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Insights of sex determination and sex differentiation in fish

Anabel Lee Martinez Bengochea
Doutoranda

Jaboticabal, São Paulo
2019

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No somos más
Que una gota de luz
Una estrella fugaz
Una chispa, tan sólo
En la edad del cielo
No somos lo
Que quisiéramos ser
Solo un breve latir
En un silencio antiguo
Con la edad del cielo
Calma
Todo está en calma
Deja que el beso dure
Deja que el tiempo cure
Deja que el alma
Tenga la misma edad
Que la edad del cielo……

Jorge Drexler

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RESUMO

A decisão sobre se uma gônada bipotencial se desenvolverá em um testículo ou em um ovário é considerado um estágio crítico na diferenciação sexual dos vertebrados. A administração de esteróides exógenos durante este período pode afetar essa plasticidade, promovendo a diferenciação sexual na direção feminina ou masculina. Dessa forma, o objetivo desta tese foi avaliar os efeitos do tratamento de 17β-estradiol no desenvolvimento de *Astyanax altiparanae* (lambari), através de análises histológicas e de análises de expressão genica de possíveis genes envolvidos em vias masculinas e femininas. Para isso, larvas com gônadas indiferenciadas foram alimentadas com Artemia contendo diferentes concentrações de estradiol durante 28 dias, desde o 1 dia pós-eclosão (dpe) até o período que precede a diferenciação gonadal. Nossos resultados mostraram que o E2 modificou o fenotípo e a relação sexual histológica e, surpreendentemente, induziu intersexo com com a presença de óvulos nos testículos nas concentrações de 2 e 6 mg de E2/kg de alimento. Esses dados são uma evidência clara de que o tratamento utilizado não foi suficiente para induzir a reversão completa do sexo em *A. altiparanae*. Em termos de expressão gênica, o tratamento com E2 (6 mg/kg de alimento) produziu uma notável plasticidade gonadal entre machos e fêmeas aos 90 dias após a eclosão (dph). Os machos, denominados "machos resistentes ao estradiol", superexpressaram os genes masculinos, como *dmrt1*, *sox9* e *amh*. Dessa forma, nós sugerimos esses machos foram capazes de resistir à feminização induzida por estradiol, aumentando a expressão de genes relacionados à diferenciação testicular. Interessantemente, também encontramos fêmeas derivadas de estradiol com maior expressão de genes masculinos como *dmrt1* e *sox9*. Em conclusão, nossos resultados mostraram que menores concentrações de E2 administradas durante o desenvolvimento larval induziram uma reversão incompleta do sexo masculino para feminino em *A. altiparanae* e produziram uma complexa plasticidade gonadal genética a 90 dph.

Palavras-chave: *Astyanax altiparanae* (lambari), diferenciação sexual, 17β-estradiol

Destaques

- A dieta com 17β-estradiol modificou as proporções fenotípicas e histológicas de sexo e intersex
- Machos resistem à feminização induzida por estradiol, aumentando a expressão de genes relacionados à diferenciação testicular;
- A fêmea derivada do tratamento com estradiol mostrou morfologia ovariana normal, mas exibiu um perfil de expressão gênica "semelhante ao masculino".

ABSTRACT

The decision whether a bipotential gonad will become a testis or ovary is considered a critical stage in vertebrate sex determination. Administration of exogenous steroids can affect this plasticity by skewing the sex gonadal differentiation towards a male or female. The aim of this study is to evaluate the effects of 17β-estradiol (E2) diet on *Astyanax altiparanae* (lambari) development, focusing on the gonadal development and gene expression analysis of possible candidate genes involved in either male or female pathways. Larvae with undifferentiated gonads were fed with steroid diet containing different concentrations of E2 during 28 days, from the mouth opening until a period that precedes the gonadal differentiation. Animals were sampled at 90 days post-hatching (dhp) for histology and gene expression analysis. Our results showed that E2 disrupted both phenotypic and histological sex ratios, and surprisingly, induced intersex with testis-ova in the concentrations of 2 and 6 mg E2/Kg food. This data is a clear evidence that the treatment used was not enough to induce complete sex reversal in *A. altiparanae*. However, in terms of gene expression, E2 (6mg/Kg food) induced a remarkable gonadal plasticity between males and females at 90 dph. The males, named as E2 resistant males, overexpressed the male-biased genes, such as *dmrt1, sox9* and *amh*. We suggested that these males were able to resist the E2-induced feminization by the expression of genes related to testis differentiation. Strikingly, we also found E2-derived females with normal ovarian morphology but with higher or lower expression of *dmrt1* and *sox9*. In conclusion, our results showed that lower E2 concentrations administered during larval development induced an incomplete male-to-female sex reversal in *A. altiparanae* and produced a complex genetic gonadal plasticity at 90 dph

Keywords: *Astyanax altiparanae* (lambari), sex differentiation, 17β-estradiol

Highlights

- 17β-estradiol (E2) diet disrupted both phenotypic and histological sex ratios and induced intersex
- Males resist to the E2-induced feminization by enhancing the expression of genes related to testis differentiation;
- Female derived from E2 treatment showed a normal ovarian morphology but exhibited a "male-like" gene expression profile.

CHAPTER 1
1. GENERAL INTRODUCTION

Sexual development is one of the most fundamental and intriguing developmental processes shaping the life history of the vast majority of vertebrates. It is classically composed of two processes, sex determination and sex differentiation. The sex determination is defined as the master switch or primary mechanism governing the fate of the phenotypic sex while the sex differentiation is the developmental consequence of the sex determination process (Herpin and Schartl, 2015). Sex differentiation can be disrupted changing the original pathway that was assuming by the sex determining gene. In addition, it seems that the sex differentiating pathway in male appears more labile between the species but implicates more players than in female allowing probably a more strict control (Cutting et al., 2013).

1.1 Genetic sex determination

In vertebrates, sexual reproduction is the oldest and most widespread form of propagation used by vertebrates. The mechanisms that control sex determination and differentiation are diverse and depend on a wide variety of genetic factors as well as in some cases on environmental factors (Von Hofsten and Olsson 2005, Trukhina et al., 2013). Genetic sex can be defined by hereditary genetic factors (XX/XY and ZZ/ZW, among others) that are determined at the moment of the fertilization. On the other hand, gonadal differentiation depends on the activation of transcriptional factors that are expressed before the window of sex differentiation. These factors may have their expression altered by environmental conditions such as temperature, osmolarity, and others (Kikuchi and Hamaguchi, 2013, Herpin et al., 2013, Heule et al., 2014). In fish genetic sex determination can involve monogenic or polygenic systems, with factors located on the autosomes or on sex chromosomes. Sex chromosomes are found in approximately 10% of fish species studied, and sex-linked phenotypic traits, and protein and molecular genetic markers have been identified in several fish systems. Several gene families known to be involved in sex determination in other vertebrates have recently been shown to be similarly involved in fish, suggesting conservation of sex determination pathways (Devlin and Nagahama, 2002). In the monogenic system, genetic sex determination (GSD) presented both XX/XY and ZZ/ZW systems and variations, environmental sex determination such as temperature-dependent sex determination (TSD) like in reptiles, or social variables (the size of an organism relative to other members of its population) (Charlesworth 2002). Contrary to many other developmental processes, the mechanisms of sex determination exhibit a very large diversity at the top of the cascade among various organismic groups and even in closely related group. This suggests that the events triggering sex determination have evolved quickly, repeatedly and independently during evolution (Herpin and Schartl, 2015).

1.2 Sex determination in vertebrates

In vertebrates, male heterogametic system is called XX-XY system (where the Y chromosome determines the male sex) and a female heterogametic system is denoted ZZ-ZW (where the W chromosome determine the female sex) (Heule et al., 2014). Most mammals have an XX/XY sex determining system and the key components of the sex determining cascade have been identified, although their position in the hierarchy and their modes of interactions are still unknown (See review: Capel, 2017; Koopman et al., 1990). Sex determination in mammals is established at the moment of the fertilization. The Y chromosome carries the sex determining region Y (*Sry*) gene, which encodes a transcription factor that initiates testis development in the bipotential gonad. Evidences came from mice, in which *Sry* is deleted in XY female and transgenic XX male carrying a genomic fragment containing the Sry gene (See review: Capel 2017). In mammals, the male heterogametic system (XY) drives the sex determination whereas in birds and in the African clawed frog *Xenopus laevis* a female heterogametic (ZW) system is present. In fish, the sex chromosomes are often homomorphic and considered not differentiated (Kikuchi and Hamaguchi, 2013). The master switch or master sex determining gene acts as a presence/absence signal. The presence of this sex determining gene initiates a cascade that will ultimately lead to the development of one sex, whereas its absence will lead to the other sexual differentiation program (Kikuchi and Hamaguchi, 2013; Penman and Piferrer, 2008). Transcriptome analysis of early gonadal populations and studies from single cell analysis indicates that the cells in XX and XY bipotential gonads are initially nearly identical. The only differences at early stages arise from genes that are specific to the sex chromosomes such as Xist, Utx and Eif2s3x (only present in XX) and Ddx3y, Eif2s3y and Jarid1d (only present in XY) (See review; Capel, 2019).

In mice, sex-determining gene. *Sry* encodes a transcription factor that acts by binding to and activating the testis-specific enhancer (TES) of the related gene *Sox9* (See review: Capel, 2017; 2019). A network of gene activity downstream of *Sox9* then promotes male development while simultaneously impeding the gene network that drives ovarian development. These two alternative gene regulatory networks give the bipotential gonad its unique ability to differentiate into two morphologically and functionally distinct organs (See review: Capel, 2017). The regulation of *Sry* expression has to be tightly controlled. Mice with a "weak" *Sry* allele, that expresses *Sry* too late, show ovotestis formation. Additionally, *Sry* expression has to reach a certain threshold to induce testis development (Bullejos and Koopman 2005). The primary function of *Sry* is to induce differentiation of pre-Sertoli cells which is essential for testis differentiation of the bipotential gonad. The fate of the of the expression of the sex determining gene *Sry* in the bipotential gonad, determines the sex of an individual and the differentiation of the germ cells. In testes, germ cells differentiate into sperm, whereas in ovaries, germ cells

differentiate into oocytes (Tanaka and Nishinakamura 2014). In male gonads, several factors implicated in regulating *Sry* in XY gonadal cells, one of this factor is *Sox9* and the ability to repress the *Wnt/βcatenin* pathway that drives the ovary fate (See review; Capel, 2019). Wnt4 and Rspo1 may be involved in the establishment of cells in the gonad field. Together these genes regulate proliferation of coelomic epithelium precursors (dividing cells) that give rise to Sertoli cells and loss of *Wnt4* was previously shown to affect the *Sox9* population (See review; Capel, 2019). However, once this population is established, repression of *Wnt4* signaling is required to stabilize testis fate. Loss of *Fgf9* leads to the reversion to ovary fate after SOX9 expression is established, but if *Wnt4* is also lost, SOX9 expression and the testis pathway are rescued (See review; Capel, 2019). These results strongly suggest that there is a second stabilization step controlling testis fate governed by repression of *Wnt4* signaling. This occurs at multiple levels including through the *Wnt* antagonist, ZNRF3 (**Figure 1**) (See review; Capel, 2019).

Interestingly, orthologous genes that are downstream of SRY in the mammalian sex determination cascade have been identified in non-mammalian vertebrates. However, non-mammalian vertebrates have a tremendous variety of sex determination mechanisms, especially in fish (Herpin and Schartl 2015; Pan et al., 2016).

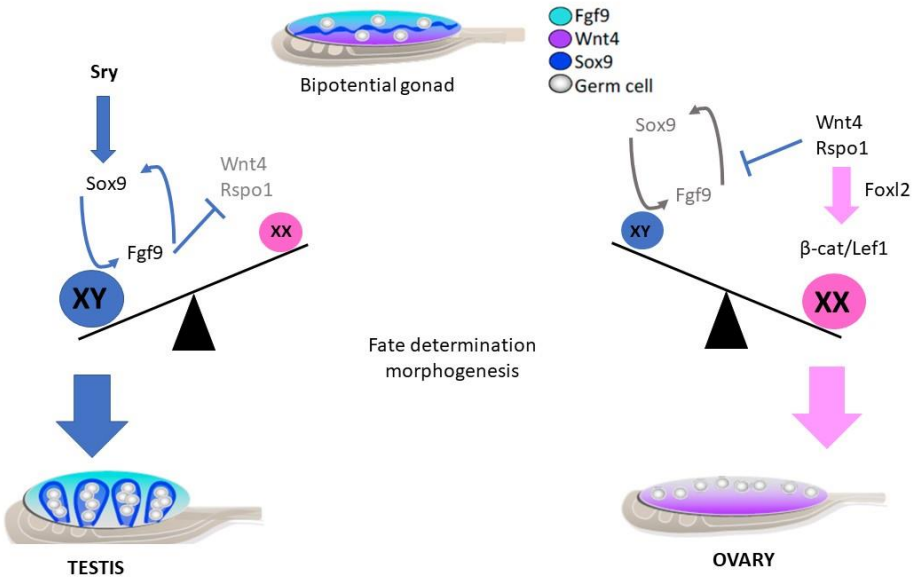


Figure 1. Signals control the fate of the gonad. When SRY triggers SOX9 upregulation, FGF9 is expressed and represses Wnt4 and the female pathway. In the absence of SOX9 upregulation, ovarian development ensues, based on Wnt and downstream signaling.. (Adapted from Capel., 2019).

1.3 Sex determination in fish

Sex determination in fish is a very flexible process in terms of evolutionary patterns. In fish species, all kinds of sex determination systems have been observed, ranging from hermaphroditism to gonochorism and from environmental to genetic sex determination (Devlin and Nagahama 2002). Fish are the largest known group of vertebrates with approximately 32,000 species, 50 % of them correspond to vertebrates (Nelson, 2016). It is possible that among genera and families sex determination varies and almost all-different forms of genetic sex determination can be found in fish, including XX/XY male heterogamety, ZZ/ZW female heterogamety, and multiple sex chromosomes (Kikuchi and Hamaguchi, 2013). Sex determining genes arose from two main independent mechanisms: gene duplication and allelic diversification. A duplicated copy of an ancestral gene and its insertion in the proto-sex chromosome constitute the gene duplication mechanism. Allelic diversification comes from a stepwise diversification of one or two loci of a sex chromosome pair, where one allele became the sex determiner while the other allele at the same locus retained a non sex determination function or favored the development of the opposite sex (Kikuchi and Hamaguchi, 2013) (**Figure 2**). In fish, different sex-determining genes have been discovered with a chromosome system XX/XY (**Figure 3**). In 2002, the first sex-determining gene was discovered in fish, in two species of medaka (*Oryzias latipes* and *Oryzias curvinotus*) (Nanda et al., 2002; Matsuda et al. 2002). This sex determining gene is *dmrt1bY* or *dmY*, and is a paralog of *dmrt1* (important gene involved in testicular development of vertebrates and invertebrates), linked to the Y chromosome. However, this gene was not found in other species of fishes, even in other species of the same genus *Oryzias* (Volff et al., 2003). Phylogenetic studies have been released that *dmrt1bY* appear belatedly than SRY (Zhang 2004). In 2012, two other major sex-determining genes were reported: *amhY* in the Pejerrey (Hattori et al., 2012), recently *amhbY* in Northern Pike (*Esox lucius*) (Pan et al., 2019), *gsdfY* in another medaka specie (*Oryzias luzonensis*) (Myosho et al., 2012), *amhr2* in the Fugu (*Takifugu rubipes*) (Kamiya et al., 2012) and *sdY* in rainbow trout (*Oncorhynchus mykiss*) (Yano et al., 2012).). In Nile tilapia (*Oreochromis niloticus*), a XX-XY specie commercially important in which the sex is mainly controlled genetically, a tandem duplication of *amh* located on the Y (sex determining gene *amhY*) (Li et al., 2015a). In the genus *Oryzias*, an allelic diversification of the *sox3* gene triggers the sex determination in *Oryzias dancena* (Takehana et al., 2014). The male sex determining gene was named *sox3Y* but this gene is not different from its X allelic copy. However, on the Y chromosome, a specific *cis* regulatory DNA segment downstream of *sox3Y* is able to induce male differentiation (Takehana et al., 2014) (**Table 1**). Sex determination in *Astyanax altiparanae* is still unknown as well as for the most Neotropical fishes (Fernandino and Hattori, 2019).

Table 1. Master sex-determining genes in Vertebrates (Adapted from Herpin and Schartl, 2015)

Master SD gene	Organism	SD system	SD gene ancestor	SD gene generated from ancestor by	Gene ancestor function
SRY	Therian mammals	XY	Sox3	Allelic diversification	Transcription factor, required in formation of the hypothalamo–pituitary axis, functions in neuronal differentiation, expressed in developing gonads
Dmrt1	Birds	WZ	Dmrt1	Allelic diversification	Transcription factor, key role in male sex dermination and differentiation
DM-W	Frog (*Xenopus laevis*)	WZ	Dmrt1	Gene duplication	Transcription factor, key role in male sex dermination and differentiation
Dmrt1bY	Medaka (*O. latipes, O. curvinotus*)	XY	*Dmrt1*	Gene duplication	Transcription factor, key role in male sex dermination and differentiation
sdY	Rainbow trout (*Onchorynchus mykiss*)	XY	*Irf9*	Gene duplication	Interfereron response factor, no gonadal function known
gsdfY	*Oryzias luzonensis*	XY	*Gsdf*	Allelic diversification	TGF-b factor, important role in fish gonad development
sox3Y	*Oryzias dancena*	XY	*Sox3*	Allelic diversification	Transcription factor, required in formation of the hypothalamo–pituitary axis, functions in neuronal differentiation, expressed in developing gonads
amhY	Pejerrey (*Odonthesthes hatcheri*)	XY	*Amh*	Gene duplication	Anti-Muellerian hormone, growth factor
amhY	Nile tilapia (*Oreochromis niloticus*)	XY	*Amh*	Gene duplication	Anti-Muellerian hormone, growth factor
amhr2Y	Fugu (*Takifugu rubripes*)	WZ	*Amh receptor*	Allelic diversification	Type II receptor for Amh, important function in gonad development
Dmrt1	Chinese tongue sole (*Cynoglossus semilaevis*)	XY	*Dmrt1*	Allelic diversification	Transcription factor, key role in male sex dermination and differentiation

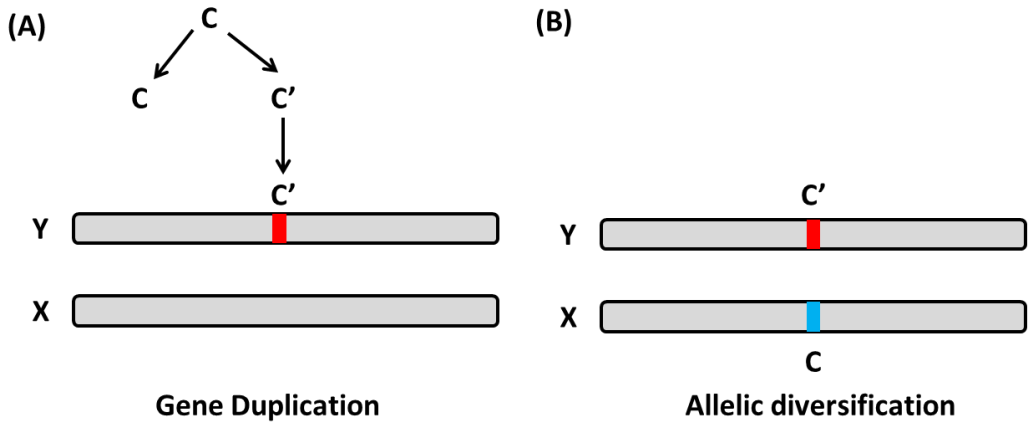


Figure 2. Schematic representation of the two main mechanisms leading to monofactorial genetic sex determination. **(A)** The gene C is duplicated give rise to gene C and C'. The gene C' is inserted in the Y chromosome. **(B)** The gene C is present on the X chromosome that give rise to the gene C' inserted in the Y chromosome (Adapted from Kikuchi and Hamaguchi, 2013).

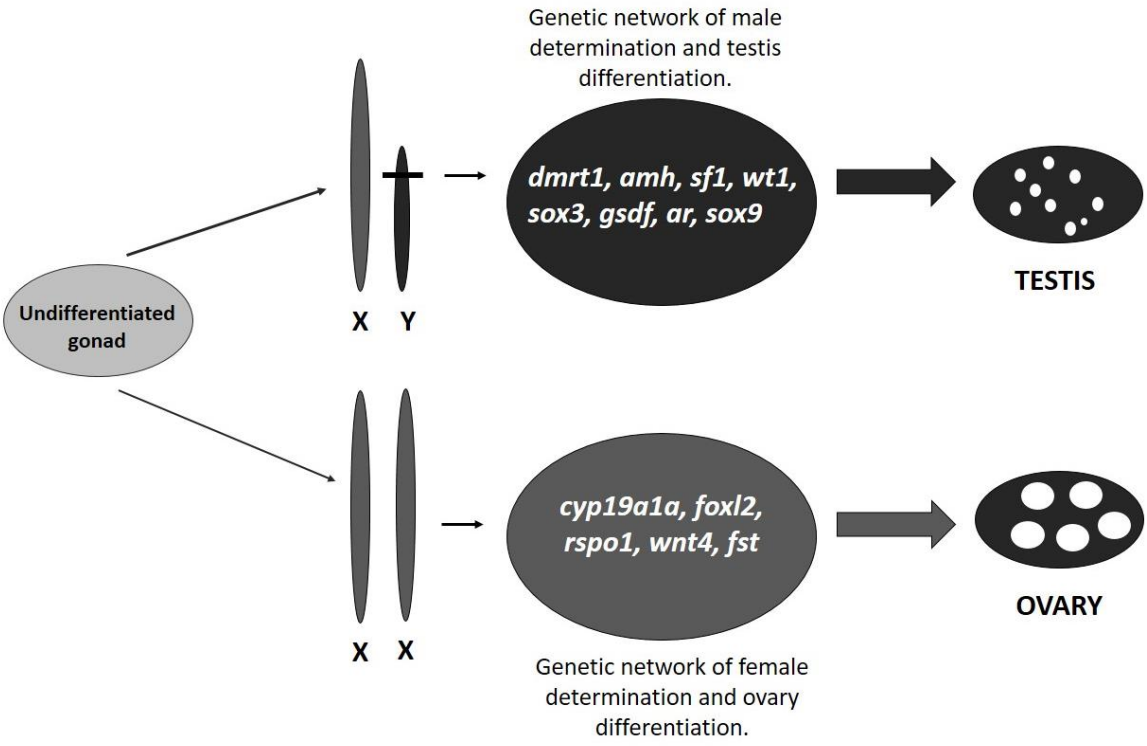


Figure 3. A schematic diagram of sex determination and gonad (testis or ovary) differentiation in fish with XX/XY sex determination system (Adapted from Smirnov and Trukhina 2019)

1.4 Sex differentiation in fish

A complex series of interacting biochemical processes are involved after the expression of the sex determining gene. At some point early in the cascade a primary sex determining gene is activated, resulting in a commitment to the production of either male or female patways programs (**Figure 3**). Although much is known about the process of sex differentiation in fish, the precise mechanisms underpinning sex differentiation as well as those involved in primary sex-determination remain undefined (Devlin and Nagahama, 2002). Fish gonadal sex differentiation exhibits much diversified strategies and physiological regulations than the one found in mammals (Baron and Guiguen, 2003). The differentiation of the bipotential gonad to either testis or ovary relies on the regulation of the steroidogenic pathway. In basal fish, sex steroid (estrogens and androgens) play a critical role in gonadal differentiation (Devlin and Nagahama, 2002, Stürssmann and Nakamura, 2002). Unlike sex-determining systems, the genes involved in gonadal differentiation appear to be relatively conserved. The genes involved in the regulatory network of sexual development described so far come mainly from mammalian studies and can be divided into functional groups as follows: 1) genes required for bipotential gonad development [Wilm's tumour suppressor-1 (WT1), steroidogenic factor-1 (SF-1), and GATA-binding protein 4 (GATA-4)]; 2) genes involved in male sex determination [double sex and mab-3 related transcription factor 1 (DMRT1), SRY-related box 9 (SOX9), dosage-sensitive sex-reversal-adrenal hypoplasia congenital critical region of X chromosome, gene 1 (DAX1), fibroblast growth factor 9 (FGF9), and desert hedgehog (DHH)]; 3) genes involved in male sex differentiation [AMH, AMHR2, and androgen receptor (AR)]; 4) genes involved in female sex determination [Wingless-type MMTV integration site family member 4 (WNT4), R-spondin-1 (RSPO-1), cateninb-1 (CTNNB1), forkhead box transcription factor L2 (FOXL2), and follistatin (FST)]; 5) genes involved in female sex differentiation [aromatase (also known as Cyp19A1 or P450arom), αestrogen receptor a (ERa), and αestrogen receptor b (ERb)] (Forconi et al., 2013) (**Figure 4**).

