Nóbrega, R.H., Schartl, M., 2019b. Intersex, hermaphroditism, and gonadal plasticity in vertebrates: evolution of the Müllerian duct and Amh/Amhr2 Signaling. Ann. Rev. Animal Biosci. 7 (1), 149–172. https://doi.org/10.1146/annurev-animal-020518-114955.

Bahamonde, P.A., Munkittrick, K.R., Martyniuk, C.J., 2013. Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena? Gen. Comp. Endocrinol. 192, 25–35. https://doi.org/10.1016/j.ygcen.2013.04.005.

Beardmore, J.A., Mair, G.C., Lewis, R.I., 2001. Monosex male production in finfish as exemplified by tilapia: Applications, problems, and prospects. In: Reproductive Biotechnology in Finfish Aquaculture, pp. 283–301. https://doi.org/10.1016/B978-0-444-50913-0.50015-1.

Biscotti, M.A., Adolfi, M.C., Barucca, M., Forconi, M., Pallavicini, A., Gerdol, M., Canapa, A., Schartl, M.., 2018. A comparative view on sex differentiation and gametogenesis genes in lungfish and coelacanths. Genome. Biol. Evol. 10 (6), 1430–1444. https://doi.org/10.1093/gbe/evy101.

Bjerregaard, L.B., Lindholst, C., Korsgaard, B., Bjerregaard, P., 2008. Sex hormone concentrations and gonad histology in brown trout (*Salmo trutta*) exposed to 17β-estradiol and bisphenol a. Ecotoxicology 17 (4), 252–263. https://doi.org/10.1007/s10646-008-0192-2.

Branco, G.S., Melo, A.G., Ricci, J.M.B., Digmayer, M., de Jesus, L.W.O., Habibi, H.R., Nóbrega, R.H., 2019. Effects of GnRH and the dual regulatory actions of GnIH in the pituitary explants and brain slices of *Astyanax altiparanae* males. Gen. Comp. Endocrinol. 273, 209–217. https://doi.org/10.1016/j.ygcen.2018.08.006.

Brennan, J., Capel, B., 2004. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nat. Rev. Genet. 5 (7), 509–521. https://doi.org/10.1038/nrg1381.

Capel, B., 2017. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat. Rev. Genet. 18 (11), 675.

Castañeda Cortés, D.C., Padilla, L.F.A., Langlois, V.S., Somoza, G.M., Fernandino, J.I., 2019. The central nervous system acts as a transducer of stress-induced masculinization through corticotropin-releasing hormone B. Development. 146, 8. pii: dev172866. https://doi.org/10.1242/dev.172866.

Chang, C.F., Lee, M.F., Chen, G.R., 1994. Estradiol-17β associated with the sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*. J. Exp. Zool. 268 (1), 53–58.

Costa, F.G., Adolfi, M.C., Gomes, C.C., Jesus, L.W.O., Batlouni, S.R., Borella, M.I., 2014. Testes of *Astyanax altiparanae*: the Sertoli cell functions in a semicystic spermatogenesis. Micron 61, 20–27. https://doi.org/10.1016/j.micron.2014.02.004.

de Bem, J.C., Fontanetti, C.S., Senhorini, J.A., Parise-Maltempi, P.P., 2012. Effectiveness of estradiol valerate on sex reversion in *Astyanax altiparanae* (Characiformes, Characidae). Braz. Arch. Biol. Technol. 55 (2), 283–290. https://doi.org/10.1590/S1516-89132012000200015.

De Paiva Camargo, M., Cassel, M., Oliveira de Jesus, L.W., Nóbrega, R.H., Borella, M.I., 2017. Characterization of undifferentiated spermatogonia and the spermatogonial niche in the Lambari fish *Astyanax altiparanae*. Theriogenology 96, 97–102. https://doi.org/10.1016/j.theriogenology.2017.03.027.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208 (3–4), 191–364. https://doi.org/10.1016/S0044-8486(02)00057-1.

Díaz, N., Piferrer, F., 2017. Estrogen exposure overrides the masculinizing effect of elevated temperature by a downregulation of the key genes implicated in sexual differentiation in a fish with mixed genetic and environmental sex determination. BMC Genomics 18 (1), 973. https://doi.org/10.1186/s12864-017-4345-7.

