Molecular studies on the genotypic and temperature-dependent sex determination of pejerrey Odontesthes bonariensis

Doctoral Dissertation

MOLECULAR STUDIES ON THE GENOTYPIC AND TEMPERATURE-DEPENDENT SEX DETERMINATION OF PEJERREY *Odontesthes bonariensis*

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Declaration

I hereby declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research. It has neither been accepted, not submitted for any other degrees. All sources of information have been duly acknowledged.

Yan Zhang

09-09-2016

People are illogical, unreasonable, and self-centered.
Love them anyway.
If you do good, people will accuse you of selfish ulterior motives.
Do good anyway.
If you are successful, you will win false friends and true enemies.
Succeed anyway.
The good you do today will be forgotten tomorrow.
Do good anyway.
Honesty and frankness make you vulnerable.
Be honest and frank anyway.
The biggest men and women with the biggest ideas can be shot down by the smallest men and women with the smallest minds.
Think big anyway.
People favor underdogs but follow only top dogs.
Fight for a few underdogs anyway.
What you spend years building may be destroyed overnight.
Build anyway.
People really need help but may attack you if you do help them.
Help people anyway.
Give the world the best you have and you'll get kicked in the teeth.
Give the world the best you have anyway.

From Dr. Kent M. Keith – The Paradoxical Commandments

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General Abstract

Pejerrey *Odontesthes bonariensis* is an excellent species for the study of temperature-dependent sex determination (TSD) in teleosts. In this species, sex ratios reach 100% female or 100% male at the environmentally relevant temperatures of 17°C (female producing temperature, FPT) and 29°C (male producing temperature, MPT) respectively, when the thermal exposure occurs between hatching and the onset of histological differentiation of the gonads (around 5 weeks post hatch). At intermediate temperatures (around 24-26°C; mixed sex-producing temperatures, MixPT), a large variation in sex ratios (e.g. 20-80%) is observed between progenies from different parents at a given temperature. These observations suggest a potential genetic involvement in the sex determination of pejerrey. In the context of the recent discovery of the presence of a sex determining gene *amhy* (male-specific duplication of the autosomal anti-Müllerian hormone gene) in the congeneric species *O. hatcheri* (Patagonian pejerrey), the purpose of this study was to determine the presence of this sex determining gene and its involvement in the variable sex ratios observed at intermediate temperatures.

In order to study the genetic contribution of *amhy* in the process of gonadal sex determination/differentiation in pejerrey, this thesis was divided into three chapters as follows. I first verified the presence of *amhy* and its paralogue, the autosomal *amha*, in this species. I then investigated the transcriptional profiles of *amhy* and *amha* at feminizing and masculinizing temperatures during early larval development with the aim of evaluating their relationship with TSD and testis formation. The last chapter of my thesis focused on the regulation of *amhy* and *amha in vitro* by cortisol and 11-Ketotestosterone (11-KT)

using *amhy* and *amha* presumptive promoters.

First, an *amhy* homologue was successfully isolated and cloned from wild and laboratory-reared pejerrey. Screening of wild and laboratory-reared pejerrey for *amhy* revealed a high, although not complete, linkage with phenotypic sex. The sex ratio in an *amhy*+/*amhy*- full sibling progeny reared during the thermolabile period of sex determination at an intermediate temperature of 25ºC was 68.7% male: 31.3% female; all *amhy*+/- fish developed as males whereas about 2/3 and 1/3 of the *amhy*-/- were female and male, respectively. At 25°C, transcription of *amhy* in *amhy*+/- animals persisted in larvae throughout the period of sex determination and histological gonadal differentiation. The autosomal *amha* was expressed in the gonads of all *amhy*+/- but only in part of the *amhy*-/- animals and seemed to be related to maleness in the latter. These observations suggest that both *amhy* and *amha* are important for testicular differentiation in pejerrey. These findings also represent the first clear genomic evidence that genetic and environmental sex determinants can coexist in species with marked TSD such as the pejerrey.