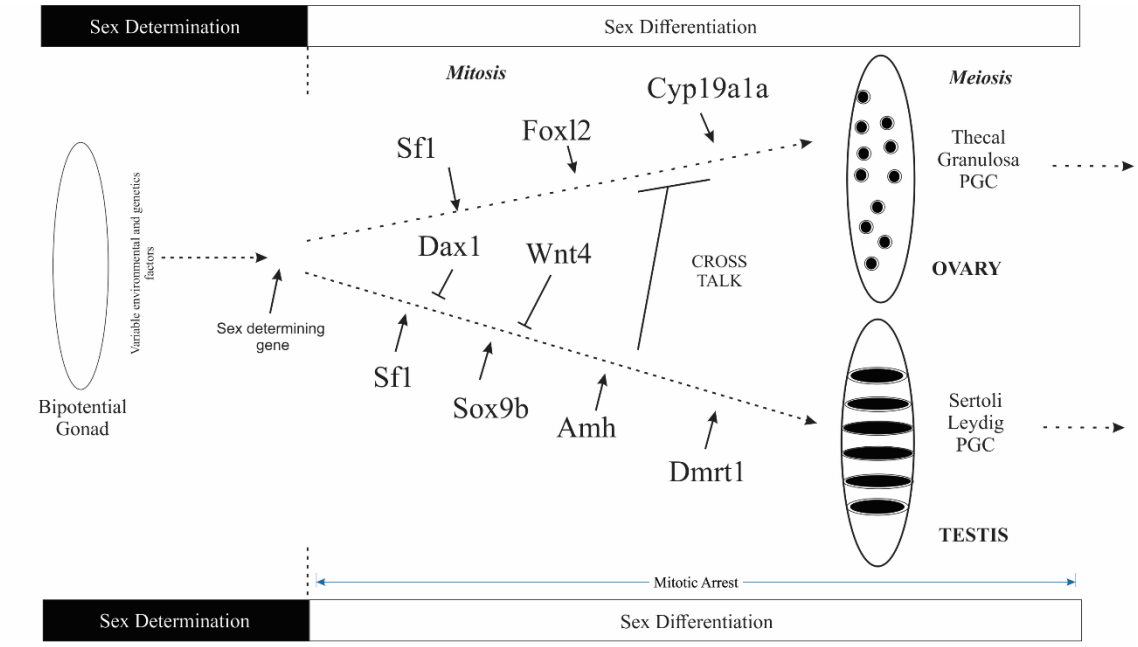


Figure 4. Putative mechanisms of fish sex determination and differentiation (Arrow-activation; Bars-repression). Adapted from Matsuda et al., 2003)

1.5 Candidate genes

1.5.1 Male pathway program

Dmrt1a (Doublesex and mab-3 related transcription factor 1)

Dmrt1 is a transcription factor implicated in testis differentiation and it is evolutionary conserved from cnidarian to mammals (Herpin and Schartl, 2011b, 2015). In mouse, DMRT1 is present during embryogenesis and at adult stage. DMRT1 holds a key position in sex differentiation. DMRT1 directs the cellular fate of male somatic cells by promoting the male specific pathway and meanwhile repressing female genes such as FOXL2 (Zhao et al., 2015b). In addition, DMRT1 maintains the identity of the male somatic cells in mouse (Zhao et al., 2015b). A transgenic study demonstrated that over-expression of *dmrtl* in the gonads of XX medaka caused a reduction in aromatase expression resulting in degeneration of ovaries and development of testicular tissue, with complete sex reversal in some individuals (Nagahama, 2005). The mechanism of this action was explored using luciferase assays, suggesting that Dmrt1 bound to Sf-1 itself, repressing Sf-1 activated transcription of aromatase (Nagahama, 2005). In medaka, Dmrt1 also plays the decisive role in maintaining the cellular identity of the adult testis, most obvious from the fact that its malfunction in adult mutant mice gonads leads to trans-differentiation of Sertoli to granulosa-like cells and feminization of a fully developed testis (Herpin et al. 2019). Consequently, the action range of Dmrt1 is not restricted to initiation of the male gonadal phenotype during early development but also contributes to the active suppression of the female networks via repression of two ‘anti-testis’ pathways, Foxl2 and Wnt family

member 4 (WNT4)/β-catenin (Herpin et al. 2019). In lambari this gene was studied by Adolfi and collaborators (2015) who found that *dmrt1* sequence is conserved and closely related to sequences of other basal teleost. Expression analysis revealed that *dmrt1* display male-specific functions in adult and probably is involved in gonad maintenance (Adolfi et al., 2015). The expression pattern of *dmrt1* change throughout the male reproductive cycle, with a significant down-regulation after spermiation, indicating a possible role of *dmrt1* in spermatogenesis. During gonadal formation, *dmrt1* expression ocurrs at 78 days after hatching, later than ovary development. This indicates that the male sex ddifferentiation period occurs before this stage (Adolfi et al., 2015). Phylogenetic tree designed by comparing the amino acid sequence of the Dmrt1 of *A. altiparanae* with 17 Dmrt1 sequences from different vertebrate groups using fugu Dmrt2 sequence as the outgroup shows a high homology with the Southern and African catfish, all belonging to the superorder Ostariophysi (**Figure 5**) (Adolfi et al., 2015).

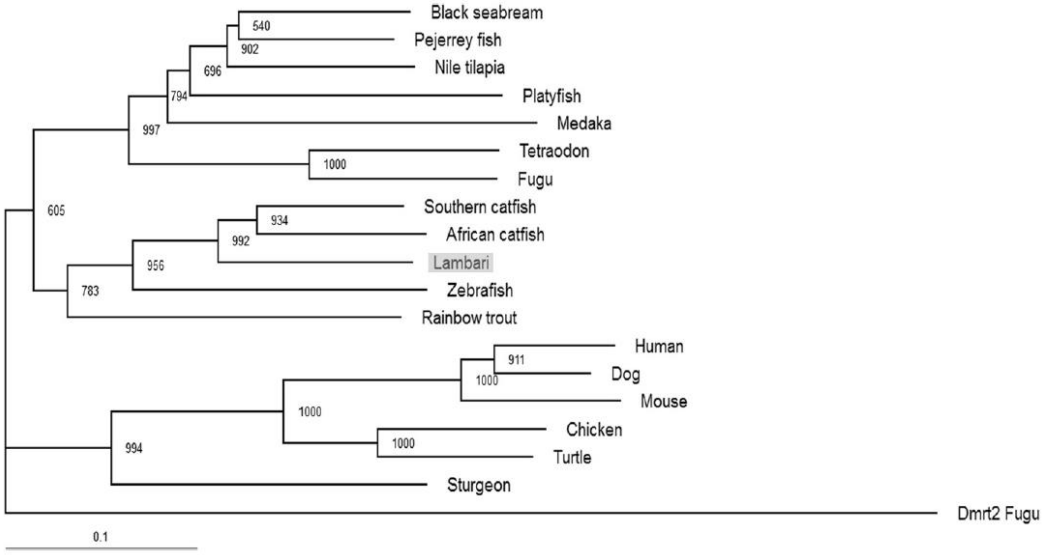


Figure 5. Phylogenetic tree of Dmrt1 in vertebrates. The tree was rooted using the fugu Dmrt2 as outgroup. Numbers on each node are the bootstrap values in thousand runs (Adolfi et al., 2015).

Sox9 (SRY box transcription factor 9)

Two *sox9* genes, *sox9a* and *sox9b* have been found in several teleost fish; zebrafish (Plavicki et al., 2015), three-spined stickleback (Kluver et al., 2005), fugu (Koopman et al., 2004), medaka (Yokoi et al., 2002), rice field eel (Zhou et al., 2003) and rainbow trout (Takamatsu et al., 1997). Investigations on these species showed that not only *sox9* has been duplicated, but also its functions have been partitioned in lineage-specific ways. Interestingly, in zebrafish, *sox9a* is a putative regulator of the gonadal expression of an SF-1 homologue. However based on expression pattern studies in medaka and rice field eel indicate that the function of *sox9* genes in cartilage development (chondrogenesis) is conserved in fish, but its role in male sex determination and

differentiation may not be (Zhou et al., 2003). In zebrafish, *sox9a* is expressed in adult testis while *sox9b* is expressed in adult ovary. However, in adult medaka, *sox9a* was expressed predominantly in the ovary and much less expressed in the testis than *sox9b* (Kluver et al., 2005). In the genus *Astyanax*, the genome of *A. mexicanus* provides a predicted sequence of a second copy of the *sox9* gene, and together with phylogenetic analyses using other species, *sox9* gene of *A. altiparanae* showed higher identity with other teleost of *sox9b* genes (Adolfi et al., 2015). However, the presence of another *sox9* gene copy in lambari can only be confirmed by isolation and characterization of this gene in this species. The *sox9a* and *sox9b* of zebrafish are more divergent in comparison to other teleost sequences, and even lambari being closer phylogenetically to zebrafish, the Sox9 sequence does not cluster together with the zebrafish sequence. However, the sequence of Sox9 from *A. altiparanae* clusters in the group of basal teleost although, it is possible the discrimination between *sox9a* or *sox9b* (**Figure 6**) (Adolfi et al., 2015).

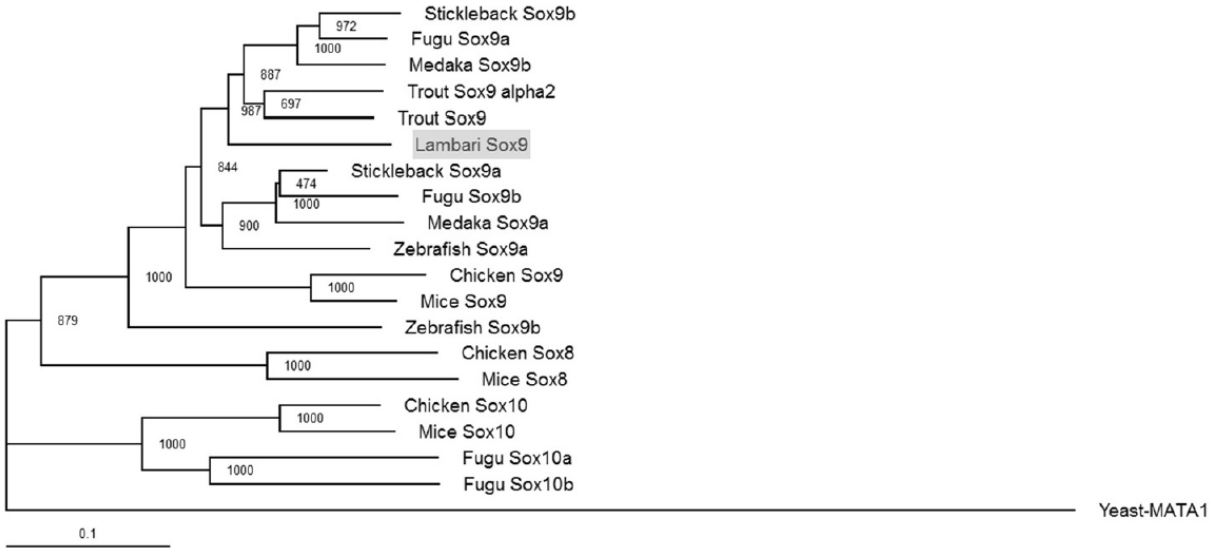


Figure 6. Phylogenetic tree of Sox E group proteins in vertebrates. The tree was rooted using yeast-MATA1 (CAA24622.1) as outgroup. Numbers on each node are the bootstrap values in thousand runs (Adolfi et al., 2015).

Amh (Anti-Mullerian Hormone)

Amh is a secreted glycoprotein belonging to TGF-β family which is expressed in Sertoli cells and in post-natal granulosa cells in mammals (Adolfi et al., 2018). In mammals, its function is to promote the regression of Müllerian ducts in embryonic testis but Amh is also implicated in testicular differentiation by suppressing Cyp19a1 (aromatase) in both male and female somatic cells in the fetal gonad (Adolfi et al., 2018). In teleost fish, the expression of *amh* is higher in males than in females, as shown in sole (Yoshinaga et al. 2004), Nile tilapia (Ijiri et al., 2008) and zebrafish (Rodríguez-Marí et al. 2005). For example, in hermaphrodites such as black snapper (*Acanthopagrus schlegeli*), high levels of *amh* are associated with the onset of testicular

differentiation (Wu et al., 2010; Wu and Chang, 2013), and its expression pattern is maintained during the period of female-to-male sex reversal. On the other hand, in male-to-female reversal, amh levels decreased dramatically (Wu et al., 2010; Wu and Chang, 2013). Although Amh is differencially expressed between males and females, little is known about the role of Amh in spermatogenesis and testicular function in adult male fish. In zebrafish, Amh inhibits the production and secretion of androgens by Leydig cells (Skaar et al., 2011). Androgens are important by stimulating spermatogonial proliferation and the entry of meiosis (Morais et al., 2017). In zebrafish, Amh neutralizes the effects of spermatogenesis stimulatory growth factors, such as Igf-3 (insulin-like growth factor 3) (Morais et al., 2017). On the other hand, Amh increases the production of inhibitory growth factors produced by Sertoli cells, such as inhibin-a (*inha*), DNA-binding transcription factor (*id3*) inhibitor and prostaglandin E2 (PGE2) (Morais et al., 2017). Together, these mechanisms lead to an inhibition of cell proliferation and differentiation indicating that Amh plays a role in the maintenance of spermatogonia in its undifferentiated state (Morais et al., 2017).

In zebrafish normal *amh* prevents female-biased sex ratios. Amh null alleles showed in adult male enormous testes, half of which contained immature oocytes, demonstrating that Amh regulates male germ cell accumulation and inhibits oocyte development or survival. Mutant males formed sperm ducts and some produced a few offspring. Young female mutants laid a few fertile eggs, so they also had functional sex ducts (Yan et al., 2019b). In medaka, mutation of the Anti-mullerian hormone receptor II (*amhrII*), known as *hotei*, showed a number of remarkable phenotypic abnormalities including: (i) excessive proliferation of germ cells; (ii) initiation of premature meiosis in phenotypically males; (iii) male-to-female sex reversal in 50% of XY males; and (iv) arrest of follicular development at early stages (Morinaga et al., 2007). Nakamura and colaborators (2012) found that Amh in medaka regulates the proliferation of mitotically active, self-renewing, type I germ cells. In *Odontesthes hatcheri*, Hattori and colaborators (2012) have shown that males have a duplicate copy of the *amh* gene, *amhY*, which is bound to the Y chromosome and is required for the sex determination of this species. In this sense, Amh and its receptor should play an important role in male sex differentiation, as well as in testicular development of vertebrates in general (Hattori et al., 2012).

Ar (androgen receptor)

Steroid hormones are known to regulate cellular processes through two distinct receptor mechanisms (Saravanan et al., 2016). Androgens are necessary for normal male phenotype expression, including the initiation and maintenance of spermatogenesis. Many physiological actions of androgens are mediated by the androgen receptor (AR), a member of the nuclear

receptor super family (Saravanan et al., 2016). AR functions as a ligand-dependent transcription factor, regulating expression of an array of target genes that are important in male development and fertility (Saravanan et al., 2016). In Actinopterygians, several duplicates of AR were characterized in the rainbow trout, (*Oncorhynchus mykiss*), the mosquitofish (*Gambusia affinis*), *Astatotilapia burtoni*, the Nile tilapia (*Oreochromis niloticus*), the Japanese eel (*Anguilla japonica*) and in *Gasterosteus aculeatus* (Douard et al., 2008).Two duplicated genes were found for AR. The different forms of ARs, termed AR-A and AR-B, have also been biochemically characterized based on their binding affinities for different androgen ligands in the Atlantic croaker (*Micropogonias undulate*s) and the kelp bass (*Paralabrax clathratus*). These two receptors display different tissue distributions with AR-A present only in the brain and AR-B found both in the brain and the gonads (Douard et al., 2008). Studies using as a model mouse confirmed the need for *ar* in reproductive development, particularly spermatogenesis (Yu et al., 2018). In zebrafish, targeted disruption of *ar* causes male infertility via defective spermatogenesis and female premature ovarian failure during growth. Mechanistic assays suggest that these effects are caused by fewer proliferated cells and more apoptotic cells in *ar*-null testes (Yu et al., 2018). Moreover, genes involved in reproductive development, estradiol induction and hormone synthesis were dys-regulated in testes and ovaries and disordered the reproductive-endocrine axis. In zebrafish, *ar* is required for spermatogenesis and maintenance of ovarian function, which confirms evolutionarily conserved functions of *ar* in other vertebrates (Yu et al., 2018).

1.5.2 Female pathway program

Cyp19a1a (Cytochrome P450, family 19, subfamily A, polypeptide 1a)

Steroids such as estrogens (E2) play an important role in ovarian differentiation and maintenance in fish (Guiguen et al., 2010; Paul-Prasanth et al., 2013). The product of CYP19a1 gene is called aromatase, a key microsomal enzyme that catalyzes the synthesis of estrogens from androgens in the smooth endoplasmic reticulum of steroidogenic cells (Devlin and Nagahama, 2002). In fish two *cyp19a1* genes have been generated by the teleost specific whole genome duplication with *cyp19a1a* known as the gonadal form and *cyp19a1b* as the brain form. In some fish species, *cyp19a1a* expression is female specific and occurs prior sex differentiation in most species studied so far (Devlin and Nagahama, 2002; Suzuki et al., 2004; Vizziano et al., 2007). During the early stage of differentiation, *cyp19a1a* localizes in a subset of unknown lineage of somatic cells and thereafter is found in both granulosa and theca cells. Aromatase inhibitors treatment induces masculinization in several fish species such as Chinook salmon (*Oncorhynchus tshawytscha*) (Piferrer et al., 1994), rainbow trout (Guiguen et al., 1999b), Nile tilapia (Guiguen et al., 1999b), fugu (Rashid et al., 2007), zebrafish (McAllister and Kime, 2003), black tetra (Mazzoni et al., 2015) and this confirmed the previously well-known important role of estrogen

for ovarian differentiation in fish. The regulation of the *cyp19a1a* promoter involves many factors such as activators Foxl2, Nr5a1, CREB (cAMP responsive element Binding), repressor Nrb01 (Dax1). Foxl2 regulates steroidogenesis especially via its binding to *cyp19a1* promoter (Bentsi-Barnes et al., 2010). Guiguen et al (2010) proposed a positive regulation loop where Foxl2 activates the *cyp19a1a* promoter, in turn the enzyme produces estrogens (E2) and the product induces the synthesis of Foxl2.

Rspo-1/R-Spondin1 (Roof plate specific spondin)

Ovarian development requires the canonical β-catenin signaling pathway (Chassot et al., 2012). In mammals, Wnt4 and R-spondin1 that are two members of the Wnt signaling pathway both drive the stabilization of β-catenin and its import to the nucleus to activate the female sex differentiation genes (Chassot et al., 2012). In fish, *wnt4* transcripts are detected in somatic cells surrounding the germ cells in rainbow trout and Rspo-1 and Rspo-2 in both somatic and germ cells of medaka (Herpin et al., 2013; Nicol et al., 2012; Zhou et al., 2012). The wnt-R-spondin1-β-catenin pathway seems to be conserved across the taxa to induce female development and should be repressed during male differentiation as shown in mammals, red eared slider turtle and zebrafish and chicken. It is evident today that the RSPO1/WNT/b-catenin pathway acts at the top of the ovarian differentiation cascade (Chassot et al., 2012). The development of male or female traits appears to be antagonistic, and two key events orient the developing gonad toward a male or a female fate: female (or male) genes need to be expressed and male (or female) genes actively repressed. The molecular mechanisms regulating the expression of Rspo1 and Wnt4 in the bipotential gonad, as well as those involved in their ovary-specific up-regulation remain not yet known (Chassot et al., 2014).

Foxl2 (Forkhead Box L2)

Another transcription factor that exhibits sexually dimorphic expression is *foxl2*. Foxl2 is a member of the winged helix/forkhead family of transcription factors known to be involved in ovarian development granulosa cell differentiation, and the proper maintenance of ovarian function (Cocquet et al., 2003). Foxl2 has been found to be correlated with the level of *cyp19* expression in a diverse range of species, including medaka (Nakamoto et al., 2006), tilapia (Yoshiura et al., 2003), rainbow trout (Baron et al., 2005b), Japanese flounder, *Paralichthys olivaceus* (Yamaguchi et al., 2007), wrinkled frog, *Rana rugosa* (Oshima et al., 2008), chicken (Govoroun et al., 2004), turtle (Ramsey et al., 2007) and mammals (Pannetier et al., 2006). At adult stages, Foxl2 protein is mainly present in follicular cells (granulosa and theca) surrounding the oocytes. In male, during sexual development, a low but significant *foxl2* expression is detected

by sensitive quantitative methods but the mRNA and/or the protein have not been visualized yet (Caulier et al., 2015). In the adult testis, Foxl2 has been detected in Leydig and germ cells of zebrafish (Caulier et al., 2015). It is worth to note that *foxl2* is absent from the developing and adult medaka testis (Nakamoto et al., 2006). The testicular function of *foxl2*/Foxl2 is unknown in most of the species.

17β-hsd (17β-hydroxysteroid dehydrogenase)

17β-hydroxysteroid dehydrogenases (17*β*-HSDs) regulate androgen and estrogen concentrations in mammals. The group of 17β-hydroxysteroid dehydrogenases (17*β*-HSD) is defined by their catalytic activity to reduce or oxidize hydroxysteroids (androgens and estrogens) at position C17 of the steroid backbone (Diotel et al., 2011). Many 17β-HSDs have overlapping substrate spectra but two of them seem to be especially important for the activation of estrogens and androgens: 17β-HSD type 1 and 17β-HSD type 3 (Diotel et al., 2011). 17*β*-HSD type 1 catalyzes the reduction of estrone to estradiol, the major biologically effective estrogen in mammals and fish (Mindnich et al., 2004). While, the main player in testosterone formation is considered to be 17β-HSD type 3, which catalyzes the reduction of androstenedione to testosterone with high efficiency and is almost exclusively expressed in testis (Mindnich et al., 2005). 17β -HSD type 3 expression was detected in various tissues of both male and female adults but displayed sexual dimorphism. Interestingly, expression was not highest in male testis but in male liver. In female adults, strongest expression was observed in ovaries. At the subcellular level, both human and zebrafish 17*β* -HSD type 3 localize to the endoplasmic reticulum (Mindnich et al., 2005). In fish 17β-HSD-1 played key roles in the regulation of estradiol synthesis (Mindnich et al., 2004; Diotel et al., 2011). Important roles for estrogens in ovarian differentiation (Guiguen et al., 1999) and for 11-oxygenated androgens in testicular differentiation (Liu et al., 2000) have been described. 17β-HSD enzyme in Japanese eel displays a substrate specificity similar to the human protein but seems to differ slightly from any known mammalian HSD17B1 in being expressed not only in female tissues but also in testis (Mindnich et al., 2004)

Esr (estrogen receptor)

Estrogen is a steroid hormone known by its reproductive roles, as well as for its non-reproductive functions in vertebrates (Socorro et al., 2000). The Esr is a nuclear receptor superfamily belonging to a steroid receptor subfamily. The Esr has been cloned in several teleost fish, including rainbow trout, killifish, *Oryzias latipes*, tilapia, channel catfish (*Ictalurus punctatus*), Japanese eel, Anguilla, red seabream (*Chrysophrys major*), gilthead seabream (*Sparus aurata*) and goldfish (*Carassius auratus*) (Socorro et al., 2000). All fish Esrs, excluding the

Japanese eel and goldfish, are more related to ERα (Socorro et al., 2000). In fish, the receptors are slightly less conserved, especially in the ligand-binding domain, and fish can have duplicate copies of the receptors. The genome of the zebrafish (*Danio rerio*) (Bondesson et al., 2016) and tilapia (Yan et al., 2019a) codes for three estrogen receptors, ESR1, ESR2a and ESR2. Estrogen also plays significant roles for normal vertebrate embryonic development (Bondesson et al., 2016). The formation of the female reproductive tract has been most studied, however, several reports suggested that estrogens have additional important functions, such as in the development of the male reproductive organs and sex differentiation of the brain (Bondesson et al., 2016). Estrogens are produced by cholesterol and it requires intermediaries and enzymes to generate androstenedione and testosterone that finally are transformed it into estrone and estradiol through the CYP19a1 enzyme (**Figure 7**).