Do Nascimento, N.F., Monzani, P.S., Pereira-Santos, M., Niedzielski, D., Senhorini, J.A., Silva, L.A., Satiko, L., Nakaghi, O., Yasui, G.S., 2019. The first case of induced gynogenesis in Neotropical fishes using the yellowtail tetra (*Astyanax altiparanae*) as a model organism. Aquaculture, 734432. https://doi.org/10.1016/j.aquaculture.2019.734432.

Donaldson, E.M., Fagerlund, U.H.M., Higgs, D.A., McBride, J.R., 1979. Hormonal enhancement of growth. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), Fish Physiology. VIII. Academic Press, New York, pp. 455–497.

Ferreira do Nascimento, N., De Siqueira-Silva, D., Pereira-Santos, M., Fujimoto, T., Senhorini, J., Nakaghi, L., Yasui, G., 2017. Stereological analysis of gonads from diploid and triploid fish yellowtail tetra Astyanax altiparanae (Garutti & Britski) in laboratory conditions. Zygote 25 (4), 537–544. https://doi.org/10.1017/S0967199417000399.

Fernandino, J.I., Hattori, R.S., 2018. Sex determination in Neotropical fish: implications ranging from aquaculture technology to ecological assessment. Gen. Comp. Endocrinol. Mar. 1 (273), 172–183. https://doi.org/10.1016/j.ygcen.2018.07.002.

Fernandino, J.I., Hattori, R.S., Shinoda, T., Kimura, H., Strobl-Mazzulla, P.H., Strüssmann, C.A., Somoza, G.M., 2008a. Dimorphic expression of *dmrt1* and

cyp19a1a (ovarian aromatase) during early gonadal development in Pejerrey, *Odontesthes bonariensis*. Sex. Dev. 2 (6), 316–324. https://doi.org/10.1159/000195681.

Fernandino, J.I., Hattori, R.S., Kimura, H., Strüssmann, C.A., Somoza, G.M., 2008b. Expression profile and estrogenic regulation of anti-Müllerian hormone during gonadal development in pejerrey *Odontesthes bonariensis*, a teleost fish with strong temperature-dependent sex determination. Dev. Dyn. 237 (11), 3192–3199. https://doi.org/10.1002/dvdy.21731.

Garutti, V., Britsky, H.A., 2000. Descrição de uma nova espécie de *Astyanax* (Teleostei: Characidae) da bacia do alto rio Paraná e considerações sobre as demais espécies do gênero na bacia. Comum. Mus. Ciênc. Tecnol. PUCRS, Sér. Zool., Porto Alegre 13, 65–88.

Godwin, J., 2010. Neuroendocrinology of sexual plasticity in teleost fishes. Front. Neuroendocrinol. 31 (2), 203–216. https://doi.org/10.1016/j.yfrne.2010.02.002.

Gomes, C.C., Costa, F.G., Borella, M.I., 2013. Distribution of GnRH in the brain of the freshwater teleost *Astyanax altiparanae*. Micron 52–53, 33–38. https://doi.org/10.1016/j.micron.2013.07.008.

Graham, P., Penn, J.K.M., Schedl, P., 2003. Masters change, slaves remain. BioEssays 25 (1), 1–4. https://doi.org/10.1002/bies.10207.

Guiguen, Y., Baroiller, J.F., Ricordel, M.J., Iseki, K., McMeel, O.M., Martin, S.A.M., Fostier, A., 1999. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol. Reprod. Dev. 54 (2), 154–162.

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. 165 (3), 352–366. https://doi.org/10.1016/j.ygcen.2009.03.002.

Herpin, A., Schartl, M., 2011. Sex determination: switch and suppress. Curr. Biol. 21 (17), R656–R659. https://doi.org/10.1016/j.cub.2011.07.026.

Herpin, A., Schartl, M., 2015. Plasticity of gene-regulatory networks controlling sex determination: of masters, slaves, usual suspects, newcomers, and usurpators. EMBO Rep. 16 (10), 1260–1274. https://doi.org/10.15252/embr.201540667.

Herpin, A., Adolfi, M.C., Nicol, B., Hinzmann, M., Schmidt, C., Klughammer, J., Engel, M., Tanaka, M., Guiguen, Y., Schartl, M., 2013. Divergent expression regulation of gonad development genes in Medaka shows incomplete conservation of the downstream regulatory network of vertebrate sex determination. Mol. Biol. Evol. 30 (10), 2328–2346. https://doi.org/10.1093/molbev/mst130.