The second chapter examined the relative contribution of *amhy* and *amha* to the TSD process of pejerrey. XY and XX larvae derived from a XX mother and a XY father were reared at 17ºC (female-promoting temperature, FPT) and 29ºC (male-promoting temperature, MPT) during the critical period of thermolabile sex determination and used for transcriptional analyses of *amhy* and *amha* by qRT-PCR. In addition, I analyzed the expression profiles of ovarian type aromatase *cyp19a1a* (critical for female development) and *amh* type II receptor *amhrII* (critical for male development), at the FPT and MPT respectively. Histological analyses at the end of experiment revealed that the MPT yielded

a single-sex male population and that the FPT yielded a mixed sex population whereby 59% of the XY fish developed as males and the remaining as females. *amhy* mRNAs were abundant in XY larvae from both the FPT and MPT groups at the beginning of the sex determination period and then declined. *amha* expression was highly correlated with maleness. During the sex determination period, *amha* was upregulated in a few of the XY larvae at the FPT and in both genotypes at the MPT. *cyp19a1a* expression was found to be inversely proportional to temperature in XX fish whereas in XY genotypes a dimorphic distribution of *cyp19a1a* was observed at the 17ºC. *amhrII* expression did not differ between XX and XY fish although it was higher at 25ºC and 29ºC than that at 17ºC in both genotypes. Thus, these results suggest that *amhy* expression is temperature-independent while *amha* and *amhrII* expression were temperature-dependent. This indicates that temperature may modulate *amha* expression though *amh* receptors which then induce masculinization in pejerrey.

A previous study has shown that the stress-related hormone cortisol promotes 11-KT production during high temperature-induced masculinization of pejerrey. The 3rd chapter focused on how the two *amh* paralogues of pejerrey interact with stress and sex steroid axis during gonadal differentiation. A luciferase reporter assay was performed with the presumptive promoters (~3kb 5' upstream fragment) of both *amh* paralogues. The glucocorticoid receptor expression plasmid was first co-transfected with luciferase reporter plasmids containing *amhy* or *amha* promoter into endothelial progenitor cells (EPCs). Transcriptional activity was then measured 48 hours post transfection in cells exposed to different cortisol and 11-KT doses. Transcriptional activity analyses showed that the *amhy* promoter did not respond to any cortisol or 11-KT doses. On the other hand, both cortisol and 11-KT activated the *amha* promoter. The transcriptional activity of *amha* promoter

revealed a cortisol dose-dependent manner, which suggests high water temperature induces *amha* expression by elevating cortisol and androgen levels in pejerrey.

This study is the first to show evidence of the co-existence of GSD and TSD in pejerrey. Although *amhy* is considered the genotypic sex determinant, the autosomal *amha* may also be involved in testis formation in pejerrey. In addition, my study also reveals the significance of cortisol and androgen signaling, especially at high temperatures, as transcriptional regulators for the *amha* gene during the process of masculinization.

General Introduction

Many poikilothermic vertebrates, including reptiles, amphibians, and fishes, exhibit a sex determination system greatly influenced by environmental factors, such as temperature, pH and social conditions (Crews, 1996; Rubin, 1985; Baroiller et al., 1999; Munday et al., 2006a, b; Conover and Kynard, 1981; Janzen and Phillips, 2006; Sandra and Norma, 2010). In fishes, the most important environmental determinant of sex is temperature (temperature-dependent sex determination or TSD) (Devlin and Nagahama, 2002) and was first evidenced in the Atlantic silverside *Menidia menidia*, a gonochoristic atherinopsid from West Atlantic. To date, TSD has been found in many other species of genus *Apsitogramma* (South American Cychlids) (Römer and Beisenherz, 1996), Japanese flounder, sea bass, medaka and tilapia with high temperatures leading to male-skewed sex ratios (Yamaguchi et al., 2010; Pavlidis et al., 2000; Hattori et al., 2007; Abucay et al., 1996).

Among teleosts, the strongest sexual thermolability is found in the pejerrey *Odontesthes bonariensis*, a South American atherinopsid (Fig. 1). In this model, monosex populations of female and male can be consistently obtained when the larvae are raised between hatching and the onset of histological differentiation of the gonads (Fig. 2) at low (17°C; FPT, female producing temperature) and high (29°C; MPT, male producing temperature) temperatures, respectively, and intermediate temperatures (24~25°C; MixPT, mixed-sex producing temperature) yield mixed sexes (Strüssmann et al., 1996a, 1997). To unravel the molecular pathways underlying the TSD mechanism, a series of experiments including the expression profiles of genes universally implicated in the sex differentiation