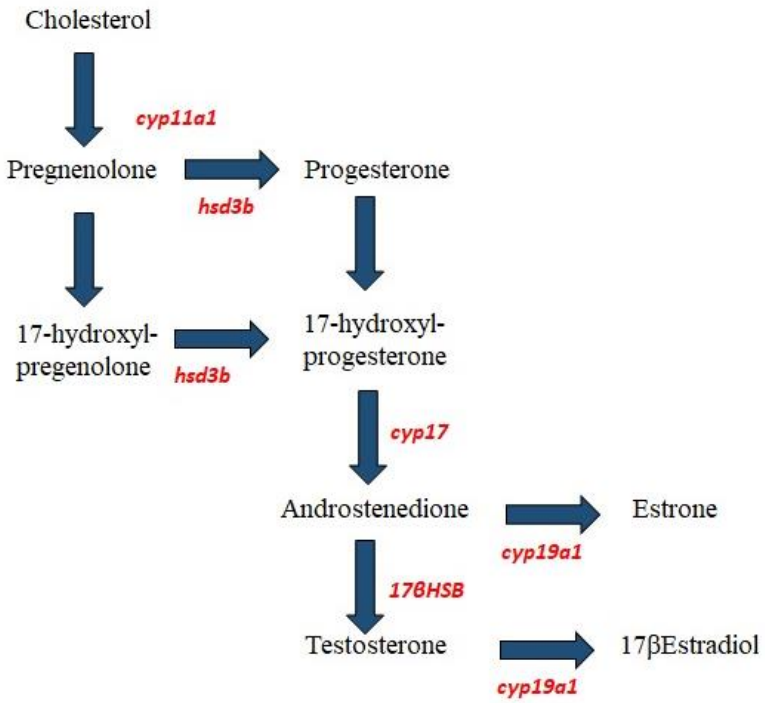


Figure 7. Synthesis of 17β-estradiol. In red are the enzymes involved in the production of estrone and 17β-estradiol. (Vizziano com pers.)

The above mention genes *dmrtl, sox9, amh, ar, cyp19a1, rspo, foxl2, 17β-hsd* and *esr* have been identified as players in mammalian sex differentiation as well as in some fish species and other vertebrates studied, albeit some species specific differences are exhibited. The large majority of these molecules had not been characterized in Neotropical species, such as lambari. The figure 9 summarizes the main effectors involved in either male or female pathway program of sex differenciation in vertebrates (**Figure 8**).

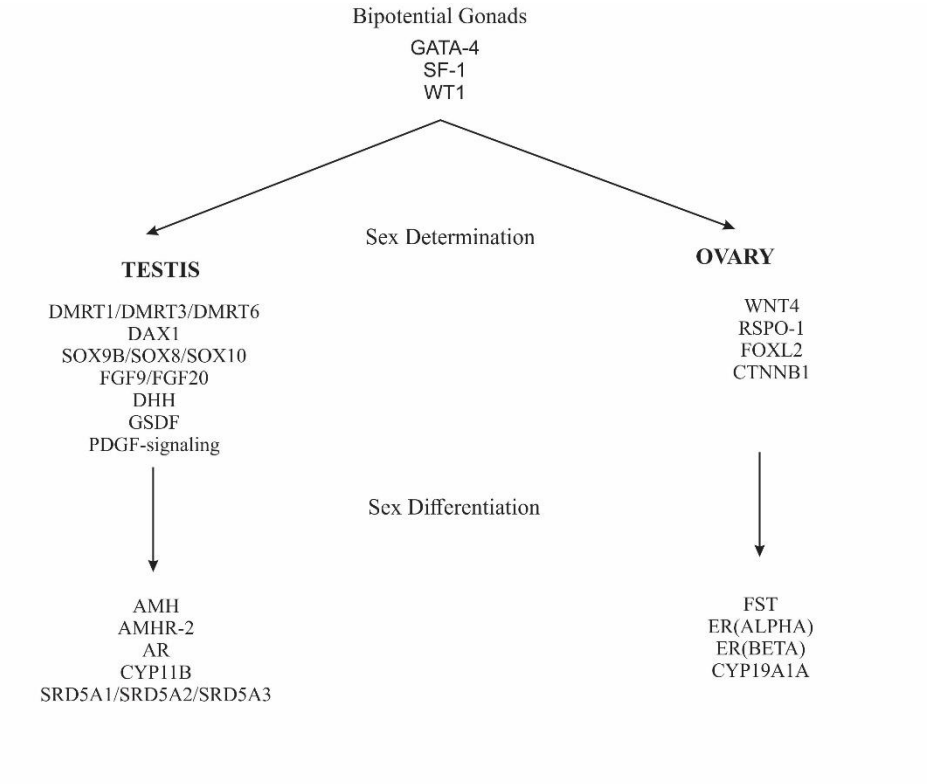


Figure 8. Main downstream effectors involved in male or female pathway programs of sex differentiation (own scheme).

1.6 Gonad plasticity and the effect of estrogens

Fish is characterized by plasticity of germ and somatic cells. This plasticity is maintained throughout the life cycle. Furthermore, the influence of factors in this process such as temperature, pH, density of population, etc, have been described. In some fish, temperature can play a role in sex determination, althought TSD in fish is less common than previously thought when compaed to reptiles, for example (Devlin and Nagahama 2002). Ribas and colaborators (2016) evaluated the effects of elevated temperature on zebrafish sex differentiation with respect to transcriptome gonadal analysis. The authors found neomales (female-to-male sex reversal) and, strikingly, females with a "male-like" transcriptome (Ribas et al., 2016). In addition, some heat-treated fish showed a gene expression profile similar to untreated controls of the same sex, indicating that they were resistant to thermal effects (Ribas et al., 2016). This study showed how diverse is the gonadal plasticity in teleost fish and functional consequences of elevated temperature in natural populations exposed to global warming.

Sex reversal can be achieved with hormonal treatment during sex differentiation. The ability to control sex in fish is quite relevant for aquaculture to obatain monosex cultures (Devlin and Nagahama 2002; Smirnov and Trukhina 2019). This process is very complex, but it can be manipulated using androgens or estrogens. These steroids control gonad differentiation and

maintain sexual phenotypes in teleost fish, wherein 17β-estradiol (E2) and 11-ketotestosterone (11-KT) function as the major estrogen and androgen, respectively (Smirnov and Trukhina 2019). The balance between estrogen and androgen production is expected to control sexual fate of the gonads during sex change. For example, factors regulating *cyp19a1a* expression are strong candidates for the trigger that initiates gonadal sex change; *cyp19a1a* promoter regions contain binding motifs for numerous factors that potentially regulate its expression (Smirnov and Trukhina 2019). The brain-pituitary-gonad (BPG) axis can be affected by different external factors, and this can provoke changes in phenotype fish, independent of the genotype. In the BPG axsis, hypothalamic neuron secrete gonadotropin releasing-hormone (Gnrh) that induces the gonadotrophs to synthesize and secrete gonadotropins. Fish possess two gonadotropins similar to those in higher vertebrates, follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) (Sullivan and Yilmaz 2018). Depending on species and reproductive status, one or both of these gonadotropins stimulate the synthesis and secretion of the estrogen, 17β-estradiol (E2). Circulating E2 then induces hepatocytes in the liver to synthesize and secrete vitellogenin (Vtgs). E2 also feeds back to act on the brain and pituitary gland, providing homeostatic control of vitellogenesis and later maturational processes (Sullivan and Yilmaz 2018). Xenoestrogens present in the environment can 'short-circuit' the brain-pituitary-gonad axis and act directly on the liver to induce abnormal vitellogenesis in male and juvenile fish, sometimes resulting in the appearance of ‘intersex’ males bearing previtellogenic oocytes in their testes, and in reproductive failure (Sullivan and Yilmaz 2018). Vitellogenesis is highly responsive to synthetic and natural estrogens, and fish Vtgs have been globally adopted as biomarkers of the presence of estrogenic chemical contaminants in aquatic environments, which is indicated by elevated Vtg levels in the blood or tissues of male and/or and juvenile fish (Sullivan and Yilmaz 2018).

In order to induce sex change, steroid hormones should be administered consistently before the period of sex differentiation. Steroid hormones are applied via immersion or treated feed. Ethinylestradiol (EE2) is often found to be more potent than estradiol (E2), but this ratio may change with exposure duration, probably due to differences in uptake and different metabolization rate, and E2 becomes more potent than EE2 as treatment duration increases (Piferrer and Donaldson 1992). Both female and male vertebrates produce estrogens. The production and excretion varies throughout life, reproductive cycle and between the two sexes. Estradiol is both metabolized reversibly and irreversibly. In the reversible metabolism, estradiol is transformed to estrone, while estradiol is transformed to catechol estrogens or estriol in the irreversible metabolism (Hoga et al., 2018). The main part of the produced metabolites are finally conjugated with sulphate and glucuronides and excreted in the urine and are as such an important source of natural estrogens in the municipal sewage system. A minor amount is excreted via feces as un-conjugated metabolites (Hoga et al., 2018).

Therefore, the observation of feminized fish in many parts of the world have raised the question whether the fertility or reproductive capacity of the fish is reduced and populations in danger of decreasing (Hoga et al., 2018). Although in most teleosts the gonadal sex can be easily manipulated by the appropriate treatment, sex control by steroid treatment is not fully effective in some species. As an example, in brook trout (*Salvelinus fontinalis*) androgen treatment did not result in an increase of the proportion of males, nor did it induce intersex, although feminization could be achieved by estrogen treatment (Díaz and Piferrer 2017). The exact timing and duration of this sensitive period differs from species to species, depending on the species-specific time of sex determination, which can lay before or after hatching, before or after onset of feeding (Díaz and Piferrer 2017). According to Pandian and Sheela (1995), fish species can be divided into two groups according to the period of sex differentiation. In one group, differentiation occurs for a short period following hatching, which makes exact timing of any hormonal treatment very important. In the other group, differentiation occurs during the late juvenile stage and is not always restricted to a single physiological stage, extending even to adult stages (Pandian and Sheela, 1995). These species maintain their sexual bipotentiality for a considerate period and may be influenced by hormonal treatment over several intervals (Adolfi et al., 2019). In a number of species, it is known that females differentiate earlier than males, as for example in sea bass, barfin flounder, schwartz's catfish, zebra cichlid, tanictis and pejerrey (Díaz and Piferrer 2017; Mazzoni and Quagio Grassiotto, 2017; Koger et al. 2000; Piferrer and Donalson 1992).

Yamamoto (1969) postulated that sex steroids were in fact the natural male and female sex inducers in fish. These include the more classic sex steroid hormones related with the gender of the fish, namely androgens (males) and estrogens (female). However, it has become clear that sexual differentiation depends on the balance between sex hormones in the organism, for example the balance between 11-ketotestosterone (11KT), 17β-estradiol (E2) and others. If the balance is altered and there is excess 11KT, then this scenario may favor masculine differentiation, while excess E2 induces feminine differentiation as in the cases of Eurasian perch (*Perca fluviatilis*) (Rougeot et al., 2007), and Japanese medaka (*Oryzias latipes*) (Bahamonde et al., 2013).

Bem and collaborators (2012) investigated the effectiveness of estradiol valerate on *A. altiparanae* feminization with the intention to contribute data that could advance the knowledge about the production and standardization of a sex reversion technique in a native fish. During 30 days, estradiol valerate was administered in different dosages (20, 40, and 80 mg/kg of diet); the control group did not receive hormone in the diet. The hormone treatments were effective in feminizing *A. altiparanae*, achieving 70-76% of the desired sex, while the control treatment had 44% females (Bem et al., 2012). The hormone treatment did not affect the growth of fish. According to Devlin and Nagahama (2002), incomplete sex transformations are regularly observed with steroid treatments in fish, with the development of both testicular and ovarian tissue

in the same individual. Also, according to these authors, in gonochorists species it is difficult to determine whether or not intersexuality arises from insufficient treatment (dosage, duration, or timing) or because the particular species is unable to fully respond to exogenous hormone treatment (Devlin and Nagahama 2002).

1.7 Intersex

Intersex is defined as the simultaneous presence of male and female gonadal tissue in an individual of a gonochoristic (fixed-sex) species (Adolfi et al., 2018). The most frequently reported manifestation of intersex is the presence of single or multiple oocytes within the testes of sub-adult or adult males. However, several other manifestations, such as the presence of testicular tissue within ovaries or the feminization of male gonadal ducts, have also been documented (Adolfi et al., 2018). In terms of aquatic contaminants, intersex can vary according to the exposure and it could be a feminization process (i.e. the presence of oocytes in the testes or a masculinization process (i.e. the presence of spermatozoa with previtellogenic oocytes) (Kavanagh et al., 2004). An important point to make is that not all gonochoristic fish species develop intersex condition (even at contaminated sites). It is unknown if there are sensitive developmental stages in which male gonochoristic species develop intersex (Kavanagh et al., 2004). For examples, no intersex cases were observed in goldfish (*Carassius auratus*), gizzard shad (*Dorosoma cepedianum*), brown bullhead (*Ictalurus ameiurus*), pumpkinseed (*Lepomis gibbosus*), and bluegill (*Lepomis macrochirus*) (Kavanagh et al., 2004).

In a characiformes species like *G. ternetzi* it was found a juvenile hermaphroditism. Gonadal aromatase was detected in interstitial cells in the early steps of the gonadal differentiation in both sexes. The authors believe that Leydig cells have a critical role in gonadal inversion. Apoptotic oocytes were observed and afterward degenerated (Mazzoni et al., 2015).

During larval life in zebrafish in the period of sex differentiation, gonads go through a transient female-like phase (approx. 13–25 days post fertilization), where all larvae produce some early-stage oocytes (sex determination occurs during this period) (See review; Kossackand Draper, 2019). For animals that were determined as females, the early-stage oocytes continue to maturation. By contrast, for animals that were determined as males, their early-stage oocytes undergo apoptosis as the gonads transition from a proto-ovary into a functional testis (See review; Kossack and Draper, 2019). Because gonads during this transition period can contain both female- and male-type germ cells, zebrafish have been referred to as juvenile hermaphrodites. The transitioning phase of testis development appeared to be over by 30 dpf, when the majority of oocytes had been cleared from gonads that will differentiate as testes (See review; Kossack and Draper, 2019).

1.8 *Astyanax* genus: *A. altiparanae*

Astyanax is a genus from the family Characidae widely distributed throughout South and Central America and previously assigned to the subfamily Tetragonopterinae (Géry, 1977; Ferreira Neto et al., 2009). The order Characiformes contains at least 24 families, about 520 genera, and about 2,300 species. The genus *Astyanax* is comprised of freshwater fish that are widely distributed in the Neotropical region (Garutti and Britski, 2000). This group has been recently considered for not exhibiting consistent evidence of monophyletism. *Astyanax altiparanae* (=*A. lacustris* (Pereira et al., 2016)) also called lambari is a typical species of the upper Paraná River Basin above Sete Quedas, in Brazil, with the exception of one population from a location in the Iguaçu River situated below this region (Ferreira Neto et al., 2009).

The available cytogenetic data on *Astyanax* indicate a high karyotypic diversity, with diploid number of 36–52 chromosomes, presence of B chromosomes, heterochromatin polymorphism, and variations with respect to the number and localization of nucleolar organizer regions (NORs) and 18S and 5S ribosomal DNA sites. Studies revealed four different diploid chromosome number, 2n = 36 for A. schubarti, 2n = 46 for A. fasciatus, 2n = 48 for A. marionae, and 2n = 50 for A. altiparanae, with karyotypes dominated by bi-armed chromosomes. Analysis in the numerical variation of the 18S rDNA sites of *Astyanax* species, shown that the terminal chromosomal position did not change among the different species, suggesting the ancestral state that diverged about 11.2 Mya (Piscor et al., 2019) (**Figure 9**).

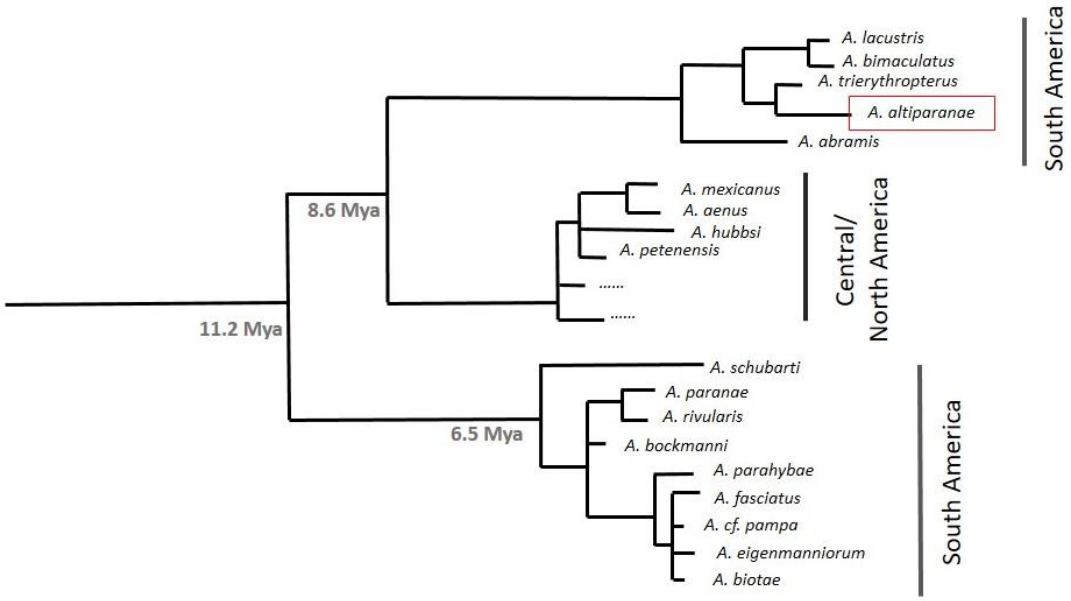


Figure 9. Cladogram adapted from Piscor and collaborators 2019. The gray numbers indicate the separation in million years ago.

Astyanax altiparanae become an attractive commercial fish for fish farmers because of the ease of fish-seed production, fast growth and resistance, and the simple handling required for this species (Cassel et al., 2017). There are about one hundred nominal species and subspecies described in *Astyanax* genus, but many aspects of their taxonomy are still unknown.

In the Iguaçu River basin, Paraná State, Brazil, *Astyanax* is represented mainly by seven or eight endemic species. The Iguaçu ichthyo fauna has a peculiar evolutionary history, with a high rate of endemism in a cataract-ridden fluvial environment (Prioli et al., 2002). *Astyanax* species have a long reproductive period, fast maturation, multiple spawn, small eggs, higher fecundity and absence of parental care (Suzuki et al., 2005). It addition to its ecological and academic importance, the order is of substantial commercial importance as a food, particularly in the case of the species of the genus *Astyanax* (Cassel et al., 2017). Since some years, *A. altiparanae* has become a relevant species for aquiculture, due to its attractive traits such as good survival rate for larvae and juveniles, easy reproduction, faster growth rate especially for the females and good resistance (Porto-Foresti et al., 2005; Gonçalves et al., 2014) which makes this specie a good study model.

Reproductive analysis showed that *A. altiparanae* has paired and ovoid ovaries, as it has been described for other species of this genus. Ovaries are classified as cystovarian, according the description by Grier et al., (2007). The germ cells are arranged along the entire lamella and occur in various stages of development. The development of the oogonia, (into mature oocytes) seems to be consistent among different teleost species. *A. altiparanae* has a long spawning period with reproductive peak from October to February (Cassel et al., 2017: Mazzoni and Quagio Grassiotto 2017). The asynchronous development of oocytes, with postovulatory complexes and vitellogenic oocytes at the same time, indicates that the species has multiple spawning events (Cassel et al., 2017). *A. altiparanae* males also appear to exhibit multiple spermiation. *A. altiparanae* testes have distinct areas with different phases of development, and the maintenance of the testicular regions during the reproductive cycle seems to enable the males to produce and storage sperm throughout the year (Costa et al., 2014). This allows the species to provide multiple releases of sperm, which is likely to be associated with more favorable environmental conditions (Cassel et al., 2017) (**Figure 10**).

All this characteristics make this specie important for commercial purposes and as a model in studies concerning fish reproduction and genotoxicity (Silva et al., 2011; Weber et al.,2013; Gomes et al., 2013; Adolfi et al., 2015; Chehade et al., 2015; Yasui et al., 2015; Santana et al., 2015; Kida et al., 2016; Levy-Pereira et al., 2019; Francisco et al., 2019). Due to its recent use as a biological model, genome and transcriptomic data base is not available, as well as there

are not so many sequences available in NCBI. Sex determining gene is still unknown, for this reason genotyping is not possible and sex was considering using external characters (phenotype: females smooth anal fin and males rough anal fin), gonad shape and morphology.

Figure 10. *Astyanax altiparanae* female adult specimen (Adapted from Cassel 2017).

1.9 BIBLIOGRAPHY

Adolfi MC, Nakajima RT, Nóbrega RH, Schartl M. (2018). Intersex, Hermaphroditism, and Gonadal Plasticity in Vertebrates: Evolution of the Müllerian Duct and Amh/Amhr2 Signaling. Annu Rev Anim Biosci. 15;7:149-172. doi: 10.1146/annurev-animal-020518-114955.

Adolfi MC, Carreira AC, Jesus LW, Bogerd J, Funes RM, Schartl M, Sogayar MC, Borella MI. (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. 11;13:2. doi: 10.1186/1477-7827-13-2

Bahamonde PA, Munkittrick KR, Martyniuk CJ. (2013). Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena? Gen Comp Endocrinol. 1;192:25-35. doi: 10.1016/j.ygcen.2013.04.005.

Baron, D. and Guiguen, Y. (2003). Gene expression during gonadal sex differentation in rainbow trout (Orcorhynchus mykiss): from candidate genes studies to high thoughout genomic approach. Fish Physiol Biochem, 28, 119-123.

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). Brazilian Archives of Biology and Technology. 55, 283-290.

Bentsi-Barnes, I.K., Kuo, F.T., Barlow, G.M., and Pisarska, M.D. (2010). Human forkhead L2 represses key genes in granulosa cell differentiation including aromatase, P450scc, and cyclin D2. Fertil Steril 94, 353-356.

Bondesson M, Hao R, Lin ChY, Williams C, and Gustafsson JA. Estrogen receptor signaling during vertebrate development. Biochim Biophys Acta. 1849(2): 142–151.