Kim, D.S., Nam, Y.K., Jo, J.-Y., 1997. Effect of oestradiol-17h immersion treatment on sex reversal of mud loach, *Misgurnus mizolepis* (Günther). Aquac. Res. 28, 941–946.

Kobayashi, Y., Nagahama, Y., Nakamura, M., 2013. Diversity and plasticity of sex determination and differentiation in fishes. Sex. Dev. 7 (1–3), 115–125. https://doi.org/10.1159/000342009.

Le Page, Y., Diotel, N., Vaillant, C., Pellegrini, E., Anglade, I., Mérot, Y., Kah, O., 2010. Aromatase, brain sexualization and plasticity: the fish paradigm. Eur. J. Neurosci. 32 (12), 2105–2115. https://doi.org/10.1111/j.1460-9568.2010.07519.x.

Lei, B., Kang, J., Yu, Y., Zha, J., Li, W., Wang, Z.J.A.Tt., 2013. β-Estradiol 17-Valerate Affects Embryonic Development and Sexual Differentiation in Japanese Medaka (*Oryzias latipes*). 134. pp. 128–134.

Li, M., Sun, L., Wang, D., 2019. Roles of estrogens in fish sexual plasticity and sex differentiation. Gen. Comp. Endocrinol. 277, 9–16. https://doi.org/10.1016/j.ygcen.2018.11.015.

Liu, H., Todd, E.V., Lokman, P.M., Lamm, M.S., Godwin, J.R., Gemmell, N.J., 2017. Sexual plasticity: a fishy tale. Mol. Reprod. Dev. 84 (2), 171–194. https://doi.org/10.1002/mrd.22691.

Malabarba, L.R., Weitzman, S.H., 2003. Description of a new genus with six new species from southern Brazil, Uruguay and Argentina, with a discussion of a putative *Characidae clade* (Teleostei: Characiformes: Characidae). Comunicações do Museu de Ciências e Tecnologia da PUCRS, Série Zoologia, Porto Alegre 16 (1), 67–151.

Martin-Robichaud, D.J., Peterson, R.H., Benfey, T.J., Crimc, L.W., 1994. Direct feminization of lumpfish (*Cyclopterus lumpus* L.) using 17β-oestradiol-enriched Artemia as food. Aquaculture 123 (1–2), 137–151. https://doi.org/10.1016/0044-8486(94)90126-0.

Morinaga, C., Saito, D., Nakamura, S., Sasaki, T., Asakawa, S., Shimizu, N., Kondoh, H., 2007. The hotei mutation of medaka in the anti-Mullerian hormone receptor causes the dysregulation of germ cell and sexual development. Proc. Natl. Acad. Sci. 104 (23), 9691–9696. https://doi.org/10.1073/pnas.0611379104.

Nagahama, Y., 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38 (2), 217–229.

Nagahama, Y., 2002. Ontogeny and plasticity of sex determination/gonadal differentiation in fishes. Biol. Reprod. 66, 75–76.

Nakamura, S., Watakabe, I., Nishimura, T., Picard, J.-Y., Toyoda, A., Taniguchi, Y., Tanaka, M., 2012. Hyperproliferation of mitotically active germ cells due to defective anti-Mullerian hormone signaling mediates sex reversal in medaka. Development 139 (13), 2283–2287. https://doi.org/10.1242/dev.076307.

Navarro-Martín, L., Blázquez, M., Piferrer, F., 2009. Masculinization of the European sea bass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. Gen. Comp. Endocrinol. 160, 3–11. https://doi.org/10.1016/j.ygcen.2008.10.012.

Pandian, T.J., Sheela, S.G., 1995. Hormonal induction of sex reversal in fish. Aquaculture 138, 1–4, 1–22. https://doi.org/10.1016/0044-8486(95)01075-0.

Park, I., Kimb, J., Choa, S.H., Kimc, D.S., 2004. Sex differentiation and hormonal sex reversal in the bagrid catfish *Pseudobagrus fulvidraco* (Richardson). Aquaculture 232, 183–193.