process and sex differentiation cascade in fish have been carried out and several molecular processes have been described so far. Larvae at the FPT had lower follicle stimulating hormone beta *fshb* and luteinizing hormone beta *lhb* expression but higher luteinizing hormone receptor *lhr* expression during the sex determining period than those at the MPT and thus suggested that temperature may signal through the pituitary (differential expression of *fshb* and *lhb*) down to the gonads (differential expression of *lhr*), probably affecting the regulation of steroidogenesis during the TSD process of pejerrey (Shinoda et al., 2010). The gonad-specific sex-related genes *cyp19a1a*, *dmrt1*, and *amh* were also proved to be involved in primary sex differentiation process during TSD (Fernandino et al., 2008a, b). In addition, temperature-induced masculinization of pejerrey was mediated via stress hormone, cortisol, as well as in other teleosts (Hayashi et al., 2010; Yamaguchi et al., 2010). During the critical period of sex determination, pejerrey larvae at MPT consistently had higher cortisol, 11-ketotestoterone (11-KT), and testosterone (T) titres than those at a FPT (citation). Moreover, cortisol-treated animals had elevated 11-KT and T, and showed typical molecular signatures of masculinization and higher proportion of males (citation). These reports provides a possible link between stress and testicular differentiation in gonochoristic TSD species and support the notion that stress responses might be involved in various forms of environmental sex determination (Hattori et al., 2009). However, while reproducible sex proportions are obtained at both FPT and MPT, at MixPT (25°C) large deviations occurs among different crosses, which could be related to the existence of a very weak genetic component acting on gonadal fate (Strüssmann et al., 1996b, 1997).

In a recent study on the genetic sex determining mechanism in the congeneric species Patagonian pejerrey *O. hatcheri*, the male-specific *amhy* (Y-linked anti-Müllerian hormone duplication) gene was identified and showed to be implicated in the triggering of

testicular development in this model (Hattori et al., 2012). Because Patagonian pejjerey and pejerrey are closely related species and share a high genetic identity (Strüssmann et al., 1997), it is conceivable that *amhy* could exist in pejerrey and be behind the variable sex ratios observed at the MixPT, as it would be the case for example, if any of the parents is a (thermally) sex-reversed animal.

In order to probe the existence of genotypic sex determinant and its relative contribution for temperature-dependent sex determination in pejerrey, I first identified *amhy* paralogue in pejerrey and examined the expression profile of *amhy* and autosomal amh, *amha* during sex determination/differentiation period at male-, female-, and mix-sex producing temperatures, respectively. Further, to examine the relevance of cortisol to *amhy* and *amha* gene expressions, I conducted reporter gene assay.

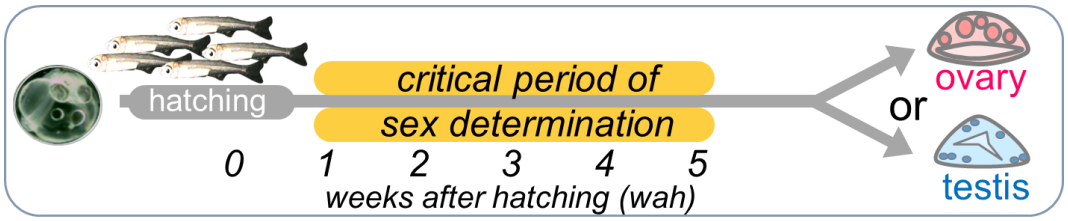

Figure 1. Schematic representation of period of sex determination in pejerrey

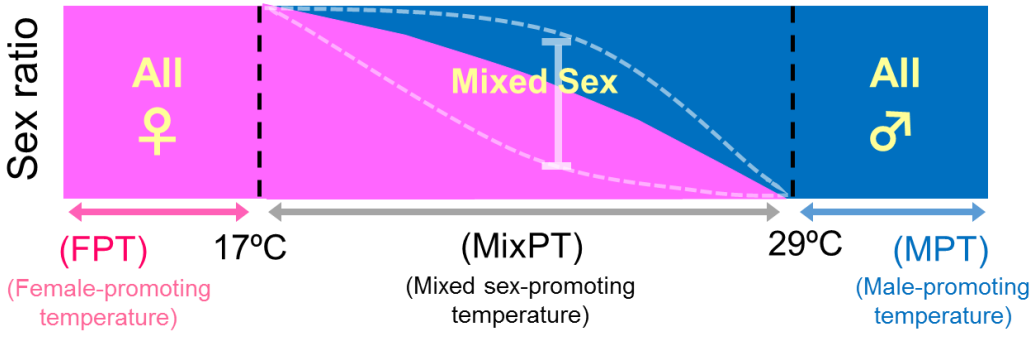

Figure 2. Sex ratios of pejerrey under different thermal treatments after hatching

Chapter 1

Genotypic sex determination in pejerrey *O. bonariensis*: evidences from the testis-determining gene *amhy*