Brennan, J, Capel, B. (2004). One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nat Rev Genet.5(7):509-21. DOI:10.1038/nrg1381.

Bullejos, M. and P. Koopman. (2005). Delayed Sry and Sox9 expression in developing mouse gonads underlies B6-Y(DOM) sex reversal. Dev Biol 278: 473-481.

Capel, B. (2017). Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nature Reviews Genetics, 18(11), 675–689. doi:10.1038/nrg.2017.60

Capel, B. (2019). To Be or Not To Be a Testis. Reproduction. REP-19-0151.R1. doi: 10.1530/REP-19-0151

Cassel M, Chehade C, Souza Branco G, Caneppele D., Romagosa E, and Borella, M. I. (2017). Ovarian development and the reproductive profile of *Astyanax altiparanae* (Teleostei, Characidae) over one year: Applications in fish farming. Theriogenology, 98, 1–15. doi:10.1016/j.theriogenology.2017.04.044

Cassel M. 2017. Desenvolvimento das modificacoes morfofuncionais em ovários de *Astyanax altiparanae* GARUTTI E BRITSKI 2000 (Teleostei, Characidae). Tese de Doutorado. DOI: https://doi.org/10.11606/T.42.2017.tde-10072017-084505.

Caulier, M., Brion, F., Chadili, E., Turies, C., Piccini, B., Porcher, J.M., Guiguen, Y., and Hinfray, N. (2015). Localization of steroidogenic enzymes and Foxl2a in the gonads of mature zebrafish (Danio rerio). Comp Biochem Phys A 188, 96-106.

Cocquet, J., De Baere, E., Gareil, M., Pannetier, M., Xia, X., Fellous, M., Veitia, R.A. (2003). Structure, evolution and expression of the FOXL2 transcription unit. Cytogen. Genome Res., 101, 206-211.

Costa FG, Adolfi MC, Gomes CC, Jesus LWO, Batlouni SR, Borella MI. Testes of Astyanaxaltiparanae: The Sertoli cell functions in a semicystic spermatogenesis. Micron 2014; 61:20–7.

Charlesworth, B. 2002. The evolution of chromosomal sex determination. Novartis Found Symp 244: 207-219; discussion 220-204, 253-207.

Chassot AA, Gillot I, Chaboissier MC. (2014). R-spondin1, WNT4, and the CTNNB1 signaling pathway: strict control over ovarian differentiation. Reproduction. 148(6):R97-110. doi: 10.1530/REP-14-0177

Chassot, A.A., Bradford, S.T., Auguste, A., Gregoire, E.P., Pailhoux, E., de Rooij, D.G., Schedl, A., and Chaboissier, M.C. (2012). WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad. Development 139, 4461-4472.

Chassot, A.A., Ranc, F., Gregoire, E.P., Roepers-Gajadien, H.L., Taketo, M.M., Camerino, G., de Rooij, D.G., Schedl, A., and Chaboissier, M.C. (2008). Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. Hum Mol Genet 17, 1264-1277.

Chehade, C.H.; Cassel, M.; Borella, I. (2015). Induced reproduction in a migratory teleost species by wáter level drawdown. Neotropical Ichthyology, 13(1): 205-212. http://dx.doi.org/10.1590/1982-0224-20140028.

Devlin, R.H. and Y. Nagahama. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191-364.

Díaz N and Piferrer F. (2017). Estrogen exposure overrides the masculinizing effect of elevated temperature by a down-regulation of the key genes implicated in sexual differentiation in a fish with mixed genetic and environmental sex determination. Biochim Biophys Acta. 1849(2): 142–151

Diotel N, Do Rego JL, Anglade I, Vaillant C, Pellegrini E. (2011). The brain of teleost fish, a source, and a target of sexual steroids.. Frontiers in Neuroscience, Frontiers, 2011, 5, pp.137. ff10.3389/fnins.2011.00137ff.

Douard V, Brunet F, Boussau B, Ahrens-Fath I, Vlaeminck-Guillem V, Haendler B, Laudet V, Guiguen Y. (2008). The fate of the duplicated androgen receptor in fishes: a late neofunctionalization event? BMC Evol Biol.18;8:336. doi: 10.1186/1471-2148-8-336.

Fernandino JI and Hattori RS. (2019). Sex determination in Neotropical fish: Implications ranging from aquaculture technology to ecological assessment. Gen Comp Endocrinol. 1;273:172-183. doi: 10.1016/j.ygcen.2018.07.002.

Francisco CM, Bertolino SM, De Oliveira Júnior RJ, Morelli S, Pereira BB. (2019). Genotoxicity assessment of polluted urban streams using a native fish *Astyanax altiparanae*. J Toxicol Environ Health A. 2019;82(8):514-523. doi: 10.1080/15287394.2019.1624235.

Ferreira Neto M, Vicari MR, de Camargo EF, ArtoniRF, Moreira-Filho O. 2009. Comparative cytogenetics among populations of *Astyanax altiparanae* (Characiformes, Characidae, Incertae sedis). Genetics and Molecular Biology, 32, 4, 792-796.

Forconi M1, Canapa A, Barucca M, Biscotti MA, Capriglione T, Buonocore F, Fausto AM, Makapedua DM, Pallavicini A, Gerdol M, De Moro G, Scapigliati G, Olmo E, Schartl M. (2013). Characterization of sex determination and sex differentiation genes in Latimeria. PLoS One. 25;8(4):e56006. doi: 10.1371/journal.pone.0056006. Print 2013.

Garutti V, Britski HA. Descrição de espécie nova de *Astyanax* (Teleostei: Characidae) da bacia do alto rio Paraná e considerações sobre as demais espécies do gênero na bacia. Comun Mus Ciênc Tecnol PUCRS 2000; 13:65–88.

Géry J (1977) Characoids of the World. TFH Publications, Neptune, 672 pp.

Gomes, C.C.; Costa, F.G.; Borella, M.I. (2013). Distribution of GnRH in the brain of the freshwater teleost *Astyanax altiparanae* (Garutti and Britski, 2000). Micron, 52-53: 33-38. http://dx.doi.org/10.1016/j.micron.2013.07.008. PMid:23972603.

Gonçalves LU, Parisi G, Bonelli A, Sussel FR, Viegas EMM. The fatty acid compositions of total, neutral and polar lipids in wild and farmed lambari (*Astyanax altiparanae*). Aquacult Res. 45(2):195-203.

Govoroun, M.S., Pannetier, M., Pailhoux, E., Cocquet, J., Brillard, J.-P., Couty, I., Batellier, F., Cotinot, C. (2004). Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev. Dynam., 231, 859-870.

Grier HJ, Uribe MC, Parenti LR. (2007) Germinal epithelium, folliculogenesis, and postovulatory follicles in ovaries of rainbow trout, Oncorhynchus mykiss (Walbaum, 1792) (Teleostei, Protacanthopterygii, Salmoniformes). J Morphol 2007; 268:293–310.

Guiguen, Y., Fostier, A., Piferrer, F., and Chang, C.F. (2010). Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol 165, 352-366.

Guiguen, Y., Baroiller, J.F., Ricordel, M.J., Iseki, K., McMeel, O.M., Martin, S.A.M., and Fostier, A. (1999b). Involvement of estrogens in the process of sex differentiation in two fish species: The rainbow trout (Oncorhynchus mykiss) and a Tilapia (Oreochromis niloticus). Molecular Reproduction and Development 54, 154-162.

Hattori R, Muraia Y, Ouraa M, Masudaa S, Majhia SK, Sakamotoa T, Fernandino J, Somoza GM, Yokotaa M, and Strüssmanna CA. (2012). A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. PNAS Vol:109, N°8, 2955-2959.

Herpin, A., and Schartl, M. (2011b). Dmrt1 genes at the crossroads: a widespread and central class of sexual development factors in fish. Febs Journal 278, 1010-1019.

Herpin A, Adolfi MC, 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. Molecular biology and evolution 30:2328-2346.

Herpin, A., and Schartl, M. (2015). Plasticity of gene-regulatory networks controlling sex determination: of masters, slaves, usual suspects, newcomers, and usurpators. EMBO Rep 16, 1260-1274.

Heule, C., Salzburger, W., and Bohne, A. (2014). Genetics of sexual development: an evolutionary playground for fish. Genetics 196, 579-591.

Hoga CA, Almeida FL, and Reyes FGR. (2018). A review on the use of hormones in fish farming: Analytical methods to determine their residues. CyTA - Journalof Food, 16:1, 679-691, DOI: 10.1080/19476337.2018.1475423

Ijiri, S.; Kaneko, H.; Kobayashi, T.; Wang, D. S.; Sakai, F.; Paul-Prasanth, B.; Nakamura, M.; Nagahama, Y. (2008). Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia, Oreochromis niloticus. Biology of reproduction, vol.78, p.333–341.

Kamiya T, Kai W, Tasumi S, Oka A, Matsunaga T, Mizuno N, Fujita M, Suetake H, Suzuki S, Hosoya S, Tohari S, Brenner S, Miyadai T, Venkatesh B, Suzuki Y, Kikuchi K. (2012). A Trans-Species Missense SNP in Amhr2 Is Associated with Sex Determination in the Tiger Pufferfish, Takifu gurubripes (Fugu). PLoS Genet 8(7): e1002798. doi:10.1371/journal.pgen.1002798.

Kavanagh, R.J., Balch, G.C., Kiparissis, Y., Niimi, A.J., Sherry, J., Tinson, C., Metcalfe, C.D. (2004). Endocrine disruption and altered gonadal development in white perch (*Morone americana*) from the Lower Great Lakes Region. Environ. Health Perspect. 112, 898–902.

Kida, B.M.S.; Abdalla, R.P.; Moreira, R.G. (2016). Effects of acidic water, aluminum, and manganese on testicular steroidogenesis in *Astyanax altiparanae*. Fish Physiology and Biochemistry, 42(5): 1347-1356. http://dx.doi.org/10.1007/s10695-016-0222-6. PMid:27025723.

Kikuchi, K., and Hamaguchi, S. (2013). Novel sex-determining genes in fish and sex chromosome evolution. Dev Dyn 242, 339-353.

Kluver, N., Kondo, M., Herpin, A., Mitani, H., Schartl, M., 2005. Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific sub-functionalization. Dev. Genes Evol., 215, 297-305.

Koger,C.S., Teh,S.J. and Hinton,D.E. (2000). Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17β-estradiol or testosterone. Marine Environmental Research 50, 201-206.

Koopman, P., A. Munsterberg, B. Capel, N. Vivian, and R. Lovell-Badge. 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348: 450-452.

Kossack M. and Draper BW. (2019). Kossack, M. E., and Draper, B. W. (2019). Genetic regulation of sex determination and maintenance in zebrafish (Danio rerio). Current Topics in Developmental Biology. doi:10.1016/bs.ctdb.2019.02.004

Koopman, P., Schepers, G., Brenner, S., Venkatesh, B., 2004. Origin and diversity of the Sox transcription factor gene family: genome-wide analysis in Fugu rubripes. Gene, 328, 177-186.

Larney Ch, Bailey TL, Koopman P. (2014). Switching on sex: transcriptional regulation of the testis-determining gene Sry. Development. 141(11): 2195–2205. doi: 10.1242/dev.107052.

Levy-Pereira N, Yasui GS, Evangelista MM, Nascimento NF, Santos MP, Siqueira-Silva DH, Monzani PS, Senhorini JA, Pilarski F. (2019). In vivo phagocytosis and hematology in *Astyanax altiparanae*, a potential model for surrogate technology. Braz J Biol. 2019 Oct 7. pii: S1519-69842019005013101. doi: 10.1590/1519-6984.205893.

Li, M., Sun, Y., Zhao, J., Shi, H., Zeng, S., Ye, K., Jiang, D., Zhou, L., Sun, L., Tao, W., et al. (2015a). A Tandem Duplicate of Anti-Mullerian Hormone with a Missense SNP on the Y Chromosome Is Essential for Male Sex Determination in Nile Tilapia, *Oreochromis niloticus*. PLoS Genet 11, e1005678.

Liu, S., Govoroun, M., D'Cotta, H., Ricordel, M.J., Lareyre, J.J., McMeel, O.M., Smith, T., Nagahama, Y., Guigue, Y. (2000). Expression of cytochrome P450(11β) (11β-hydroxylase) gene during sex differentiation and spermatogenesis in rainbow trout, Oncorhynchus mykiss. J. Steroid Biochem. Mol. Biol. 75, 291–298.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417: 559-563.

Matsuda M. (2003). Sex determination in fish: Lessons from the sex-determining gene of the teleost medaka, *Oryzias latipes*. Dev Growth Differ. 45(5-6):397-403.

Mazzoni, T. S., and Grassiotto, I. Q. (2017). Ovary Differentiation and Activity in Teleostei Fish. Theriogenology. doi:10.5772/intechopen.69022the

Mazzoni TS, Grier HJ, Quagio-Grassiotto. (2015). The basement membrane and the sex establishment in the juvenile hermaphroditism during gonadal differentiation of the *Gymnocorymbus ternetzi* (Teleostei: Characiformes: Characidae).

McAllister, B.G., and Kime, D.E. (2003). Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). Aquat Toxicol 65, 309-316.

Mindnich R, Deluca D, Adamski J. (2004). Identification and characterization of 17 beta-hydroxysteroid dehydrogenases in the zebrafish, Danio rerio. Mol Cell Endocrinol. 27;215(1-2):19-30.

Mindnich R, Haller F, Halbach F, Moeller G, Hrabé de Angelis M, Adamski J. (2005). Androgen metabolism via 17beta-hydroxysteroid dehydrogenase type 3 in mammalian and non-mammalian vertebrates: comparison of the human and the zebrafish enzyme. J Mol Endocrinol. 2005 Oct;35(2):305-16.

Morais, RDV. S,Crespo, D, Nóbrega, RH.; Lemos, M. S,Van De Kan, THJG, De França, LR Male, R, Bogerd, J, Schulz, R. W. (2017). Antagonistic regulation of spermatogonial differentiation in zebrafish (Danio rerio) by Igf3 and Amh. Molecular and Cellular Endocrinology, vol.454, p.112-124, 2017. doi: 10.1016/j.mce.2017.06.017.

Morinaga C, Saito D, Nakamura S, Sasaki T, Asakawa S, Shimizu N, Mitani H, Furutani-Seiki M, Tanaka M, 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 U S A. 5;104(23):9691-6.

Myosho T, Otake H, Masuyama H, MatsudaM, Kuroki Y, Fujiyama A, Naruse K, Hamaguchi S, and Sakaizumi M. 2012.Tracing the Emergence of a Novel Sex-Determining Gene in Medaka, *Oryzias luzonensis*. Genetics, Vol. 191, 163–170.

Nagahama, Y., 2005. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol Biochem, 31, 105-109.

Nakamoto, M., Matsuda, M., Wang, D.-S., Nagahama, Y., Shibata, N., (2006). Molecular cloning and analysis of gonadal expression of Fox12 in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun, 344, 353-361.

Nakamura S, Watakabe I, Nishimura T, Picard JY, Toyoda A, Taniguchi Y, di Clemente N, Tanaka M. (2012). Hyperproliferation of mitotically active germ cells due to defective anti-Müllerian hormone signaling mediates sex reversal in medaka. Development. 139(13):2283-7. doi: 10.1242/dev.076307.

Nanda I, Kondo M, Homung U, Asakawa S, Winkler C, Shimizu A, ShanZ,Haaf T, Shimizu N, ShimaA,Schmid M, and Schartl M. (2002). A duplicated copy of DMRT1 in the sexdetermining region of the Y chromosome of the medaka, *Oryzias latipes*.Proc. Natl. Acad. Sci. USA 99:11778-11783.

Nelson, J.S.; Grande, T.; Wilson, M.V.H. (2016). Fishes of the world 5th ed. Wiley – Interscience, New Jersey. 752p.

Nicol, B., Guerin, A., Fostier, A., and Guiguen, Y. (2012). Ovary-predominant wnt4 expression during gonadal differentiation is not conserved in the rainbow trout (Oncorhynchus mykiss). Mol Reprod Dev 79, 51-63.

Oshima, Y., Uno, Y., Matsuda, Y., Kobayashi, T., Nakamura, M. (2008). Molecular cloning and gene expression of Fox12 in the frog Rana rugosa. Gen Comp Endocrinol, 159, 170-177.

Pan, Q, Anderson, J, Bertho, S, Herpin, A, Wilson, C, Postlethwait, JH, Schartl, M, Guiguen, Y. (2016). Vertebrate sex-determining genes play musical chairs. C R Biol. 339(7-8):258-62. doi: 10.1016/j.crvi.2016.05.010.

Pandian,T.J. and Sheela,S.G. (1995). Hormonal induction of sex reversal in fish. Aquaculture 138, 1-22.

Pannetier, M., Fabre, S., Batista, F., Kocer, A., Renault, L., Jolivet, G., MandonPepin, B., Cotinot, C., Veitia, R., Pailhoux, E.(2006). FOXL2 activates P450aromatase gene transcription: towards a better characterization of the earlysteps of mammalian ovarian development. J Mol Endocrinol, 36, 399-413.

Paul-Prasanth, B., Bhandari, R.K., Kobayashi, T., Horiguchi, R., Kobayashi, Y., Nakamoto, M., Shibata, Y., Sakai, F., Nakamura, M., and Nagahama, Y. (2013). Estrogen oversees the maintenance of the female genetic program in terminally differentiated gonochorists. Sci Rep 3, 2862.

Penman, D.J., and Piferrer, F. (2008). Fish Gonadogenesis. Part I: Genetic and Environmental Mechanisms of Sex Determination. Rev Fish Sci 16, 16-34.

Pereira, L. S., Agostinho, A. A., and Delariva, R. L. (2016). Effects of river damming in Neotropical piscivorous and omnivorous fish: feeding, body condition and abundances. Neotropical Ichthyology, 14(1). doi:10.1590/1982-0224-20150044

Piferrer, F., Zanuy, S., Carrillo, M., Solar, I.I., Devlin, R.H., and Donaldson, E.M. (1994). Brief Treatment with an Aromatase Inhibitor during Sex-Differentiation Causes Chromosomally Female Salmon to Develop as Normal, Functional Males. J Exp Zool 270, 255-262.

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

Piferrer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. Aquaculture, 197, 229–281.

Piscor D, Pozzobon APB, Fernandes CA, Centofante L, Parise-Maltempi PP. (2019). Molecular Clock as Insight to Estimate the Evolutionary History and Times of Divergence for 10 Nominal *Astyanax* Species (Characiformes, Characidae): An Evolutionary Approach in Species with 2n = 36, 46, 48, and 50 Chromosomes. Zebrafish.16(1):98-105. doi: 10.1089/zeb.2018.1647

Plavicki JS, Baker TR, Burns FR, Xiong KM, Gooding AJ, Hofsteen P, Peterson RE, and Heideman W. (2015). Construction and characterization of a sox9b transgenic reporter line. Int J Dev Biol. 2014; 58(9): 693–699. doi: 10.1387/ijdb.140288jp

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

Ramsey, M., Shoemaker, C., Crews, D. (2007). Gonadal expression of Sfl and aromatase during sex determination in the red-eared slider turtle (Trachemys scripta), a reptile with temperature-dependent sex determination. Differentiation, 75, 978-991.

Rashid, H., Kitano, H., Lee, K.H., Nii, S., Shigematsu, T., Kadomura, K., Yamaguchi, A., and Matsuyama, M. (2007). Fugu (Takifugu rubripes) sexual differentiation: CYP19 regulation and aromatase inhibitor induced testicular development. Sexual Development 1, 311-322.

Rodríguez-Marí, A.; Yan, Y.-L.; Bremiller, R. A.; Wilson, C.; Cañestro, C.; Postlethwait, J. H. (2005). Characterization and expression pattern of zebrafish anti- Müllerian hormone (amh) relative to sox9a, sox9b, and cyp19a1a, during gonad development. Gene Expression Patterns, v.5, p.655–667.

Rougeot, C., Krim, A., Mandiki, S.N.M., Kestemont, P., Mélard, C. (2007). Sex steroid dynamics during embryogenesis and sexual differentiation in eurasian perch, Perca fluviatilis. Theriogenology 67, 1046–1052.

Santana, C.A.; Andrade, L.H.C.; Súarez, Y.R.; Yukimitu, K.; Moraes, J.C.S.; Lima, S.M. (2015) Fourier transform-infrared photoacoustic spectroscopy applied in fish scales to access environmental integrity: a case study of *Astyanax altiparanae* species. Infrared Physics & Technology, 72: 84-89. http://dx.doi.org/10.1016/j.infrared.2015.07.005

Saravanan UT, Priya N and Narendiran JN. (2016). Comparative expression of androgen receptor in gonads and brain during different stages of gonadal development in Cirrhinus mrigala. Journal of Entomology and Zoology Studies 4(5): 532-536.

Silva, N.J.R.; Lopes, M.C.; Fernandes, J.B.K.; Henriques, M.B. (2011). Caracterização dos sistemas de criação e da cadeia produtiva do *A. altiparanae* no estado de São Paulo, Brasil. Informações Econômicas, 41(9): 17-28

Skaar, K. S.; Nóbrega, R. H.; Magaraki, A. Olsen, L. C.; Schulz, R. W.; Male, R. (2011). Proteolytically activated, recombinant Anti-Müllerian Hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. Endocrinology, v.152, n.9, p.3527–3540.

Smirnov AF and Trukhina AV. (2019). Comparison of Sex Determination in Vertebrates (Nonmammals). Sex-determination System IntechOpen, edited by Alina Maria Sisu. DOI: 10.5772/intechopen.83831

Socorro S, Power DM, Olsson PE, Canario AV. (2000). Two estrogen receptors expressed in the teleost fish, Sparus aurata: cDNA cloning, characterization and tissue distribution. J Endocrinol. 166(2):293-306.

Strüssmann, C.A. and Nakamura, M. (2002). Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes.Fish Physiol Biochem, 26, 13-29.

Sullivan CV, and Yilmaz O. (2018). Vitellogenesis and Yolk Proteins, Fish. Encyclopedia of Reproduction, Second Edition. Elsevier. DOI: 10.1016/B978-0-12-809633-8.20567-0

Suzuki HI, Vazzoler AEAM, Marques EE, Lizama MAP, INADA P. (2004). The upper Paraná River and its floodplain: physical aspects, ecology and conservation. Backhuys Publisher; p271-91.

Takamatsu, N., Kanda, H., Ito, M., Yamashita, A., Yamashita, S., Shiba, T.(1997). Rainbow trout 50X9: cDNA cloning, gene structure and expression. Gene,202, 167-170.

Takehana, Y., Matsuda, M., Myosho, T., Suster, M.L., Kawakami, K., Shin, I.T., Kohara, Y., Kuroki, Y., Toyoda, A., Fujiyama, A., et al. (2014). Co-option of Sox3 as the male-determining factor on the Y chromosome in the fish *Oryzias dancena*. Nat Commun 5, 4157.

Tanaka SS and Nishinakamura R. 2014. Regulation of male sex determination: genital ridge formation and Sry activation in mice. Cellular and Molecular Life Sciences. Volume 71, Issue 24, pp 4781–4802.

Vizziano, D., Baron, D., Randuineau, G., Mahe, S., Cauty, C., and Guiguen, Y. (2008). Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more

physiological than masculinization induced by androgen supplementation. Biol Reprod 78, 939-946.

Vizziano, D., Randuineau, G., Baron, D., Cauty, C., and Guiguen, Y. (2007). Characterization of early molecular sex differentiation in rainbow trout, Oncorhynchus mykiss. Dev Dyn 236, 2198-2206.

Volff JN., Kondo M., Schartl M. (2003). MedakadmY/dmrt1Y is not the universal primary sexdetermining gene in fish. Trends in genetics 19(4): 196-199.