Pfennig, F., Standke, A., Gutzeit, H.O., 2015. The role of *Amh* signaling in teleost fish – multiple functions not restricted to the gonads. Gen. Comp. Endocrinol. 223, 87–107. https://doi.org/10.1016/j.ygcen.2015.09.025.

Piferrer, F, et al., 1994. Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. J. Exp. Zool. 270 (3), 255–262. https://doi.org/10.1002/jez.1402700304.

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197 (1–4), 229–281. https://doi.org/10.1016/S0044-8486(01)00589-0.

Piferrer, F., Donaldson, E.M., 1992. The comparative effectiveness of the natural and a synthetic estrogen for the direct feminization of Chinook salmon (*Oncorhynchus tashawytscha*). Aquaculture 106, 183–193.

Popma, T.J., Green, B.W., 1990. Reversão sexual de tilápias em tanques de terra. In: Manual de Produção Em Aquacultura. Universitu Aurburn, Florida (EUA, pp. 52.

Porto-Foresti, F., Castilho-Almedia, R.B., Foresti, F., 2005. Biologia e criação do lambari-do-rabo-amarelo. Espécies Nativas Para Piscicultura No Brasil. Santa Maria: UFSM, (Santa Maria: UFSM). pp. 101–116.

Pradhan, A., Olsson, P.E., 2016. Regulation of zebrafish gonadal sex differentiation. AIMS Mol. Sci. 3, 567–584. https://doi.org/10.3934/molsci.2016.4.567.

Ribas, L., Liew, W.C., Díaz, N., Sreenivasan, R., Orbán, L., Piferrer, F., 2017. Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. Proc. Natl. Acad. Sci. 114 (6), E941–E950. https://doi.org/10.1073/pnas.1609411114.

Schulz, R.W., Bogerd, J., Male, R., Ball, J., Fenske, M., Olsen, L.C., Tyler, C.R., 2007. Estrogen-induced alterations in *amh* and *dmrt1* expression signal for disruption in male sexual development in the zebrafish. Environ. Sci. Technol. 41 (17), 6305–6310. https://doi.org/10.1021/es070785+.

Specker, J.L., Chandlee, M.K., 2003. Methodology for estradiol treatment in marine larval and juvenile fish: uptake and clearance in summer flounder. Aquaculture 217, 663–672.

Stewart, A.B., Spicer, A.V., Inskeep, E.K., Dailey, R.A., 2001. Steroid hormone enrichment of *Artemia* nauplii. Aquaculture 202 (1–2), 177–181. https://doi.org/10.1016/S0044-8486(01)00598-1.

Tsai, Y.J., Lee, M.F., Chen, C.Y., Chang, C.F., 2011. Development of gonadal tissue and aromatase function in the protogynous orange-spotted grouper *Epinephelus coioides*. Zool. Stud. 50, 693–704.

Wang, D.S., Zhou, L.Y., Kobayashi, T., Matsuda, M., Shibata, Y., Sakai, F., Nagahama, Y., 2010. Doublesex- and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in Tilapia. Endocrinology 151, 1331–1340. https://doi.org/10.1210/en.2009-0999.

Wei, L., Li, X., Li, M., Tang, Y., Wei, J., Wang, D., 2019. *Dmrt1* directly regulates the transcription of the testis-biased *Sox9b* gene in Nile tilapia (*Oreochromis niloticus*). Gene 687, 109–115. https://doi.org/10.1016/j.gene.2018.11.016.

Wheeler, J.R., Segner, H., Weltje, L., Hutchinson, T.H., 2020. Interpretation of sexual secondary characteristics (SSCs) in regulatory testing for endocrine activity in fish. Chemosphere. https://doi.org/10.1016/j.chemosphere.2019.124943.

Wootton, R.J., Smith, C., 2014. Reproductive Biology of Teleost Fishes. John Wiley & Sons, Ltd, Chichester, UK. https://doi.org/10.1002/9781118891360.

Yao, H.H., Matzuk, M.M., Jorgez, C.J., Menke, D.B., Page, D.C., Swain, A., Capel, B., 2004. Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. Dev. Dyn. 230, 210–215.

Zhai, G., Shu, T., Xia, Y., Lu, Y., Shang, G., Jin, X., He, J., Nie, P., Yin, Z., 2018. Characterization of sexual trait development in cyp17a1-deficient Zebrafish. Endocrinology 159 (10), 3549–3562. https://doi.org/10.1210/en.2018-00551.