Abstract

In this study, we examined whether a homologue of the master sex determining gene *amhy* of Patagonian pejerrey *Odontesthes hatcheri* is present and plays any role in testis determination of pejerrey *O. bonariensis*, a species otherwise known for its strong temperature-dependent sex determination (TSD). Screening of wild and laboratory-reared pejerrey for *amhy* revealed a high, although not complete linkage with phenotypic sex. The sex ratio in an *amhy*+/*amhy*- full sibling progeny reared during the thermolabile period of sex determination at an intermediate temperature of 25ºC was 68.7% male:31.3% female; all *amhy*+ fish developed as males whereas about 2/3 and 1/3 of the *amhy*- were female and male, respectively. RT-PCR and ISH analyses revealed that transcription in *amhy*+ animals began during embryo stage and persisted in larvae through the period of sex determination and histological gonadal differentiation. The autosomal *amha* was present in all individuals regardless of *amhy* genotype; during this period, it was expressed in the gonads of all *amhy*+ but only in part of the *amhy*- animals. After histological gonadal differentiation, all gonads of *amhy*- animals with *amha* ISH signals were testes and those without it were ovaries. These results suggest that *amhy* is important for testicular differentiation in pejerrey, at least at intermediate temperatures. Thus, *amhy*+ animals probably differentiate as males by expression of either *amhy* alone or *amhy* and *amha* together whereas the *amhy*- rely solely on *amha* expression, which may itself be temperature-dependent.

These findings represent the first clear genomic evidence that genetic and environmental sex determinants can coexist in species with marked temperature-dependent

sex determination such as the pejerrey. The finding of *amhy* will make it possible to screen wild pejerrey populations for the effects of global warming, climate change, and anthropogenic factors on reproduction and to study the ecological relevance of TSD for this species.

Introduction

The pejerrey *Odontesthes bonariensis* is an excellent model for the study of temperature-dependent sex determination (TSD) in teleosts. In this species, sex ratios reach 100% female or 100% male at environmentally relevant temperatures of 17°C (female producing temperature, FPT) and 29°C (male producing temperature, MPT), respectively. The critical time of sex determination has been estimated between 1 and 5 weeks after hatching (wah) depending on the water temperature (Strüssmann et al., 1997a). The end of this period coincides with the beginning of the histological differentiation of the gonads, which occurs first in ovaries and then in testes (Ito et al., 2005). In addition, significant information on the molecular and biochemical processes underlying its TSD is available. For example, differential expression of *fshb* (follicle stimulating hormone beta) and *lhb* (luteinizing hormone beta) in the pituitary and of *lhr* (luteinizing hormone receptor), *cyp19a1a*, *dmrt1*, and *amh* in the gonads were found to be involved in the sex differentiation process (Shinoda et al., 2010; Fernandino et al., 2008a; Fernandino et al., 2008b). Other studies have shown a connection between environmental temperature and sex determination that is mediated by the glucocorticoid stress-related hormone cortisol, in particular during masculinization (Hattori et al., 2009; Fernandino et al., 2012). Thus, significant advances have been achieved concerning the mechanism of TSD in pejerrey but, as discussed next, the picture is far from complete.

While the reproducible sex ratios consistently obtained at the FPT (all-female) and MPT (all-male) suggest that genotypic sex determinants in *O. bonariensis* are virtually inexistent, this is not a foregone conclusion. For example, at intermediate, mixed sex-

producing temperatures (MixPT; around 24-26°C), large variability in sex ratios (e.g. 20-80%) is observed between progenies from different parents at a given temperature. Such variability could be related to subtle, hitherto unknown environmental effects besides temperature or it could be an indication that parents carry some form of genotypic gender determinant that affects sex determination at sexually neutral temperatures (Strüssmann et al., 1997a). The latter scenario has become more plausible after a recent study on the sex-determining mechanism of the congeneric species *O. hatcheri* (Patagonian pejerrey), which possesses a typically balanced (1:1) sex ratio at intermediate temperatures, revealed a male-specific duplication of the *amh* gene (called *amhy*, for Y-linked anti-Müllerian hormone) that triggers testicular development (Hattori et al., 2012). Because the two species are closely related and share a high genetic identity (Strüssmann et al., 1997b), it is conceivable that *amhy* could exist in O. bonariensis and be behind the variable sex ratios observed at the MixPT, as it would be the case for example, if any of the parents is a (thermally) sex-reversed animal. It is noteworthy that environment and genotype interactions have been implied before in sex determination of other species with TSD (Baroiller et al., 1999; Devlin and Nagahama, 2002; Strüssmann et al., 2010; Penman and Piferrer, 2008; Baroiller et al., 2009; Luckenbach et al., 2009), but a clear genotypic factor has never been identified.