Weber, A.A.; Arantes, F.P.; Sato, Y.; Rizzo, E.; Bazzoli, N. (2013). Oocyte adhesiveness and embryonic development of *Astyanax bimaculatus* (Linnaeus, 1758) (Pisces:Characidae). Zygote, 21(2): 198-202. http://dx.doi.org/10.1017/S096719941200007X. PMid:22717095.

Wu, G-C., Chiu, P. C. Lyu, Y. S, Chang, CF. (2010). The expression of Amh and Amhr2 is associated with the development of gonadal tissue and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. Biology of reproduction, vol.83, p.443–453.

Wu, GC and Chang, CF. (2013). The switch of secondary sex determination in protandrous black porgy, *Acanthopagrus schlegeli*. Fish Physiology Biochemistry, vol.39, p.33–38.

Yamamoto, T. (1969). Sex differentiation. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. 3A. Academic Press, New York, pp. 117–175.

Yan L, Feng H, Wang F, Lu B, Liu X, Sun L, Wang D. (2019a). Establishment of three estrogen receptors (esr1, esr2a, esr2b) knockout lines for functional study in Nile tilapia. J Steroid Biochem Mol Biol. 191:105379. doi: 10.1016/j.jsbmb.2019.105379

Yan L, Batzel P, Titus T, Sydes J, Desvignes T, Bremiller R, Draper B, Postlethwait JH. (2019b). The roles of Amh in zebrafish gonad development and sex determination. https://doi.org/10.1101/650218

Yano A, Guyomard R, Nicol B, Jouanno E, Quillet E, Klopp Ch, Cabau C, Bouchez O, Fostier A. and GuiguenY. (2012). An Immune-Related Gene Evolved into the Master Sex-Determining Gene in Rainbow Trout, Oncorhynchus mykiss. Current Biology 22, 1–6.

Yasui, G.S.; Senhorini, J.A.; Shimoda, E.; Pereira-Santos, M.; Nakaghi, L.S.; Fujimoto, T.; Arias-Rodriguez, L.; Silva, L.A. (2015). Improvement of gamete quality and its short-term storage:an approach for biotechnology in laboratory fish. Animal, 9(3): 464-470. http://dx.doi.org/10.1017/S1751731114002511. PMid:25391393.

Yokoi, H., Kobayashi, T., Tanaka, M., Nagahama, Y., Wakamatsu, Y., Takeda, H.,Araki, K., Morohashi, K., Ozato, K. (2002). Sox9 in a teleost fish, medaka (*Oryzias latipes*): Evidence for diversified function of Sox9 in gonad differentiation. Mol Reprod Dev, 63, 5-16.

Yoshinaga, N. Shiraishi, E. Yamamoto, T. Iguchi, T. Abe, S. Kitano, T. (2004)Sexually dimorphic expression of a teleost homologue of Müllerian inhibiting substance during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochemical and Biophysical Research Communications, vol.322, p.508– 513.

Yoshiura, Y., Senthilkumaran, B., Watanabe, M., Oba, Y., Kobayashi, T., Nagahama, Y. (2003). Synergistic expression of Ad4BP/SF-1 and cytochrome P-450 aromatase (ovarian type) in the ovary of Nile tilapia, Oreochromis niloticus, during vitellogenesis suggests transcriptional interaction 1. Biol Reprod, 68, 1545-1553.

Yu G, Zhang D, Liu W, Wang J, Liu X, Zhou C, Gui J, Xiao W. (2018). Zebrafish androgen receptor is required for spermatogenesis and maintenance of ovarian function. Oncotarget. 6;9(36):24320-24334. doi: 10.18632/oncotarget.24407

Zhao, L., Svingen, T., Ng, E.T., and Koopman, P. (2015b). Female-to-male sex reversal in mice caused by transgenic overexpression of Dmrt1. Development 142, 1083-1088.

Zhang J. (2004). Evolution of DMY, a newly emergent male sex-determination gene of medaka fish. Genetics 166(4): 1887-1895.

Zhou, R., Liu, L., Guo, Y., Yu, H., Cheng, H., Huang, X., Tiersch, T.R., Berta, P. (2003). Similar gene structure of two Sox9a genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (*Monopterus albus*). Mol Reprod Dev, 66, 211-217.

Zhou, L., Charkraborty, T., Yu, X., Wu, L., Liu, G., Mohapatra, S., Wang, D., and Nagahama, Y. (2012). R-spondins are involved in the ovarian differentiation in a teleost, medaka (*Oryzias latipes*). BMC Dev Biol 12, 36.

2. OBJECTIVE

2.1 General Objective

The aim of this thesis is to evaluate the effects of 17β-estradiol diet during the *A. altiparanae* development, focusing on gonadal development and gene expression analysis of some downstream effectors involved in male and female pathways of sex differentiation. This study will provide relevant information on whether 17β-estradiol can induce gonadal sex reversal in this species and how this hormone can modify the regulatory cascades involved on gonadal differentiation of this species. Moreover, these data would be helpful to establish a hormonal treatment protocol to produce monosex culture of this species which is extremely relevant for aquaculture.

2.2 Specific objectives

1. Evaluated the expression of selected genes in adult gonads (testes and ovaries) to find potential sex-based gene candidates for *A. altiparanae*.

2. Evaluate the effects of 17β-estradiol diet during *A. altiparanae* sex differentiation with respect to the sex ratio gonad morphology and growth rate.

3. Analysis of the effects of 17β-estradiol diet on the expression of genes involved either male or female pathway program during *A. altiparanae* gonad development.

2.3 Hypthesis

The hypothesis of this work is obtained a population of 100% rex reversal fish (from male to female) to understand how the plasticity of the downstream regulatory pathways of the population at 90dph using as a model a neotropical fish, lambari. For this purpose, we studied three different doses of 17β-estradiol (2mg/kg, 4mg/kg and 6mg/kg) to investigate which doses is the best to obtained 100% female population.

This study will provide relevant information on whether 17β-estradiol can induce gonadal sex reversal in this species and how this hormone can modify the regulatory cascades involved on gonadal differentiation in this species.

CHAPTER 2

3.Estrogen disrupted sex ratio and induced a complex gonadal plasticity in lambari, *Astyanax altiparanae*

3.1 INTRODUCTION

The decision whether the bipotential gonad anlage will become a testis or ovary is a critical stage in vertebrate sex determination. This developmental process includes fate determination and cellular differentiation which are finely regulated by a complex network of multiple regulatory genes and its interactions (Herpin et al., 2013; Herpin and Schartl, 2015). Recent studies on the molecular identification of upstream regulators and the downstream regulatory network have provided new information on how sexual development is regulated in different organisms, and how new sex determiners have evolved (see review in Herpin and Schartl, 2015). These authors demonstrated that the downstream effectors are not as strictly conserved as it was suggested by the central paradigma of sex determination “masters change, slaves remain” (Graham et al., 2003; Herpin and Schartl, 2015). Herpin and Schartl (2015) proposed a plasticity of gene regulatory-network composed of "masters", "slaves", "usual suspects", "newcomers" and "usurpators" regulating sex determination within vertebrates. In this scenario, in addition to the "masters" and "slaves", new players could be added into the regulatory network cascade, modifying the length or the entire cascade, but without necessarily impacting on the final phenotype of male or female (Herpin and Schartl, 2015). This explains the diversity of molecular mechanisms underlying sex determination and differentiation among various organismic groups, even within closely related groups or species with the same sex determining gene. Although diverse, the commonly reported effectors involved in the vertebrate male pathway program are *Fgf9, Sox9, Sox8, Sf1, Dmrt1*, while *Rspo1, Wnt4, β-catenin, Fst, Foxl2, Esr 1,2* and *Cyp19a* are the common described for the female pathway program (Brennan and Capel, 2004; Yao, 2005; Herpin and Schartl, 2015; Biscotti et al., 2018).

In addition to the specific male or female regulatory pathways, it has been shown that the development of a testis or ovary from a bipotential gonad requires a controlled antagonism of one organogenetic pathway by another (Warr and Greenfield, 2012). When the gonadal development is disrupted, it can lead to a gonadal sex reversal which is

defined as the transformation of one structure into another normally found elsewhere, in this case, in the opposite sex (Warr and Greenfield, 2012). The gonadal sex reversal sometimes can result in an incomplete differentiation of one tissue in another. This results in an intersex condition, as found in many vertebrates, including fish (Adolfi et al., 2019). Intersex is defined as the simultaneous presence of male and female gonadal tissue in an individual of a gonochoristic (fixed-sex) species (Adolfi et al., 2019). In aquatic animals, intersex is often viewed as a signature effect of exposure to endocrine disrupting compounds (EDCs), such as the estrogenic chemicals (Bahamonde et al., 2013; Adolfi et al., 2019).

17β-Estradiol (E2) is a natural estrogen hormone (Simpson et al., 1994) commonly used to induce effective feminization in some fish species, such as in Cyprinidae, Anabantidae, Poecilidae, Ictaluridae, Salmonidae and Cichlidae (Pandian and Sheela, 1995). Moreover, this hormone is widely used to obtain female monosex cultures from sex reversed fish (Hunter and Donaldson, 1983; Piferrer and Lim, 1997; Aktas and Genc, 2011). This is the case of some species where the female is more economically attractive than the male due to its faster growth rate (Beardmore et al., 2001). However, to obtain an effective sex reversal, several studies have shown that E2 hormonal treatment should be carried out before the sexual differentiation, during the time where the gonadal tissue is more sensitive to the action of the hormone (Pandian and Sheela 1995; Piferrer 2001; Devlin and Nagahama 2002; Díaz and Piferrer 2017). The molecular mechanism by which estrogens or anti-androgens exert its feminizing effects are not completely clear and systematically compared. In pejerrey, (*Odontesthes bonariensis*), for example, E2 plays an important role in the early stages of gonadal development by promoting the expression of genes related to the ovarian differentiation while repressing the testicular differentiation cascade. In this species, administration of exogenous E2 decreased the *dmrt1* mRNA levels, whereas the gonadal aromatase (*cyp19a1a)* was up-regulated (Fernandino 2008a). In addition to these genes, *amh*, which plays a critical role in testicular differentiation, is down-regulated by estrogens in pejerrey (Fernandino 2008b).

The species *Astyanax altiparanae*, known locally as lambari-do-rabo-amarelo (Nakatani et al., 2001), is a South American teleost species, which is native to the Upper Paraná basin in Brazil (Garutti and Britski, 2000). *A. altiparanae* is a fish whose traits include easy reproduction, good survival rate for larvae and juveniles and faster growth rate, especially for females (Porto-Foresti et al., 2005). For these reasons, the specie has commercial importance (Porto-Foresti et al., 2005), and scientifical relevance as

Neotropical experimental model fish (Gomes et al., 2013; Costa et al., 2014; Chehade et al., 2015; Siqueira-Silva et al., 2015; Yasui et al., 2015; Adolfi et al., 2015; Pereira-Santos et al., 2016; Jesus et al., 2017; de Paiva Camargo et al., 2017; Cassel et al., 2017; Branco et al., 2019; Levy-Pereira et al., 2019). Therefore, the aim of this study is to evaluate the effects of E2 on *A. altiparanae* gonadal development by evaluating the gonadal morphology and the E2-induced changes on male and female sexual differentiation regulatory pathways. This study will expand our knowledge on the reproductive biology of *A. altiparanae*, and also provide information on the gonadal sex differentiation in a Neotropical species.

3.2 MATERIAL AND METHODS

Ethics statement

All animal experimentation followed the guidelines for the Ethical Animal treatment and were approved by the Ethics Committee for animal experimentation of São Paulo State University (CEUA - protocol number: 9496). All efforts were made to minimize discomfort and suffering during the experimental procedures.

Animals and steroid diet treatment

The specimens used in this study were obtained from the Center of Aquaculture of São Paulo State University (CAUNESP), Jaboticabal (São Paulo State, Brazil). The artificial reproduction was carried out using *A. altiparanae* broodstocks (1 female : 2 males) that were kept in 100L tanks with constant photoperiod (14 h light:10 h dark) and temperature (28°C), Spawning was induced by a single dose of 3-6 mg carp pituitary extract/Kg of fish and the fertilized eggs were collected and incubated into a controlled incubation system until the larva-mouth opening period, which is approximately 4 days post-hatching (dph) for this species at these conditions. At this stage (start of experiment), larvae were considered undifferentiated, as previously described (Adolfi et al., 2015). The animals were then randomly distributed into groups (130 larvae/tank per group) that received different concentrations of steroid diet (0, 2, 4 or 6 mg E2/Kg food) for 28 days. Hormonal treatment lasted until 32 dph because this period precedes the gonadal sex differentiation as reported before for this species (Adolfi et al., 2015). In this species, ovaries were identified at 58 dph, while testis at 78 dph, which suggests that gonadal sex differentiation might occur before 58 dph (Adolfi et al., 2015).

After 28 days of treatment, juvenile fish were then fed *ad libitum* twice a day with commercial powdered food without any hormonal treatment. Fish were sampled at 90 dph (three replicas/group); biometric data (weight and length) were recorded and gonads dissected and collected for morphological and gene expression analysis (see below). The phenotypic sex was recorded based on the presence of secondary sexual characteristics (enlarged abdomen and smooth anal fin for females and flat abdomen and rough anal fin for males) and external gonadal parameters such as color, transparency, vascularity and presence of oocytes with a naked eye. Approximately 108, 111, 125 and 101 fish at 90

dph from the experimental groups 0, 2, 4 and 6 mg E2 per Kg food, respectively, were evaluated for the phenotypic sex.

During the whole experiment, water quality parameters were monitored daily. The 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), while ammonia (< 0.1 mg/L) and nitrite (0.11 mg/L) concentrations were analyzed weekly. In addition to the abiotic parameters, mortality rate among the groups was assessed during the whole experiment.

Steroid diet preparation

For the larval stages, steroid diet was prepared according to Stewart and collaborators (2001). In this method, decapsulated Artemia cysts were incubated in the hatching medium at proportion of 3 g of cysts per L of medium (Stewart et al., 2001). A stock solution of 500 mg of E2 (Cayman Chemical, 1180 E, MI 48108) in 75 ml of 70% ethyl alcohol was prepared. Subsequently, 300, 600 and 900 µl of stock solution was added to 1 L hatching medium to prepare 2, 4 and 6 mg E2 per L as final concentration, respectively. Artemia cysts were cultivated at 18–20°C for 24 h and nauplii were collected by siphoning, filtered and rinsed in 1 L tap water. Stewart and collaborators (2001) have confirmed that E2 is efficiently incorporated into Artemia prior to the first feeding instar II stage. In our experiment, larvae were fed *ad libitum* 4 times a day with the Artemia nauplii exposed to 0, 2, 4 or 6 mg E2 per L.

For juvenile stages, fish were fed with commercial powdered food containing 0, 2, 4, 6 mg E2 per Kg of food, as described by Popma and Green (1990). For this purpose, a hormone–alcohol mixture at 2, 4, 6 mg E2/L was sprayed onto the powdered food. The food was left in a dark room for 12 h to volatilize the solvent and afterwards the powdered food was stored at 4°C. Juvenile fish were fed *ad libitum* 4 times a day with commercial powdered food containing 2, 4, 6 mg E2 per Kg of food. The control diet was impregnated with 96% ethyl alcohol, processed the same way as the treated feed, and stored at 4°C until the day of use.

Histology

Gonads from the different experimental groups 0 (n = 12), 2 (n = 24), 4 (n = 29) and 6mg E2/Kg food (n = 44) were analyzed at 90 dph by histology. For this purpose, gonads were 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 3μm thickness. Some samples were also fixed in buffered 4% paraformaldehyde solution, embedded in Paraplast (Paraplast High Melt, Leica) and sectioned with 5μm thickness. The sections were stained with hematoxylin and eosin. The gonads were analyzed and classified as ovary, testis or testis-ova. The histological sections were examined and documented using Leica DMI6000 microscope from the Reproductive and Molecular Biology group (UNESP/Botucatu).

Gene expression analysis

Gene expression analysis were performed in adult male and female fish tissues (380 dph) to examine the tissue distribution expression for the selected genes. We also analyzed the expression of the selected genes in adult testes and ovaries (380 dph) to identify putative sex biased genes in this species. In the first approach, different tissues (pituitary, muscle, liver, gonads and intestine) were used from males (n = 3) and females (n = 3), while in the second one, testes (n = 6) and ovaries (n = 6), confirmed by histology, were used. All individuals were not subjected to any hormonal treatment.

We also evaluated the E2-induced changes on gene expression in the gonads of juvenile fish (90 dph) (n = 5 males, 5 females per group, both confirmed by histology prior the analysis) in the control (0 mg E2/Kg) and 6 mg E2/Kg food group. We selected this group because this concentration (6 mg E2/Kg) induced more phenotypic and histological females after the treatment (Fig. 1).

Total RNA (adults and juveniles) was extracted 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 5x iScript reaction mix for 5 min at 25°C. Reverse transcription (RT) was performed at 46°C for 20 min using iScript Reverse Transcriptase (Bio-Rad Laboratories, Hercules, CA, EUA), as described by the manufacturer.

qRT-PCRs reactions (10 μL) were performed in a StepOne Plus system (Life Technologies), 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 sec and 60°C for 1 min, and at the end, 95°C for 15 sec. Primers of *dmrt1, elf1a* and *sox9* (Table 1) are specific to *A. altiparanae* and were obtained from Adolfi et al., (2015). Primers for *amh, ar, cyp19a1a, foxl2, esr* and *hsd17β1* were designed based on highly conserved nucleotide regions of *A. mexicanus*. (Table 1). Sequences to design the primers for *rspo1* and *esr* were based on the transcript variant X1 of the available genome of cave fish, *A. mexicanus*, a related species of *A. altiparanae*. These sequences correspond to *rspo1* and *esr1* or *esr alpha* in other species. In addition, based on *A. mexicanus* genome, only one isoform was found for *ar*, *esr* and the rest of the evaluated genes. All primers for qRT-PCR amplification were designed with Primer Express version 3.0 software (Applied Biosystems). The levels of *elf1a* mRNA, chosen as endogenous control mRNA, remained stably expressed under the different experimental conditions. The validity of the qRT-PCR reactions were confirmed through the analysis of the melting curves (Supplementary Fig. 1A) and by checking the amplified fragments in an agarose gel. The relative mRNA levels of the target genes were determined using the $2^{-(\Delta\Delta CT)}$ method, in which the expression of the studied gene was normalized using *ef1a* as the reference gene and subsequently calibrated to its average expression value found in all tissues/conditions.

Statistical Analysis

Results were expressed as mean values ± SEM. Significant differences between two groups were identified using unpaired Student t-test ($p < 0.05$). Comparisons of more than two groups were performed with one-way ANOVA followed by Student-Newman-Keuls test ($p < 0.05$). Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, (http://www.graphpad.com) was used for all statistical analysis.

3.3 RESULTS

E2 disrupted the sex ratio and induced intersex

The E2 treatment disrupted the sex ratio by increasing the number of phenotypic females in a dose dependent manner (Fig. 1A). For the phenotypic identification, as mentioned before, animals were recorded as females if presented enlarged abdomen, smooth anal fin and gonads with higher vasculature and oocytes at naked eye. The higher percentage of phenotypic females (~80%) was observed in the 6 mg E2/Kg food (Fig. 1A). The histological analysis revealed that E2 treatment imbalanced the sex ratio, and surprisingly, induced intersex individuals with testis-ova in the groups of 2 and 6 mg E2/Kg food (Fig. 1B). We found 45,8% testes, 37,5% ovaries and 16,7% testes-ova in the treatment of 2 mg E2/Kg (Fig. 1B). For the intermediate concentration (4 mg/Kg), we observed 65,5% testes, 34,5% ovaries and no intersex among the individuals (Fig. 1B). While the highest concentration of E2 (6 mg/Kg) produced 52,3% ovaries, 40,9% testes and 6,8% testes-ova (Fig. 1B). There was no intersex condition in the control fish (0 mg E2/Kg) (Fig. 1B).

With respect to the gonadal histological characteristics, no differences were observed between control and treatment groups (Fig. 2). In females, the ovaries presented numerous lamellae that project into the ovarian lumen, and also oocytes at different stages of development, especially at primary and secondary growth phases (Fig. 2A,D,F). For males, the testes showed an anastomosing tubular structure and a germinal epithelium composed of cysts at different stages of germ cell development (Fig. 2B,E,G). The testicular lumen was fulfilled of spermatozoa and secretion (Fig. 2B,E,G). The intersex individuals presented a testis-ova gonad with oocytes intermixed with male germ cell cysts (Fig. 2C,H).

Interestingly, the hormonal treatments did not induce significant fish mortality, and no differences in length and weight were observed between control and the experimental groups (Table 2).

Tissue screening and sex-biased gene expression in adult gonads

Expression analysis for the selected genes were evaluated by qRT-PCR in adult tissues (pituitary, muscle, liver, gonads and intestine) from males and females (Fig. 3).

Among the evaluated genes, only *amh*, *dmrt1* and *cyp19a1a* were highly expressed in the testes when compared to other tissues (Fig. 3A). Most of the selected genes showed lower or not detectable expression levels in adult tissues from males and females (Fig. 3). For the females, none of the selected genes were highly expressed, except the *esr*, which was highly expressed in the liver (Fig. 3B).

Further analysis compared the expression of the selected genes in the adult testes and ovaries in order to identify sex-biased genes for this species (Fig. 4). This identification would help to understand the E2-induced changes on gene expression (see below). Therefore, the qRT-PCR revealed a dimorphic expression for *dmrt1, amh, sox9*, showing that these transcripts are highly expressed in the testes than in ovaries (Fig. 4). We did not find any dimorphic expression for *esr*, *rspo*, *cyp19a1a* and *foxl2*, considered as female genes (Fig. 4).

E2 induced changes on gonadal gene expression at 90 dph

Gene expression analysis were performed in testes and ovaries from control and 6 mg E2/Kg food group. Prior the analysis, testes and ovaries from control and 6 mg E2/Kg food were first confirmed by histology. We selected the highest concentration (6 mg/kg), because this dose induced more phenotypic and histological females. Intersex individuals were not subjected for gene expression analysis, due to their low number in the samples collected for gene expression analysis. Our results showed that E2 induced changes on the expression of the target genes when compared to the control (Fig. 5; Supplemetary Fig. 1B,C). The males that received E2 (6 mg/Kg) showed higher testicular expression of *dmrt1*, *amh* and *sox9* when compared to the non-treated males (Fig 5A,B,D). The 17β-HSD enzyme, which is involved in the conversion of dihydrotestosterone into androstenediol, androstenedione into testosterone and estrone into estradiol, showed lower mRNA levels in the E2 (6 mg/Kg) group (Fig. 5E). On the other hand, genes related to the female pathway program, such as *esr* and *foxl2* were up-regulated in the males from the E2 group (Fig. 5G,I).

When evaluating the females, steroid treatment also changed the expression of target genes when compared to the control (Fig. 5, Supplemetary Fig. 1C). Surprisingly, the testicular-biased-genes *dmrt1* and *sox9* were overexpressed in the ovaries of the E2-derived females than in the control (Fig. 5A,D). On the other hand, *amh*, which is also a

male-biased gene, did not change its expression levels in the steroid group when compared to the control (Fig. 5B). For the genes related to the female pathway, E2 diet significantly increased the *esr* and *rspo* mRNA levels when compared to the control (Fig. 5G,H), whereas the gene encoding the gonadal aromatase (*cyp19a1a)* was down-regulated in the hormone diet group (Fig. 5F).