In this context, this study was designed to probe the presence of *amhy* in the pejerrey genome and whether it has a role in gonadal sex determination of this species. We successfully cloned an *amhy* homolog in laboratory-reared pejerrey, genotyped broodstock and wild fish based on *amhy*, and carried out progeny tests to confirm its sex linkage and Mendelian inheritance. In addition, we examined the ontogeny of *amhy* expression in relation to that of the autosomal form *amha* and to time of histological gonadal sex

differentiation. The results clearly show that *amhy* is functionally implicated in testicular differentiation in pejerrey at intermediate, temperatures, and prove the coexistence of environmental and genotypic sex determination in this species.

Materials and Methods

Ethical statement

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology (TUMSAT). Experiments with fish at TUMSAT do not require any special authorization as long as they adhere to the institutional guidelines, which is the case of this study. Laboratory fish were procured from the Aquatic Animal Rearing Facilities of TUMSAT, which is licensed to keep broodstock and propagate fish, and were sacrificed by anesthetic overdose in order to minimize animal suffering prior to any sampling. All samples of wild fish used in this study were a kind donation from Dr. Seiichi Kasuga, National Institute for Environmental Studies (NIES), Ibaraki, Japan and were already dead when provided to us. These samples were taken in 2001 during routine fisheries resource assessments conducted by the NIES and have been kept frozen until use. Pejerrey is not an endangered species and its collection is not subject to permit requirement.

Cloning and sequencing of pejerrey amhy

To obtain the complete cDNA sequence of the *amhy* gene in *O. bonariensis*, total mRNA extracted from the gonad of a laboratory-reared, *amhy*-positive (amhy+) was used. Extraction of mRNA and synthesis of cDNA were performed according to previous studies (Hattori et al., 2012). 5' and 3' UTR fragments were amplified by the primers listed in Table S1 using GeneRacer (Invitrogen, Carlsbad, CA) and Smart RACE cDNA

amplification (Clontech, Mountain View, CA) kits, following manufacturer's instructions. Genomic DNA was extracted following the protocol described by Aljanabi and Martinez (Aljanabi and Martinez, 1997) and used for intron sequencing. PCR was performed using primers designed on the basis of the *O. hatcheri amhy* (Table S1; NCBI accession code HM153803). All amplifications were done according to the following conditions: 3 min at 94°C, 30 cycles of 30 sec at 94°C, 45 sec at 60°C and 2.5 min at 72°C, then followed by a final elongation for 5 min at 72°C. PCR products were electrophoresed in 1% agarose gel, purified, and sequenced in an ABI PRISM 3100 capillary sequencer (Life Technologies, Carlsbad, CA) using the BigDye Terminator method. Sequences were analyzed with GENETYX version 11.0 (GENETYX, Tokyo, Japan).

Phylogenetic analysis

The predicted amino acid sequences of pejerrey Amhy and Amha (GeneBank accession numbers KC847082 and AY763406, respectively) were compared to the Amh sequences of other teleosts available at GenBank using the software GENETYX version 11.0. The following sequences were compared: Patagonian pejerrey Amhy and Amha (Odontesthes hatcheri, DQ441594 and HM153803, respectively), Atlantic salmon Amh (Salmo salar, AY722411), zebrafish Amh (Danio rerio, AY721604), Japanese flounder Amh (Paralichthys olivaceus, AB166791), blue tilapia Amh (Oreochromis aureus, DQ257618) and Japanese medaka Amh (Oryzias latipes, AB214971). The phylogenetic tree was constructed by the Neighbor-Joining method (Saitou and Nei, 1987) using MEGA software (vers. 5.2.2) (Tamura et al., 2011) with 10000 replicates.

amhy genotyping of wild fish and laboratory broodstock

A random sample of 90 pejerrey juveniles collected by seine net in the Lake Kasumigaura (Ibaraki, Japan) on September 2001 and 24 laboratory-reared broodstock fish from the Aquatic Animal Rearing Facilities, Tokyo University of Marine Science and Technology (Shinagawa Campus, Tokyo, Japan), were screened for the presence of *amhy* using primers designed on the basis of the 5' flanking region of *O. hatcheri amhy* (Table S1; NCBI accession code HM153804). The autosomal *amh* homolog of *O. bonariensis* (*amha*; NCBI accession code AY763406) was analyzed using the primers indicated in Table S1 as a positive control. Animals carrying the *amhy* gene (*amhy*-positives) were represented by *amhy*+ when the exact genotype could not be determined and by *amhy*+/+ or *amhy*+/- when they were confirmed as homozygous or heterozygous, respectively. Those without *amhy* (*amhy*-negative) were represented by *amhy*-/-. Genomic DNA extraction and amplification followed the protocols described in the previous section. Gonadal sex of each individual was asserted by dissection and visual inspection of the gonads for wild fish, after sacrificing them through procedures described above, or manual stripping of gametes/gonadal cannulation for laboratory broodstock.