3.4 DISCUSSION

Several studies have demonstrated that estrogens or anti-androgens can skew the sex ratio towards females in different species of fish (Pandian and Sheela 1995; Piferrer 2001; Devlin and Nagahama 2002; Vizziano et al., 2008, Budd et al., 2015, Díaz and Piferrer, 2017). The hormonal treatment with E2 has been reported as the most efficient method to induce feminization and produce sex reversed fish to obtain monosex populations in aquaculture (Hunter and Donaldson, 1983; Piferrer and Lim, 1997; Piferrer, 2001; Aktas and Genc, 2011). This is also applied for *A. altiparanae*, a neotropical model species with economical and ecological relevance (Bem et al., 2012). In the present study, we evaluated whether E2 could skew the sex ratio in *A. altiparanae*, and how this hormone could modify the regulatory cascades involved on the gonadal sex differentiation. Our results demonstrated that E2 increased the number of phenotypic females at 90 dph in a dose dependent manner. The concentration of 6 mg E2/Kg food was the most efficient to disrupt the sex ratio according to the phenotypic characteristics (~80% females out of 101 sampled fish in this group) and histological analysis (52,3% of ovaries out of 44 sampled fish). For the other concentrations, histology revealed 45,8% testes, 37,5% ovaries and, surprisingly, 16,7% testes-ova in the 2 mg E2/Kg group (n = 22). While for 4 mg E2/Kg group, we observed 65,5% testes, 34,5% ovaries and no intersex among the individuals (n = 29). While the highest concentration (6 mg E2/Kg) produced 52,3% ovaries, 40,9% testes and 6,8% testes-ova (n = 44). Based on these results, our data indicate that E2 can disrupt the phenotypic and histological sex ratios in *A. altiparanae*. Another interesting finding is the presence of intersex individuals with testes-ova at the lowest (16,7%) and highest (6,8%) concentration of E2. From our knowledge, this is the first study that reports the occurrence of intersex in lambari. Intersex is defined as the simultaneous presence of male and female gonadal tissue in a gonochoristic (fixed-sex) species and it is often viewed as an effect of exposure to estrogenic chemicals (Bahamonde et al. 2013). When the gonadal development is disrupted by estrogens, a complete male-to-female sex reversal is reported (Warr and Greenfield, 2012), but sometimes it can lead to an incomplete differentiation of one tissue in another, producing intersex animals (Adolfi et al., 2019). Therefore, our results indicate that treatment used was not enough to induce a complete male-to-female sex reversal in *A. altiparanae*, producing intersex individuals with testes-ova at 90 dph. In another study with the same species, Bem and collaborators (2012) used at least 10 times higher

concentration of estradiol valerate to produce 70-76% male-to-female sex reversal without intersex at 161 dph. This leads us to conclude that the concentration used in our work could be considered lower to induce a complete male-to-female sex reversal. Despite the species-specific sensitivity, other studies also reported intersex after using a considerably lower concentration of E2. 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*), an exposure to 10 ng/L E2 for approximately 100 days after hatching caused 10% intersex but no change in the sex ratio (Bjerregaard et al., 2008). Interestingly, in our study, the percentage of intersex decreased from 16,7% to 6,8%, using the lowest and highest E2 dose, respectively. This is another evidence that if used in higher concentrations, E2 could be more effective to induce a complete sex reversal in *A. altiparanae.* Despite of 29 animals from the intermediate concentration (4 mg/Kg) had their gonads observed at histology, we did not find any intersex animal in this group. This is intriguing and deserves more attention in future experiments.

In this study, we also examined the E2-induced changes on gene expression to understand how E2 could affect the regulatory cascade involved on gonadal sex differentiation of *A. altiparanae*. For this purpose, we first evaluated the existence of sex-biased genes by analyzing the expression of some selected candidate genes in adult gonads (380 dph). Our data revealed a testis-biased expression for *dmrt1* ($p < 0.01$) and *sox9* ($p < 0.05$), which is in accordance with previous studies for this species (Adolfi et al., 2015). In several fish species, both genes, *dmrt1* and *sox9*, are expressed during the early stages of development, playing an essential role in testis differentiation (Marchand et al., 2000; Kluver et al., 2005; Masuyama et al., 2012; Herpin and Schartl, 2015; Adolfi et al., 2015; Cui et al., 2017; Adolfi et al., 2019a). We do not know if *A. altiparanae* presents different isoforms of *dmrt1* and *sox9* (Adolfi et al., 2015). Moreover, the *sox9* sequence used here shows higher identity with other teleost *sox9b* genes (Adolfi et al., 2015), which is the copy exclusively expressed in the testes (Kluver et al., 2005; Adolfi et al., 2015).

Our expression analysis also revealed a dimorphic expression for *amh* ($p < 0.0001$). These transcripts are highly expressed in the testes than in the ovaries of *A. altiparanae*. In fish, it has been reported that Amh, anti-Müllerian hormone, a member of transforming growth factor β, is expressed in the undifferentiated gonads of both sexes at relatively low and equal levels (Kluver et al., 2007; Pfennig et al., 2015; Adolfi et al.,

2019b). During testis differentiation, *amh* expression increases and remains higher during and after puberty in adult males (Pfennig et al., 2015; Adolfi et al., 2019b). Accumulating evidences that Amh plays a role in testis differentiation come from medaka studies, where *amhr2* 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). Based on these observations, our expression analysis suggested that *amh* could be considered a testis-biased gene ($p < 0.0001$) with potential roles on testicular differentiation of *A. altiparanae*. When analyzing possible candidates involved in the female pathway program, none of the selected genes (*esr*, *rspo, cyp19a1a* and *foxl2*) showed a female-biased expression pattern. This observation was also reported for other fish species, such as zebrafish and medaka where *esr* and *rspo* are not female-biased genes (Herpin et al., 2013).

When analyzing the E2-induced changes on gene expression, we found that E2-derived males overexpressed the testicular-biased genes *dmrt1*, *sox9* and *amh*. On the other hand, 17β-HSD, an enzyme involved in the production of steroids (androstenediol, testosterone and estradiol), showed lower testicular mRNA levels in the E2 group. This data suggests that steroids levels (androstenediol, testosterone and estradiol) could be reduced in these males when compared to the control. Although *esr* and *foxl2* are not female-biased genes, their mRNA levels were significantly increased in the testes of treatment group. Foxl2 is considered an important gene for the ovarian differentiation in mammals and fish (see review in Herpin and Schartl, 2015). Moreover, it has been shown in trout (*O. mykiss*) that *foxl2* expression is dependent of estrogens and is temporally co-expressed with *cyp19a1a* during the initial steps of ovarian differentiation (Vizzano et al. 2008). Therefore, we believe that the higher expression of *esr* and *foxl2* in *A. altiparanae* males could be interpreted as E2-related effects. Altogether, the testicular expression profile found in the E2-derived *A. altiparanae* males could be associated to a mechanism of resilience to the E2 treatment. We believe that these males, considered as "E2 resistance males" (Fig. 6), were able to resist the E2-induced feminization by enhancing the expression of genes related to testis differentiation, such as *dmrt1*, *sox9* and *amh.* The higher expression of these genes might be responsible for maintaining the testicular morphology under E2 treatment (Fig. 6). Despite the androgen pathway seemed to be affected, as shown by *17β-hsd* and E2-related genes (*esr* and *foxl2)*, this genetic profile was not enough to induce a complete male-to-female sex reversal (Fig. 6). Interestingly,

the same phenomenon is reported for zebrafish (Ribas et al., 2017) and European sea bass (Navarro-Martín et al., 2009) but for the opposite sex. In these species, heat-exposed females remained as phenotypic females because they were unable to be masculinized by high temperature (see below) (Navarro-Martín et al., 2009; Ribas et al., 2017).

Strikingly, we also found females derived from the E2 treatment that showed normal ovarian morphology but with altered gene expression, exhibiting higher expression of testicular-biased genes, such as *dmrt1* and *sox9* (Fig. 6). Moreover, these E2-derived females also presented lower mRNA levels for the gonadal aromatase (*cyp19a1a*), which is important for estrogen production in females (Fig. 6). In tilapia, zebrafish and rainbow trout, it has been shown that *dmrt1* down-regulates *cyp19a1a* transcription, resulting in a decreased aromatase activity and consequently lower E2 levels (Guiguen et al., 2009; Wang et al., 2010; Paradhan and Olsson 2016; Li et al., 2018). Likewise, the lower expression of *cyp19a1a* in the E2-derived females in our experiment could be attributed to the higher expression of *dmrt1*. Moreover, due to the decrease of *cyp19a1a* mRNA levels, it is probably that E2 levels are reduced in these females. Although not female-biased, *esr* and *rspo* were up-regulated in the *A. altiparanae* females treated with E2 (Fig. 6). With respect to r-spondin (*rspo*), this gene is considered a potential female-determining gene in vertebrates which regulates the Wnt4/β-catenin signaling pathway (see review in Herpin and Schartl, 2015). Altogether, our results showed that despite of the normal ovarian morphology, the E2-derived females showed a "male-like" gonadal expression (Fig. 6). This is quite interesting and demonstrates the complexity of the gonadal plasticity in teleost fish. As far as we know, this is the second evidence that reports such complexity in fish. The first evidence comes from studies in zebrafish, where Ribas and collaborators (2017) found females or so-called "superfemales" that were able to resist the heat-induced masculinization, maintaining their ovarian morphology despite changing their gonadal transcriptome to a testis-like one. These females displayed a gene expression analysis where the female related genes, such as *cyp19a1a* and *vtg5* were down-regulated, while genes related to the testicular differentiation, such as *dmrt1* and *amh* were up-regulated (Ribas et al., 2017).

In our study, we speculate whether *A. altiparanae* females with "male-like" expression could be derived from the E2 resistant males which somehow lost their resistance to E2 and undergo a complete gonadal transformation (testis to ovary) but not accompanied at the transcriptomic level (Fig. 6). The presence of intersex individuals

could support this hypothesis and would indicate a transitional stage of the gonadal transformation (Fig. 6). In this scenario, an interesting question for future studies concerns to determine which genes are involved in the E2 resilience and which genes would be involved in the transcriptional and morphological gonadal male-to-female sex reversal.

With this respect, when comparing the expression profile of the E2 resistant males with the E2-derived females, a similar pattern was found for the male-biased genes (*dmrt1* and *sox9*), except for *amh*. While *dmrt1* and *sox9* mRNA increased in both males and females treated with E2, *amh* was down-regulated only in the E2-derived females. Considering the role of Amh on testicular sex differentiation (Adolfi et al., 2019; Morinaga et al., 2007; Nakamura et al., 2012), we speculate that the down-regulation of *amh* could be associated to the gonadal transformation into ovary. Schulz and collaborators (2007) demonstrated that 17a-ethinylestradiol (EE2) exposure in early life of zebrafish caused a down-regulation of *amh* and consequently a disruption in the male gonadal development. Further studies will be necessary to address the role of Amh in *A. altiparanae* sex differentiation and to evaluate how Amh interacts with other effectors (Dmrt1 and Sox9) in the male regulatory cascade pathway of this species.

Finally, we also wonder if all E2-derived females presented a "male-like" gene expression profile. Thus, based on R-values for *dmrt1* and *sox9* (Supplementary Fig. 1C), we could observed that 1 out of 5 individuals presented lower *dmrt1* and *sox9* mRNA levels (Supplementary Fig. 1C). This indicates that some individuals among the population remain with the female expression profile independently of the E2 treatment (Fig. 6). These results are still a puzzle especially because there are no evidences of sex determining gene and the mechanisms involved in the gonadal sex differentiation of this species are unknown.

In conclusion, our results provide information on gonadal sex differentiation of a Neotropical species model, *A. altiparanae*. First, we showed that E2 concentration used was not enough to induce a complete male-to-female sex reversal, producing intersex individuals with testes-ova. We also discovered that E2 treatment induced a complex gonadal plasticity, in terms of genetic expression (Fig. 6): 1 - the E2 resistant males that overexpressed the testicular sex-biased genes *dmrt1*, *sox9* and *amh*; 2 - the E2-derived females with normal ovarian morphology but with a "male-like" gene expression; and 3- the E2-derived females with lower *dmrt1* and *sox9*. This model opens new possibilities

to study the mechanisms of gonadal sex differentiation and plasticity in a Neotropical fish model, as well as the consequences of exogenous estrogens in the natural environment at individual, populational and transgenerational levels.

3.5 FIGURES

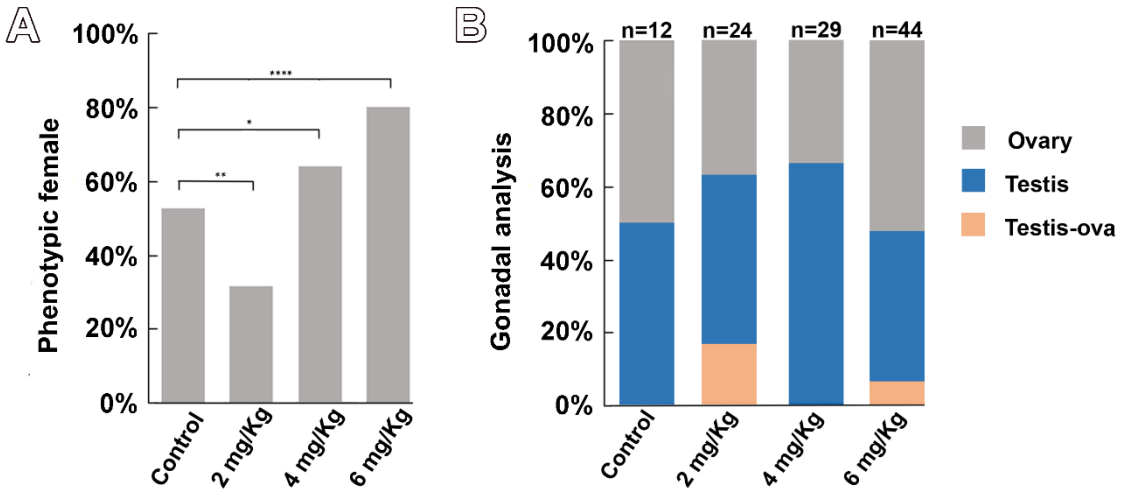


Figure 1. A. Percentage of phenotypic females derived from the steroid diet treatments. Animals received a steroid diet containing 2, 4 or 6 mg E2 per Kg food during 28 days, from the mouth opening (4 days post-hatching) until the period that precedes the gonadal sex differentiation. The asterisks denote significant differences between control and treatment groups using Chi-square (control vs 2mg/Kg: ** p = 0.0014; control vs 4 mg/Kg: * p = 0.0100; control vs 6 mg/Kg: **** p < 0.0001). The phenotypic sex was identified according to the presence of secondary sexual characteristics: enlarged abdomen and smooth anal fin for females; flat abdomen and rough anal fin for males, and also according to external gonadal parameters such as color, transparency, vascularity and presence of oocytes with a naked eye. Approximately 108, 111, 125 and 101 fish from the experimental groups 0, 2, 4 and 6 mg E2 per Kg food, respectively, were evaluated for the phenotypic sex. **B.** Percentage according to the gonadal histology: control (0 mg/Kg food) exhibited 50% ovaries, 50% testes (50%) and no intersex; 2 mg E2/Kg food showed 45,8% testes, 37,5% ovaries and 16,7% testes-ova; 4 mg E2/Kg food presented 65,5% testes, 34,5% ovaries and no intersex; 6 mg E2/Kg food produced 52,3% ovaries, 40,9% testes and 6,8% testes-ova. Sex ratio (males/females) were: 1,00 (control), 1,22 (2 mg/Kg), 1,9 (4 mg/Kg) and 0,78 (6 mg/Kg). The sample size for the histological analysis was: 12 (control), 24 (2 mg/Kg), 29 (4 mg/Kg) and 44 (6 mg/Kg).

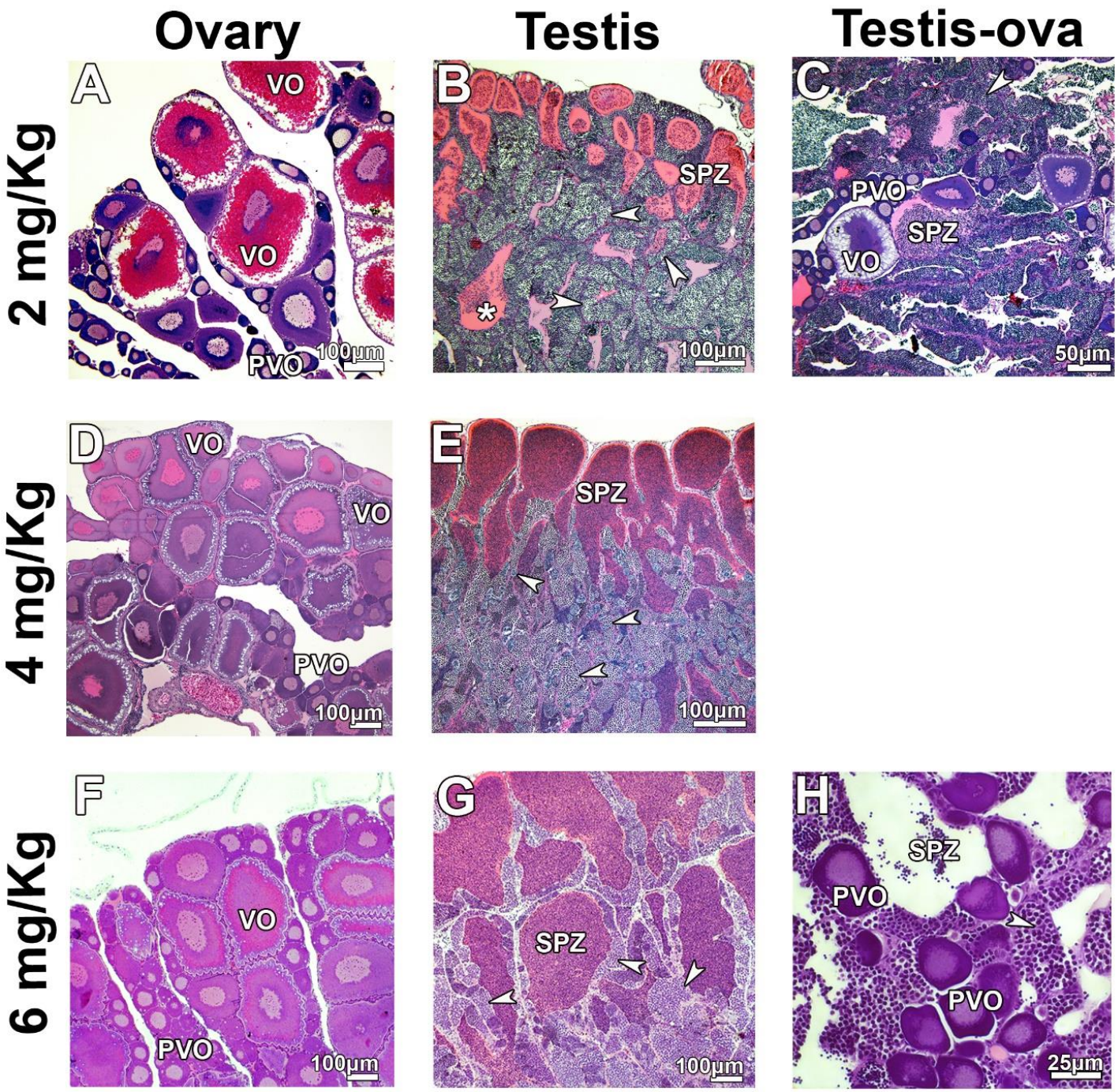


Figure 2. Gonadal histological analysis from the 2, 4 and 6 mg E2 per Kg food . The gonadal morphology among the groups were quite similar, no structural differences were observed. The females (A,D,F) showed similar morphology; numerous lamellae with oocytes at different stages of development, especially at primary and secondary growth phases, including previtellogenic oocytes (perinucleolar) (**PVO**) and vitellogenic oocytes (**VO**). The males (B,E,G) exhibited testes with anastomosing tubular structure (E) 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 (C,H) presented a testis-ova gonad with oocytes (PVO and VO) intermixed with male germ cell cysts (arrowheads). Staining: hematoxylin and eosin (H&E).

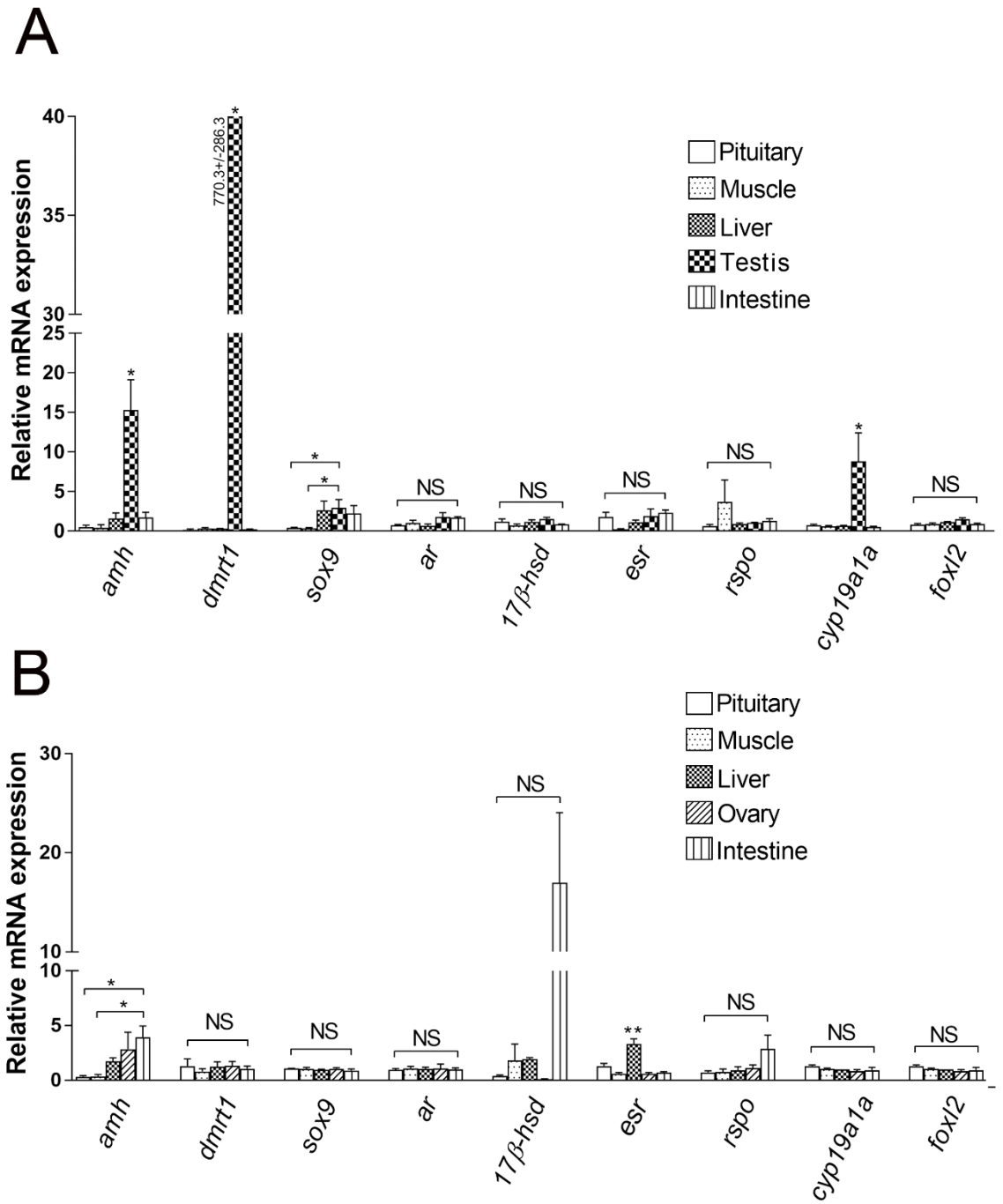


Figure 3. A,B. Relative expression of *amh* [Anti-Mullerian Hormone], *dmrt1* [doublesex and mab-3-related transcription factor 1], *sox9* [SRY-box transcription factor 9b], *ar* [androgen receptor], *17β-hsd* [17-bethydroxysteroid dehydrogenase], *esr* [estrogen

receptor], *rspo* [R-spondin 1], *cyp19a1a* [aromatase] and *foxl2a* [forkhead box L2 a] in adult tissues (pituitary, muscle, liver, gonads, intestine). **A.** Male (n = 3). **B.** Female (n = 3). Animals were sampled at 380 days post-hatching. The expression level of each gene was first normalized to the expression of *elf1a* and then expressed as relative values to its average expression in all organs. Each bar represents the mean ± SEM. Asterisks denote significant differences among the samples: * ($p < 0.05$), ** ($p < 0.01$) and NS (not significant).

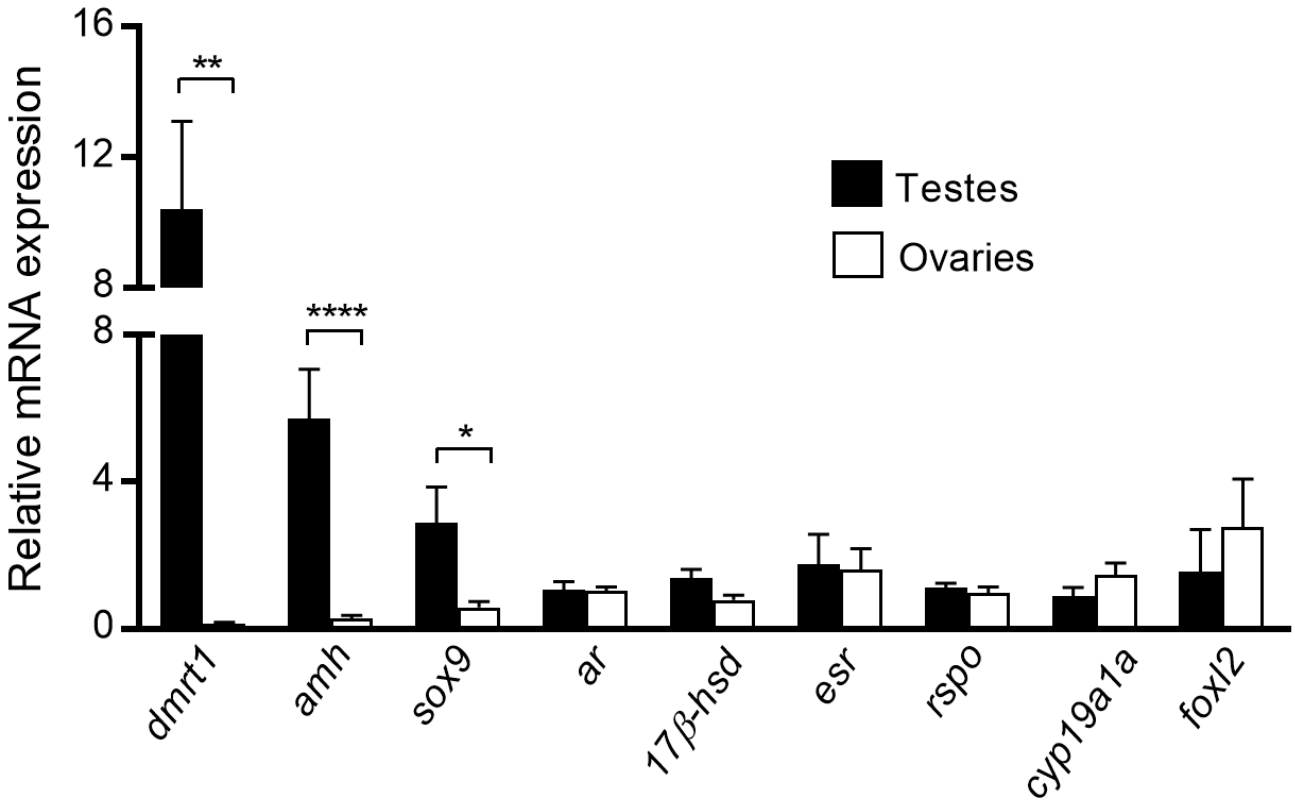


Figure 4. The relative expression of *dmrt1* [doublesex and mab-3-related transcription factor 1], *amh* [Anti-Müllerian Hormone], *sox9* [SRY-box transcription factor 9], *ar* [androgen receptor], *17β-hsd* [17-beta hydroxysteroid dehydrogenase], *esr* [estrogen receptor], *rspo* [R-spondin 1], *cyp19a1a* [aromatase] and *foxl2a* [forkhead box L2 a] in adult gonads of *A. altiparanae*; testes (n = 6) and ovaries (n = 6). Animals were sampled at 380 days post-hatching. The expression level of each gene was normalized to the expression of *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: * ($p < 0.05$); ** ($p < 0.01$); **** ($p < 0.0001$).

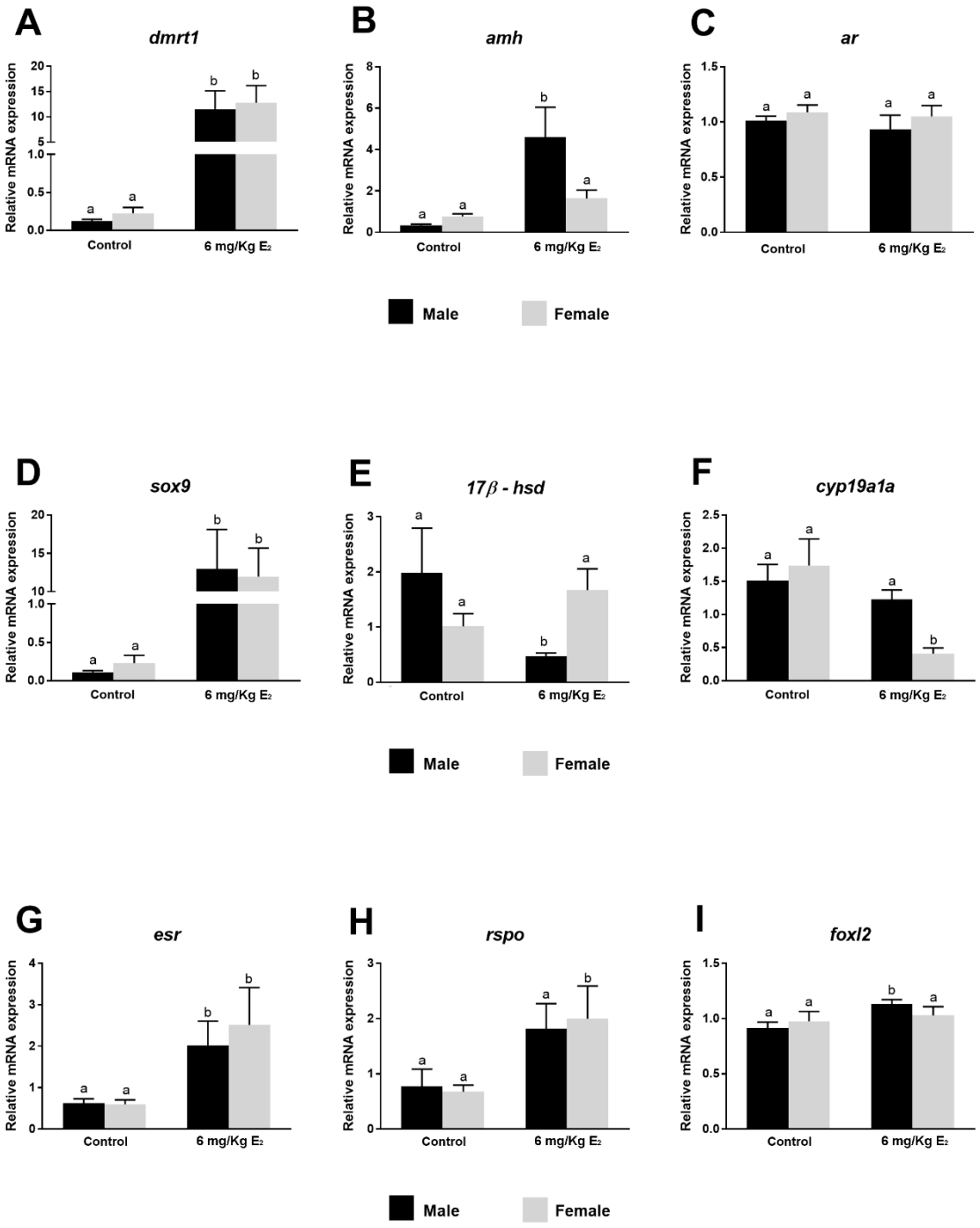


Figure 5. The relative expression of the selected genes in testes and ovaries from control and 6 mg E2/Kg food at 90 days post-hatching. Control males (n = 5); control females (n =5), 6 mg E2/Kg males (n = 5) and 6 mg E2/Kg females (n = 5). **A:** *dmrt1* [doublesex and mab-3-related transcription factor 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 the expression of *elf1a* and expressed as relative value to its average expression in both gonads. Each bar represents the mean ± SEM. Different letters denote significant differences ($p < 0.05$) between control and treatment group for the same gonad.

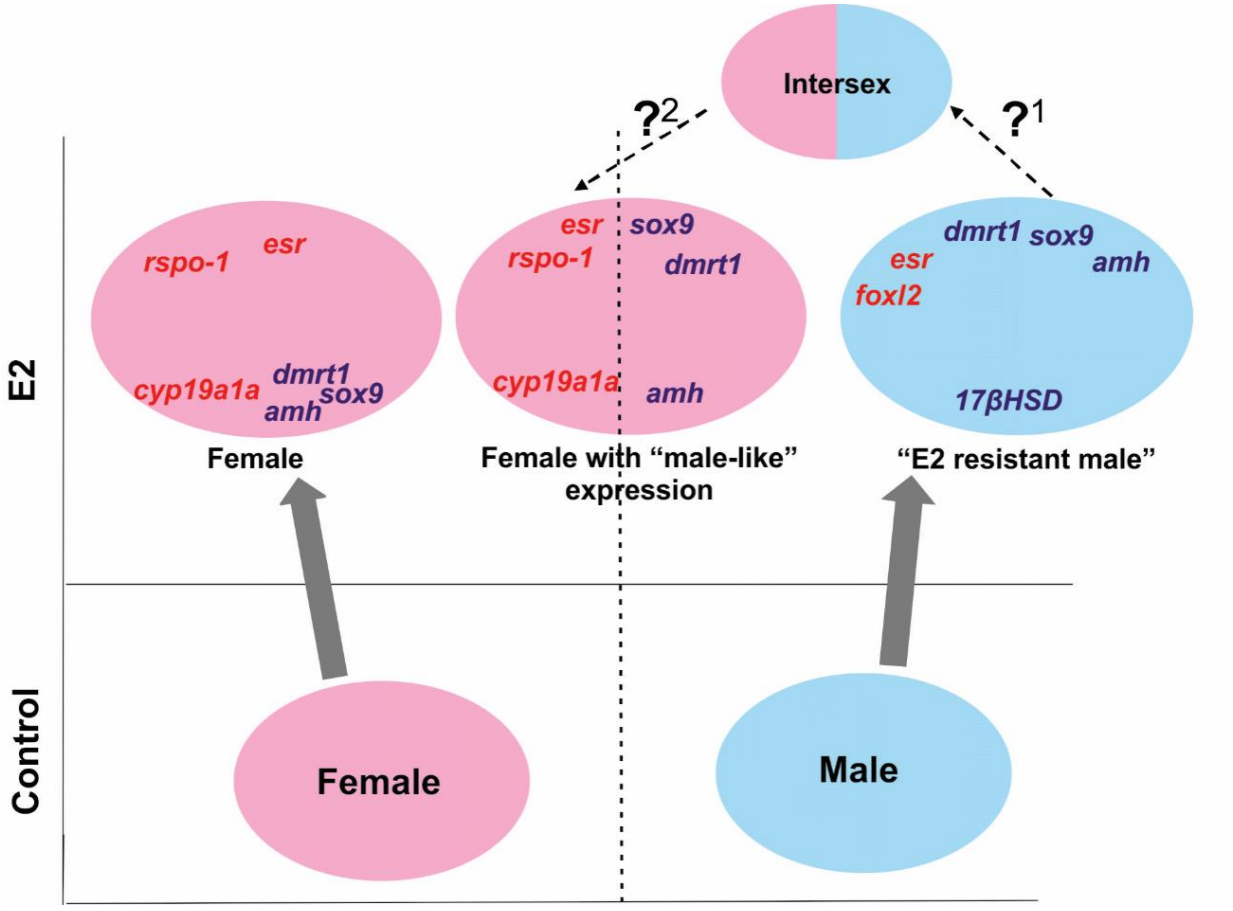


Figure 6. Gonadal plasticity in *Astyanax altiparanae*. The existing dichotomy between male and female originates a complex gonadal plasticity at 90 days post-hatching when larvae (4 days post-hatching) with undifferentiated gonads were treated with 6 mg E2/Kg food during 28 days. Four different scenarios were derived from the E2 treatment. The "E2 resistant males" which comprises males that were resilient to the E2 treatment. These males overexpressed genes related to the testicular differentiation, such as *dmrt1*, *sox9* and *amh*. On the other hand, *17β-hsd* is downregulated, while genes considered E2-related, such as *esr* and *foxl2* were up-regulated. We believe that these males become

resistant to the E2-induced feminization due to the overexpression of male-biased genes. The first question mark (?[1]) speculates whether the intersex individuals (second scenario) were originated from the E2 resistant males that lost their resistance to E2 and start to undergo sex reversal (male-to-female). Intersex individuals could indicate an incomplete gonadal sex reversal or a transitional stage to originate sex reversed females (?[2]). The third scenario comprises the females that exhibited a normal ovarian morphology but showed a "male-like" gene expression. *dmrt1*, *sox9*, *esr* and *rspo-1* were up-regulated, while *cyp19a1a* and *amh* were down-regulated in these females. The last scenario refers to the animals that developed as females independently of the E2 treatment. Within the circles, genes on the upper part are up-regulated, while the ones on the lower, are down-regulated.

3.6 TABLES

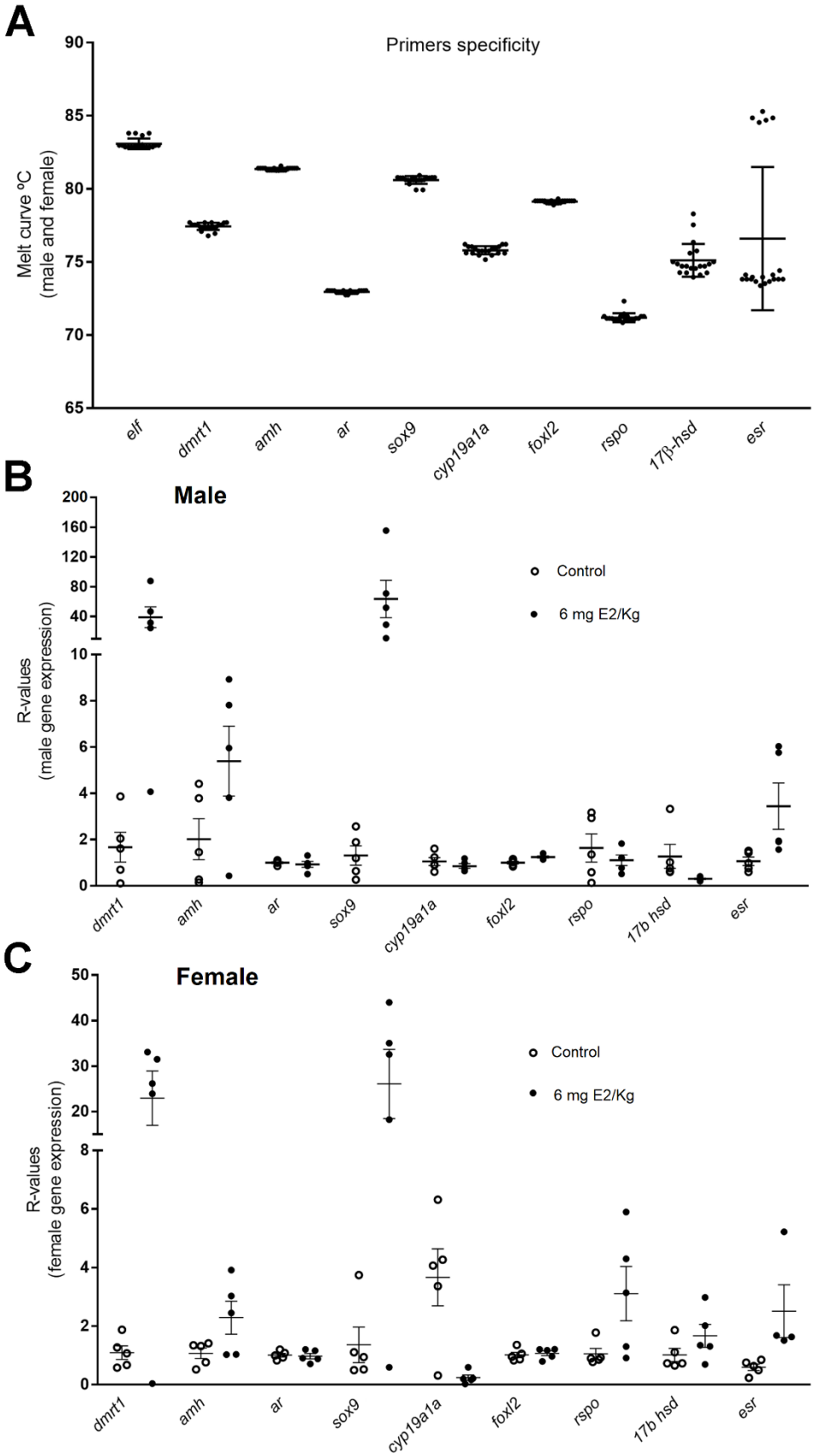
Table 01: Specifications of the primers used in qRT-PCR reactions

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

Table 02: Total length and weight of *A. altiparanae* sampled at 90 days post-hatching. T1 = control; T2 = 2 mg/L estradiol; T3 = 4 mg/L estradiol and T4 = 6 mg/L estradiol.

Treatment	Total length (cm)	Weight (g)
Control	6.95 ± 0.79	5.29 ± 1.90
2 mg E2/Kg	7.02 ± 0.84	5.29 ± 2.10
4 mg E2/Kg	6.86 ± 0.85	4.96 ± 1.81
6 mg E2/Kg	7.08 ± 0.84	5.50 ± 1.90

3.7 SUPPLEMENTAL FIGURE



Supplementary Figure 1. A. Primer specificity. The primer specificity was evaluated according to the melt curve temperature (dissociation temperature). The dissociation temperature in all qPCR reactions are shown for each primer. All primers showed high specificity. Although some variations were found for *17β-hsd* (2 samples) and *esr* (5 samples), most of the samples showed the same melt curve temperature. Each dot represents the melt curve temperature for one sample. **B,C.** R values for each evaluated gene from control and 6 mg E2/Kg group. **B.** Male. **C.** Female. Each dot represents R value for one sample per condition. For females, note that one sample showed a lower R value for *dmrt1* and *sox9*, while the rest exhibited higher R values for the same genes.

3.8 ACKNOWLEDGEMENTS

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3.9 REFERENCES

Adolfi, Mateus 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*. Reproductive Biology and Endocrinology, 13(1), 2. https://doi.org/10.1186/1477-7827-13-2

Adolfi, Mateus Contar, Fischer, P., Herpin, A., Regensburger, M., Kikuchi, M., Tanaka, M., & Schartl, M. (2019). Increase of cortisol levels after temperature stress activates *dmrt1a* causing female-to-male sex reversal and reduced germ cell number in medaka. Molecular Reproduction and Development, mrd.23177. https://doi.org/10.1002/mrd.23177

Adolfi, Mateus Contar, Nakajima, R. T., Nóbrega, R. H., & Schartl, M. (2019). Intersex, Hermaphroditism, and Gonadal Plasticity in Vertebrates: Evolution of the Müllerian Duct and Amh/Amhr2 Signaling. Annual Review of Animal Biosciences, 7(1), 149–172. https://doi.org/10.1146/annurev-animal-020518-114955

Aktas M, & Genc MA. (2011). The Effects of 17 β-Estradiol on Growth, Survival and Feminization of Green Tiger Shrimp, *P. semisulcatus* (Decapoda: Penaeidae). Journal of Animal and Veterinary Advances, 10(5), 562–565.

Bahamonde, P. A., Munkittrick, K. R., & Martyniuk, C. J. (2013). Intersex in teleost fish: Are we distinguishing endocrine disruption from natural phenomena? General and Comparative Endocrinology, 192, 25–35. https://doi.org/10.1016/j.ygcen.2013.04.005

Baron, D., Batista, F., Chaffaux, S., Cocquet, J., Cotinot, C., Cribiu, E., Fellous, M. (2005). Foxl2 gene and the development of the ovary: A story about goat, mouse, fish and woman. Reproduction Nutrition Development, 45(3), 377–382. https://doi.org/10.1051/rnd:2005028

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

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

Biscotti, M. A., Adolfi, M. C., Barucca, M., Forconi, M., Pallavicini, A., Gerdol, M., Schartl, M. (2018). A Comparative View on Sex Differentiation and Gametogenesis Genes in Lungfish and Coelacanths. Genome Biology and Evolution, 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. General and Comparative Endocrinology, 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. Nature Reviews Genetics, 5(7), 509–521. https://doi.org/10.1038/nrg1381

Budd, A., Banh, Q., Domingos, J., & Jerry, D. (2015). Sex Control in Fish: Approaches, Challenges and Opportunities for Aquaculture. Journal of Marine Science and Engineering, 3(2), 329–355. https://doi.org/10.3390/jmse3020329

Cassel, M., Chehade, C., Souza Branco, G., Caneppele, D., Romagosa, E., & Borella, M. I. (2017). Ovarian development and the reproductive profile of *Astyanax altiparanae* (Teleostei, Characidae) over one year: Applications in fish farming. Theriogenology, 98, 1–15. https://doi.org/10.1016/j.theriogenology.2017.04.044

Chehade, C., Cassel, M., & Borella, M. I. (2015). Induced reproduction in a migratory teleost species by water level drawdown. Neotropical Ichthyology, 13(1), 205–212. https://doi.org/10.1590/1982-0224-20140028

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

Cui, Z., Liu, Y., Wang, W., Wang, Q., Zhang, N., Lin, F., Chen, S. (2017). Genome editing reveals *dmrt1* as an essential male sex-determining gene in Chinese tongue sole (*Cynoglossus semilaevis*). Scientific Reports, 7(1), 42213. https://doi.org/10.1038/srep42213

de Jesus, L. W. O., Bogerd, J., Vieceli, F. M., Branco, G. S., Camargo, M. P., Cassel, M., Borella, M. I. (2017). Gonadotropin subunits of the characiform *Astyanax altiparanae*: Molecular characterization, spatiotemporal expression and their possible role on female reproductive dysfunction in captivity. General and Comparative Endocrinology, 246, 150–163. https://doi.org/10.1016/j.ygcen.2016.12.004

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

de Siqueira-Silva, D. H., dos Santos Silva, A. P., Ninhaus-Silveira, A., & Veríssimo-Silveira, R. (2015). The effects of temperature and busulfan (Myleran) on the yellowtail tetra *Astyanax altiparanae* (Pisces, Characiformes) spermatogenesis. Theriogenology, 84(6), 1033–1042. https://doi.org/10.1016/j.theriogenology.2015.06.004

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

dos Santos, M. P., Yasui, G. S., Xavier, P. L. P., de Macedo Adamov, N. S., do Nascimento, N. F., Fujimoto, T., Nakaghi, L. S. O. (2016). Morphology of gametes, post-fertilization events and the effect of temperature on the embryonic development of *Astyanax altiparanae* (Teleostei, Characidae). Zygote, 24(6), 795–807. https://doi.org/10.1017/S0967199416000101

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*. Sexual Development, 2(6), 316–324. https://doi.org/10.1159/000195681

Fernandino, Juan Ignacio, 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. Developmental Dynamics, 237(11), 3192–3199. https://doi.org/10.1002/dvdy.21731

Fernandino JI and Hattori RS. (2018). Sex determination in Neotropical fish: Implications ranging from aquaculture technology to ecological assessment. Gen Comp Endocrinol. 2019 Mar 1;273:172-183. doi: 10.1016/j.ygcen.2018.07.002.