After *amhy* genotyping, laboratory-reared broodstock were used in single-pair crosses between one *amhy*-/- female and nine *amhy*+ males were produced by artificial fertilization for testing Mendelian inheritance and whether the males were homozygous (*amhy*+/+) or heterozygous (*amhy*+/-). We also performed a progeny test with one *amhy*+ female and an *amhy*-/- male. Incubation until hatching was performed as described below. Randomly-chosen hatchlings (n= 24-98) from each cross were analyzed following the same procedures used for wild fish and broodstock genotyping.

Rearing procedures and sampling for mRNA expression analysis

One of the pairs that yielded a balanced sex ratio in the progeny test (*amhy*-/- female, F1, *amhy*+/- male, M9; Table S2) was selected and allowed to breed naturally in a 650-liter recirculated-water rearing tank under controlled temperature (20°C), photoperiod (14L/10D), and salinity (0.2-0.5% NaCl in dechlorinated tap water). Fertilized eggs were collected, cleaned of chorionic filaments, and transferred to incubators with flowing brackish water (salinity of 0.2-0.5%) at 19°C. After hatching (about 9 days after fertilization), approximately 800 to 1000 newly-hatched larvae were stocked in each of two 60-liter tanks and reared at 25°C (MixPT) (Strüssmann et al., 1997a; Ito et al., 2005) for up to 14 weeks. Fish were fed live Artemia nauplii from the first day to satiation three to four times daily and gradually weaned into powdered fish food (TetraMin flakes, Melle, Germany) from the third week. Fish were sampled daily (0 to 8 days after fertilization, or daf; n=10) and weekly (0 to 10 wah; n=20), respectively, for gene expression and histological analyses (see below for details). Larvae and juveniles were fin-clipped for genomic DNA extraction and *amhy* genotyping according to the methods described in the previous section. The remaining fish (n= 67) were collected at the end of the experiment (14 wah) for histological determination of sex ratios.

Histological analysis of gonadal sex differentiation and sex ratios

For the histological analysis of gonadal sex, trunks were fixed overnight in Bouin's fixative solution, dehydrated in ascending ethanol series, cleared in xylene, and embedded

in Paraplast Plus (McCormick Scientific, St. Louis, MO). Cross-sections were cut serially at a thickness of 5 µm, stained with Hematoxylin-Eosin, and analyzed following previously reported histological criteria (Ito et al., 2005; Strüssmann and Ito, 2005).

Tissue distribution and temporal expression analysis of amhy, amha, and cyp19a1a transcripts

The tissue distribution of *amhy* and *amha* transcripts was analyzed using total RNA extracted from testis, brain, gill, heart, trunk kidney, spleen, liver, anterior and posterior intestine, and muscle of an *amhy*+/- 20-week old juvenile. For the temporal expression analysis, whole embryos and trunks of larvae were stored in RNAlater (Sigma-Aldrich, St. Louis, MO) at -80°C until use. Trizol Reagent (Life Technologies) was used for total RNA extraction. Genomic DNA extracted from the remaining interphase was used for genotyping embryos. All procedures followed the reagent manufacturer's protocol. Synthesis of cDNA and transcription analyses of *amhy*, *amha*, and *β-actin* in whole embryos and juvenile tissues were performed by RT-PCR according to a previous study (Hattori et al., 2012). In larvae, the same genes were analyzed by qRT-PCR using the specific sets of primers and probes shown in Table S1. The suitability of *β-actin* as an endogenous control was confirmed by qRT-PCR in the same samples (Fig. S1). The specificity of the primers was confirmed by using plasmids containing *amhy* or *amha* ORFs as controls and also by direct sequencing of PCR products. The transcript levels of the ovarian differentiation marker *cyp19a1a* were analyzed at 4 and 6 wah following methods reported in our previous studies (Fernandino et al., 2008a; Fernandino et al., 2008b; Hattori et al., 2012; see also Table S1).