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.

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., Fostier, A., Piferrer, F., & Chang, C.-F. (2010). Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. General and Comparative Endocrinology, 165(3), 352–366. https://doi.org/10.1016/j.ygcen.2009.03.002

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

Herpin, Amaury, Adolfi, M. C., Nicol, B., Hinzmann, M., Schmidt, C., Klughammer, J., Schartl, M. (2013). Divergent Expression Regulation of Gonad Development Genes in Medaka Shows Incomplete Conservation of the Downstream Regulatory Network of Vertebrate Sex Determination. Molecular Biology and Evolution, 30(10), 2328–2346. https://doi.org/10.1093/molbev/mst130

Hinfray, N., Nóbrega, R. H., Caulier, M., Baudiffier, D., Maillot-Maréchal, E., Chadili, E., Brion, F. (2013). Cyp17a1 and Cyp19a1a in the zebrafish testis are differentially affected by αestradiol. Journal of Endocrinology, 216(3), 375–388. https://doi.org/10.1530/JOE-12-0509

Hunter, G. A., & Donaldson, E. M. (1983). 5 Hormonal Sex Control and its Application to Fish Culture. In Fish Physiology (Vol. 9, pp. 223–303). https://doi.org/10.1016/S1546-5098(08)60305-2

Klüver, N., Kondo, M., Herpin, A., Mitani, H., & Schartl, M. (2005). Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. Development Genes and Evolution, 215(6), 297–305. https://doi.org/10.1007/s00427-005-0477-x

Klüver, N., Pfennig, F., Pala, I., Storch, K., Schlieder, M., Froschauer, A., Schartl, M. (2007). Differential expression of anti-Müllerian hormone (*amh*) and anti-Müllerian hormone receptor type II (*amhrII*) in the teleost medaka. Developmental Dynamics, 236(1), 271–281. https://doi.org/10.1002/dvdy.20997

Levy-Pereira N, Yasui GS, Evangelista MM, Nascimento NF, Santos MP, Siqueira-Silva DH, Monzani PS, Senhorini JA, Pilarski F. (2019). In vivo phagocytosis and hematology in *Astyanax altiparanae*, a potential model for surrogate technology. Braz J Biol. 2019 Oct 7. pii: S1519-69842019005013101. doi: 10.1590/1519-6984.205893.

Li, M., Sun, L., & Wang, D. (2019). Roles of estrogens in fish sexual plasticity and sex differentiation. General and Comparative Endocrinology, 277, 9–16. https://doi.org/10.1016/j.ygcen.2018.11.015

Marchand, O., Govoroun, M., D'Cotta, H., McMeel, O., Lareyre, J.-J., Bernot, A., Guiguen, Y. (2000). DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 1493(1–2), 180–187. https://doi.org/10.1016/S0167-4781(00)00186-X

Masuyama, H., Yamada, M., Kamei, Y., Fujiwara-Ishikawa, T., Todo, T., Nagahama, Y., & Matsuda, M. (2012). *Dmrt1* mutation causes a male-to-female sex reversal after the sex determination by *Dmy* in the medaka. Chromosome Research, 20(1), 163–176. https://doi.org/10.1007/s10577-011-9264-x

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. Proceedings of the National Academy of Sciences, 104(23), 9691–9696. https://doi.org/10.1073/pnas.0611379104

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

Nakatani, K, Agostinho, A.A, Baumgartner, G, Bialetzki, A, Sanches, P.V, Makrakis, M.C, & Pavanelli, C.S. (2001). Ovos e larvas de peixes de água doce:

Desenvolvimento e manual de identificação. Maringá, PR: Eletrobrás; Uem, 2001. Il., 378p.

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

Pfennig, F., Standke, A., & Gutzeit, H. O. (2015). The role of *Amh* signaling in teleost fish – Multiple functions not restricted to the gonads. General and Comparative Endocrinology, 223, 87–107. https://doi.org/10.1016/j.ygcen.2015.09.025

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, & Lim L.C. (1997). Application of sex reversal technology in ornamental fish culture. 1(2), 113–118.

Popma, T.J, & Green, B.W. (1990). Reversão sexual de tilápias em tanques de terra. Manual de Produção Em Aquacultura, Florida(EUA: Universitu Aurburn.), 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), 101-116.

Porto-Foresti, F, Oliveira, C, & Foresti, F. (2001). Cultivo do Lambari: Uma espécie de pequeno porte e grandes possibilidades. Panorama Da Aquicultura, 11, 15–19.

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. Proceedings of the National Academy of Sciences, 114(6), E941–E950. https://doi.org/10.1073/pnas.1609411114

Sato, Y., Sampaio, E. V., Fenerich-Verani, N., & Verani, J. R. (2006). Biologia reprodutiva e reprodução induzida de duas espécies de Characidae (Osteichthyes, Characiformes) da bacia do São Francisco, Minas Gerais, Brasil. Revista Brasileira de Zoologia, 23(1), 267–273. https://doi.org/10.1590/S0101-81752006000100021

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. Environmental Science & Technology, 41(17), 6305–6310. https://doi.org/10.1021/es070785+

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Bulun, S. E. (1994). Aromatase Cytochrome P450, The Enzyme Responsible for Estrogen Biosynthesis*. Endocrine Reviews, 15(3), 342–355. https://doi.org/10.1210/edrv-15-3-342

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

Viñas, J., & Piferrer, F. (2008). Stage-Specific Gene Expression During Fish Spermatogenesis as Determined by Laser-Capture Microdissection and Quantitative-PCR in Sea Bass (Dicentrarchus labrax) Gonads1. Biology of Reproduction, 79(4), 738–747. https://doi.org/10.1095/biolreprod.108.069708

Vizziano, D., Baron, D., Randuineau, G., Mahè, S., Cauty, C., & Guiguen, Y. (2008). Rainbow Trout Gonadal Masculinization Induced by Inhibition of Estrogen Synthesis Is More Physiological Than Masculinization Induced by Androgen Supplementation1. Biology of Reproduction, 78(5), 939–946. https://doi.org/10.1095/biolreprod.107.065961

Vizziano, D., Randuineau, G., Baron, D., Cauty, C., & Guiguen, Y. (2007). Characterization of early molecular sex differentiation in rainbow trout, *Oncorhynchus mykiss*. Developmental Dynamics, 236(8), 2198–2206. https://doi.org/10.1002/dvdy.21212

Warr, N., & Greenfield, A. (2012). The molecular and cellular basis of gonadal sex reversal in mice and humans: Molecular and cellular basis of gonadal sex reversal. Wiley Interdisciplinary Reviews: Developmental Biology, 1(4), 559–577. https://doi.org/10.1002/wdev.42

Yan, Y.-L., Batzel, P., Titus, T., Sydes, J., Desvignes, T., Bremiller, R., Postlethwait, J. H. (2019). The roles of Amh in zebrafish gonad development and sex determination. BioRxiv, 650218. https://doi.org/10.1101/650218

Yao, H. H.-C. (2005). The pathway to femaleness: Current knowledge on embryonic development of the ovary. Molecular and Cellular Endocrinology, 230(1–2), 87–93. https://doi.org/10.1016/j.mce.2004.11.003

Yasui, G. S., Senhorini, J. A., Shimoda, E., Pereira-Santos, M., Nakaghi, L. S. O., Fujimoto, T., Silva, L. A. (2015). Improvement of gamete quality and its short-term storage: An approach for biotechnology in laboratory fish. Animal, 9(3), 464–470. https://doi.org/10.1017/S1751731114002511

Zhou, L., Charkraborty, T., Yu, X., Wu, L., Liu, G., Mohapatra, S., Nagahama, Y. (2012). R-spondins are involved in the ovarian differentiation in a teleost, medaka (*Oryzias latipes*). BMC Developmental Biology, 12(1), 36. https://doi.org/10.1186/1471-213X-12-36

CHAPTER 3

4.FINAL REMARK

The regulatory network involved in gonadal sex differentiation is a complex and multifactorial process even in closely related species. For example, different regulatory network are seeing in the *Oryzias* species especially in those with the same sex determining gene (*dmrt1bY*) (**Figure 6, apedix 8**). Interestingly, although, the final morphological product is the same (testis and ovary) (**Figure 1**). Lynch (2007) proposed a theoretical analysis to show the qualitative features of the transcriptional networks. The final gene product of a genetic network or cascade produces a phenotype, changes in the upstream system can occur without necessarily altering the finally expressed phenotype (Lynch, 2007). As a result, the regulatory network has changed, but the phenotype will be constant (Lynch, 2007). Similar observation were seen in this thesis where changing the regulatory pathways did not alter the morphological phenotype (female with "male-like" expression and estradiol "resistant male"). In this thesis, a battle of sexes as shown by the E2 resistant male, where the male pathway is stronger compared with the females with "male-like" gene expression (**Figure 2**). In females, *cyp19a1* expression produces aromatase, which converts testosterone to estradiol (E2) and maintains the auto-regulatory feed-forward loop sustaining the high oestrogen levels that support ovarian function (Guiguen et al., 2010). Within this loop, transcription factors like Foxl2 interact to up-regulate *cyp19a1* expression (Wang et al., 2007). An oestrogenic environment reinforces female-specific gene expression while suppressing male-promoting genes. In males, *cyp19a1* expression and aromatase production is suppressed and *hsd11b2* and *cyp11c1* promote production of 11KT; such that androgenesis prevails supporting testicular function and male-specific gene expression. Inhibitory and activating effects of androgens on Amh are reported (Pfennig et al., 2015). Dmrt1 suppresses *cyp19a1* promoter activity directly (Wang et al., 2010) and indirectly via its antagonistic relationship with *foxl2*. Thus, regulation of the androgen/oestrogen balance that determines sexual fate in fish seems to be governed predominantly by *dmrt1*, *amh*, *foxl2* and *cyp19a1* (Todd et al., 2016) as we showed in this thesis (**Figure 2**).

Our results contribute to the understanding how regulatory pathways are in related species, as well as, the complex paradigms that authors like M. Lynch (Nat. Rev. Genet. 2007) and Herpin and Schartl (2015) proposed. Also, this thesis contribute with information to obtain sex reversed fish (higher population of female) for the production of monosex in aquaculture in order to increase the production of this fish for commercial purposes.

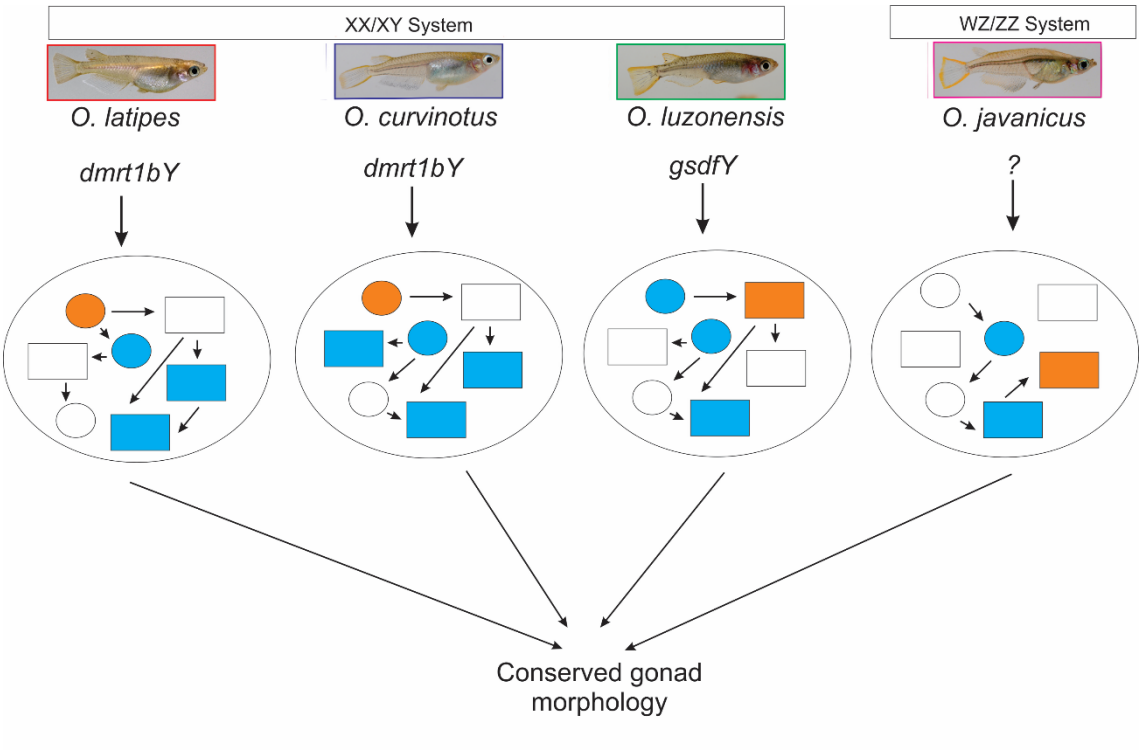

Figure 1. Regulatory network among *Oryzias* species. Regulatory network is not conserved between *O. latipes*, *O curvinotus*, *O .luzonensis* and *O. javanicus*. Figures in orange correspond to the sex determining gene, while figures in blue correspond to the same actors in the regulatory pathway with a different regulation in each specie. Even *O. latipes* and *O. curvinotus* shared the same sex determining gene (*dmrt1bY*), and the others different, the final outcome is the conserved gonadal morphology among species (Adapted from Heule et al., 2014).

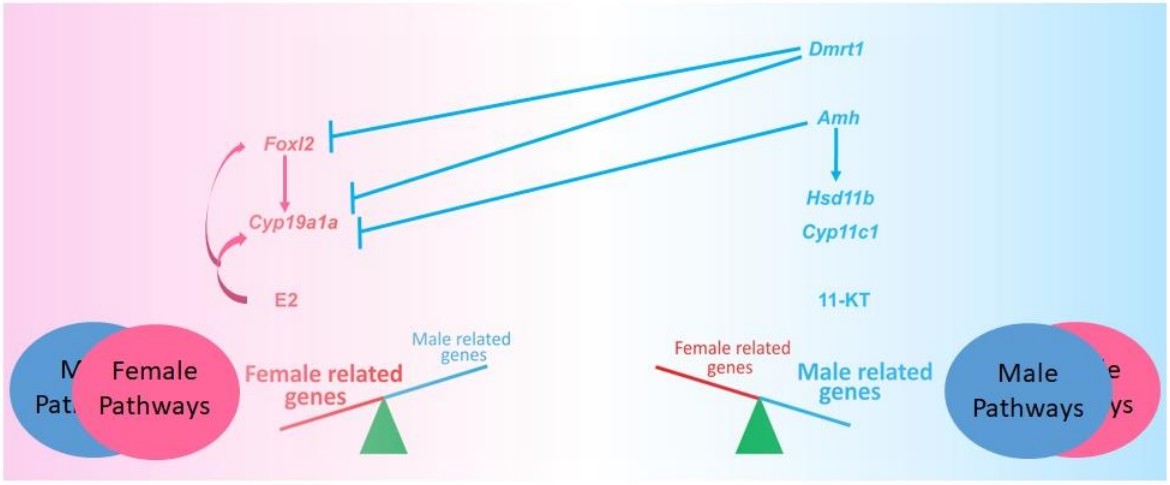

Figure 2. Antagonistic sex-specific gene networks maintain sexual fate in fishes by promoting either an oestrogenic (left) or androgenic (right) environment (own scheme).

REFERENCES

Adolfi, Mateus 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*. Reproductive Biology and Endocrinology, 13(1), 2. https://doi.org/10.1186/1477-7827-13-2

Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T.L., Hahn, M.W., Kitano, J., Mayrose, I., Ming, R., et al. (2014). Sex determination: why so many ways of doing it? PLoS Biol 12, e1001899.

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). Brazilian Archives of Biology and Technology. 55, 283-290.

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

Grossen, C., Neuenschwander, S., and Perrin, N. (2011). Temperature-dependent turnovers in sex-determination mechanisms: a quantitative model. Evolution 65, 64-78.

Guiguen Y, Fostier A, Piferrer F, Chang CF: Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol 165: 352–366 (2010).

Herpin, Amaury, Adolfi, M. C., Nicol, B., Hinzmann, M., Schmidt, C., Klughammer, J., Schartl, M. (2013). Divergent Expression Regulation of Gonad Development Genes in Medaka Shows Incomplete Conservation of the Downstream Regulatory Network of Vertebrate Sex Determination. Molecular Biology and Evolution, 30(10), 2328–2346. https://doi.org/10.1093/molbev/mst130

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

Kozielska, M., Pen, I., Beukeboom, L.W., and Weissing, F.J. (2006). Sex ratio selection and multi-factorial sex determination in the housefly: a dynamic model. J Evol Biol 19, 879-888.

Lynch, M. (2007). The evolution of genetic networks by non-adaptive processes. Nat Rev Genet. (10):803-13. doi: 10.1038/nrg2192

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

Todd EV, Liu H, Muncaster S, Gemmell NJ. (2016). Bending Genders: The Biology of Natural Sex Change in Fish. Sex Dev. 10(5-6):223-241. doi: 10.1159/000449297.

van Doorn, G.S., and Kirkpatrick, M. (2007). Turnover of sex chromosomes induced by sexual conflict. Nature 449, 909-912.

Wang Z, Gerstein M, Snyder M: RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63 (2009).

5.APENDIX

5.1 Projects in collaboration with the University of Wurzburg

1. Evolution of the sex determination pathway in the genus *Oryzias*: a comparative transcriptome analysis

INTRODUCTION

In vertebrates, fish are the group with the highest diversity of sex determining genes. This variability is observed not only in species from distant branches of the phylogeny, but also within the same genus. The genus *Oryzias* is a well studied paradigm for this phenomenon. To understand how the sex determination genetic network evolved in closely related species, we analysed the genome and the transcriptome of adult gonads of four species of *Oryzias*: (1) *O. latipes* (XX/XY system where the sex determining gene (SD) on the Y is *dmrt1bY*, (2) *O. curvinotus* (XX/XY system with the same SD gene *dmrt1bY* on a homologous Y), *O. luzonensis* (XX/XY system but with a different SD gene, *gsdfY*, on a different linkage group) and *O. javanicus* (SD gene still unknown, ZZ/ZW sex determination system).

METHODS

Three gonad samples of each sex were analyzed by RNA-seq in all four species. All sequences were mapped to the *O. latipes* reference genome using the RNA-seq aligner STAR and differentially expressed genes between testis and ovary were detected using DESeq2 (Bioconductor/R) for each species. Functionally enriched pathways and GO categories were found using the functional annotation tool DAVID (https://david.ncifcrf.gov/).

RESULTS AND DISCUSSION

Histology of the adult gonads in four species showed similar structures. A global transcript analysis revealed enriched categories for genes involved in metabolic pathways also across all four species. Histogram analysis for an overview of the transcriptome data (considering Log Fold Change (LFC)>(3), Base Mean>100) showed that *O. javanicus* ovaries and testis are more similar to each other and were separated from the other species. Genes involved in gonadal development showed unexpected divergence in expression pattern between species. Several genes that are preferentially expressed in males of one species are expressed in females in the other species. *O. javanicus* has more genes exclusive differentially expressed in females (e.g. activinRII) and males (e.g. *cyp46a1*, *ptger3*) than the other species. The *fsta* (*follistatin a*) gene, a well-characterized "female" gene is highly expressed in ovary of *O. javanicus*, while in *O. latipes* this gene is higher expressed in males. Even though *O. latipes* and *O. curvinotus* share the same SD gene, the expression pattern of several genes critically involved in gonadal development are different. Analysis between the genome of *O. latipes, O. curvinotus* and *O. luzonensis* showed a conserved synteny for *dmrt1*. *dmrt1* in *O. luzonensis* is higher expressed in females, while in *O. latipes* and *O. curvinotus* the expression pattern is similar but it is higher expressed in males. Interestingly, the transcriptome showed a LFC significantly higher for *dmrt1* in *O. javanicus* males compared with the other species.

CONCLUSION

The final outcome showed a similar morphology of the gonad, despite of having different sex determining genes and regulatory pathways. The WZ species *O. javanicus* exhibited considerable differences in gonad gene expression compared to the XY species *O. latipes*, *O. curvinotus* and *O. luzonensis*.

2. Gonadal transcriptome of hybrids derived from closely related species with the same sex determining gene: *O. latipes* and *O. curvinotus*.

In vertebrates, fish is the group with the highest diversity of sex determining genes. This variability is observed not only in species from distant branches of the phylogeny, but also within the same genus. The genus *Oryzias* is a well-studied paradigm for this phenomenon to understand how the sex determination genetic network evolved in closely related species. *O. latipes* and *O. curvinotus* share not only the same sex chromosome system, XX/XY, but also the same sex determining gene, *dmrt1bY*. However, crossings between both species causes XY female sex reversal (SR) and gonadal abnormalities. We analyzed the genome of the parental species and the transcriptome of the adult gonads of offspring from *O. latipes* and *O. curvinotus* crosses (H:Y*lat* when the Y comes from *O. latipes* and H:Y*cur* when the Y comes from *O. curvinotus*). Histological analysis of the gonads showed similar morphology between H:Y*lat* and H:Y*cur* XX females and XY females SR. All exhibited a significant decrease of pre-vitellogenic and vitellogenic oocytes compared to parental fish. Males of both hybrids were sterile showing empty seminiferous tubules. Transcriptome analysis of genes involved in gonadal sex differentiation and gametogenesis showed that XY female hybrids SR from both interspecific breedings (H:Y*lat* and H:Y*cur*) have a strikingly similar expression pattern as XX females. On the other hand, the expression pattern between H:Y*lat* and H:Y*cur* sterile males was different. Genes involved in germ cell specification (*vasa*), germ cell differentiation (*daz*l) and meiosis (*sycp3*) were highly expressed in both hybrids when compared to expression in testis of the parentals.. Interestingly, normalized read counts for *dmrt1bY* showed higher values in H:Y*lat* males and even in XY females SR. One explanation for this higher expression of *dmrt1bY* in XY females SR might be attributed to the different regulatory composition of *dmrt1bY* promoter in both parental genomes. In conclusion, gonadal structure and transcriptomes of hybrids are different from the parentals. The expression of *dmrt1bY* of one species in the different background of another species interferes with the correct development of the gonad.

5.2 Original publications

2019- Estrogen disrupted sex ratio and induced a complex gonadal plasticity in lambari, *Astyanax altiparanae*. Martinez-Bengochea A, Doretto L, Rosa IF, Oliveira MA, Silva C, Silva DMZA, Santos GR, Santos JSF, Avelar MM, Silva LV, Lucianelli-Junior D, Souza ERB, Silva RC, Stewart AB, Nakaghi LSO, Valentin FN, Nóbrega RH. *General and comparative endocrinology*. IF: 2.445 (re-submitted GCE_2019_365).

5.2.1 Articles in collaboration with University of Wurzburg

2019- Retinoic Acid Controls Gametogenesis by Regulating Sex-Related and Oogenesis Genes in Medaka. Mateus C. Adolfi1, Amaury Herpin, Anabel Lee Martinez Bengochea, Susanne Kneitz, Martina Regensburger, David J. Grunwald and Manfred Schartl. (in preparation)

2020- Insights into the molecular regulatory network underlying the XX/XY and ZW/ZZ sex chromosome systems in two fish models: Oryzias genus and Xiphophorous maculatus. Martinez-Bengochea, A.L, Roco A.S, Kneitz, S, Herpin, A, Nóbrega, R.H., Adolfi, M.C, Schartl, M. (in preparation)

2020- Genetic and gonadal disruption in hybrids derived from closely related species sharing the same sex determining gene dmrt1bY: *O. latipes* and *O curvinotus*. Martinez-Bengochea, A.L., Kneitz, S, Herpin, A, Nóbrega, R.H, Adolfi, M.C, Schartl, M. (in preparation)