Localization of amhy/amha mRNAs by ISH

Samples for in situ hybridization (ISH) in pre- and post-differentiation gonads were collected at 4 and 10 wah, fixed and processed for preparation of histological sections as described above. Body trunk sections were hybridized in the automated tissue processer Hybrimaster HS-500 (Aloka, Tokyo, Japan) using an *amh* probe that recognizes both *amhy* and *amha*, synthesized according to a previous study (Fernandino et al., 2008b). Final detection was performed manually with NBT/BCIP according to the manufacturer's (Roche Diagnostics, Basel, Schweiz) protocols.

Results

Cloning and sequence analysis of amhy gene

An *amhy* homolog was cloned from a laboratory-reared pejerrey and revealed the *amhy*-characteristic 0.5 kb fragment within the third intron (Fig. 1A). The deduced Amhy protein, including the characteristic TGF-β domain (amino acids 421–514) with seven canonical cysteine residues, comprised 514 amino acids. Phylogenetic analysis based on the amino acid sequence of the open reading frame showed that *O. bonariensis* Amhy shared the same clade with *O. hatcheri* Amhy whereas the Amha in both species were placed together in another clade (Fig. 1B). Among the outgroup species, the medaka Amh showed to have the shortest genetic distance to the *Odontesthes* species Amhs, displaying similar distances to both Amhy and Amha clades.

Genotyping of wild fish, broodstock, and progeny from specific crosses

The analysis of juveniles from Lake Kasumigaura revealed 38 $amhy^{+}$ and 52 $amhy^{-/-}$ out of 90 individuals whereas that of our *O. bonariensis* broodstock revealed 14 $amhy^{+}$ and 10 $amhy^{-/-}$ out of 24 individuals (Table 1; Fig. 1C). In both cases, there was a high but not complete concordance between genotypic and phenotypic sex. The progeny of all 9 $amhy^{+}$ males crossed pairwise with the same $amhy^{-/-}$ female showed sex ratios statistically undistinguishable from 1:1 (Fisher's exact test), indicating that all males were heterozygous ($amhy^{+/-}$) for the *amhy* gene (Table S2). No $amhy^{+/+}$ male was found among the tested fish. Likewise, the cross of an $amhy^{+}$ female with an $amhy^{-/-}$ male confirmed that

the former was heterozygous for *amhy* (Table S2). As expected, *amha* was detected in all fish regardless of phenotypic sex or *amhy* genotype (Fig. 1C).

Tissue distribution and temporal expression analysis of amhy, amha, and cyp19a1a

Transcripts of *amhy* were found in the testis and in the brain whereas *amha* was expressed only in the testis of juveniles (Fig. 2A). Transcripts of *amhy* were detected in embryos from late blastula stage until hatching in all $amhy^{+/-}$ individuals (Fig. 2B). In larvae trunks, the expression of *amhy* was highest at 1 wah and decreased until 4 wah, when it reached a low but stable plateau (Fig. 3A). *amha* mRNA expression was undetectable in $amhy^{+/-}$ embryos (Fig. 2B) and low in larvae between 1 and 3 wah (Fig. 3B) but clearly upregulated between 4 and 6 wah. *amha* mRNA expression was not detected in any of the $amhy^{-/-}$ embryos (Fig. 2B) and was consistently low in larvae between 1 and 3 wah (Fig. 3C). In contrast, between 4 and 10 wah the mRNA expression assumed a bimodal distribution thereby 7 out of 19 $amhy^{-/-}$ individuals (37%) had high values and the remaining ones had low levels (Fig. 3C).

A comparative analysis between the expression of *amha* and the ovarian differentiation marker *cyp19a1a* at 4 and 6 wah revealed that all 10 $amhy^{+/-}$ individuals had high and low transcript levels of *amha* and *cyp19a1a*, respectively (Fig. 3D). The $amhy^{-/-}$ animals, on the other hand, showed either this pattern (4 out of 10 individuals) or the opposite one with relatively high *cyp19a1a* and low *amha* levels (6 out of 10 individuals; Fig. 3D).

Localization of amha/amhy mRNAs by ISH

ISH signals for *amha/amhy* were detected exclusively in somatic cells of the medullary region of gonads of all *amhy*$^{+/-}$ (n=2 for each sampling point) and in 8 out of 14 *amhy*$^{-/-}$ individuals from 4 and 10 wah (Fig. 4). At 10 wah, when all gonads had differentiated as ovaries or testes, only the latter had ISH signals.

Relation of phenotypic sex to amhy genotype under controlled conditions

The remaining fish from the *amha*/*amhy* expression analysis at 14 wah (n=67) were 68.7% males and 31.3% females. The ratio of *amhy*$^{+/-}$ to *amhy*$^{-/-}$ fish was nearly 1:1 (49.3%:50.7%) and all of the formers (n=33) were phenotypically male. Among the 34 *amhy*$^{-/-}$ fish, 21 (61.8%) and 13 (38.2%) were female and male, respectively. The gonads of all individuals examined, including the testes of both *amhy*$^{-/-}$ and *amhy*$^{+/-}$ males, had no abnormalities or difference of any kind compared to previously reported criteria (Ito et al., 2005; Strüssmann and Ito, 2005) (data not shown).

Discussion

In this study, we examined whether a homolog of the sex determining gene *amhy* of *Odontesthes hatcheri* (Hattori et al., 2012) is present and plays any role in testis determination of pejerrey *O. bonariensis*, a species otherwise known for its strong temperature-dependent sex determination (Strüssmann et al., 1997a). Cloning of the *O. bonariensis amhy* revealed a molecule that is 98% and 97% identical in terms of the open reading frame and TGF-β domain, respectively, to its homolog in *O. hatcheri*. Wild-caught pejerrey and captive broodstock were then genotyped on the basis of *amhy*, showing its presence in about half of the individuals and, for those that were phenotypically sexed, with few exceptions, they were males. More importantly, $amhy^{+/-}$ was linked 100% to maleness in a progeny that was reared throughout the critical period of sex determination under a temperature (25ºC) known to produce mixed-sex populations (Strüssmann et al., 1997a; Ito et al., 2005). Conversely, most of the $amhy^{-/-}$ individuals were females although there were clearly more exceptions among those reared at 25ºC (e.g., approximately 1/3 of $amhy^{-/-}$ males; see further discussion below about the effects of this temperature). In this context, and keeping in mind the strong effects of water temperature on pejerrey sex determination (Strüssmann et al., 1997a), the results suggest that *amhy* is sex-linked in *O. bonariensis* and that it could be implicated in the sex determination of this species just as it is in *O. hatcheri* (Hattori et al., 2012).

To address this hypothesis, we examined the ontogeny of *amhy* expression during gonadal sex determination and histological sex differentiation in offspring from an $amhy^{-/-}$ female and an $amhy^{+/-}$ male raised under controlled laboratory conditions. During

incubation at 19ºC, *amhy* transcripts were consistently expressed from the late-blastula stage onwards in all *amhy*$^{+/-}$ genotypes. The *amhy* transcription was maintained through hatching and transfer to 25ºC, the period considered as critical for sex determination (1-5 wah) (Strüssmann et al., 1997a), and finally the appearance of histological signs of gonadal differentiation (4-7 wah) (Ito et al., 2005). This pattern of expression is consistent with a role in gonadal differentiation and, considering its sex linkage, the cellular pattern of expression described below, as well as the known involvement of Amh in testicular differentiation in several fish species including its congener *O. hatcheri* (Fernandino et al., 2008a; Hattori et al., 2012; Piferrer and Guiguen, 2008), with testicular development. Still, the expression from early embryogenesis, even before the formation of the gonad anlagen, is intriguing. This is much earlier than in *O. hatcheri* where *amhy* plays the master trigger for testicular differentiation (Hattori et al., 2012). Whether this early sex-specific expression can affect sex afterwards by epistatic effects on other genes, hence predisposing the *amhy*$^{+/-}$ genotypes to become males, remains to be assessed. Other questions concerning *amhy* that must be addressed are to what degree its expression is affected by water temperature, if it acts through or independently of *amha* (see the following discussion), and if the expression found in the brain is implicated in sex differentiation.

In contrast to *amhy*, *amha* was found in all fish regardless of gonadal phenotype, indicating that it is located in autosomal chromosomes just as it is in *O. hatcheri* (Hattori et al., 2012). Yet, it seems to be critical for masculinization in *amhy*$^{-/-}$ individuals, perhaps as a function of temperature and endocrine factors (Fernandino et al., 2008a), and may be a coadjuvant factor in *amhy*$^{+/-}$ genotypes. The first line of evidence that supports a role for *amha* is that its expression, although not as early as that of *amhy*, coincided temporally with the period when the pejerrey gonads are still sexually labile (see references above).

This pattern differs from the late *amha* expression described in *O. hatcheri*, where it is considered as irrelevant for testicular differentiation (Hattori et al., 2012). Further, both qRT-PCR and ISH revealed a bimodal pattern of *amha* expression in *amhy*$^{-/-}$ individuals where the proportion of animals with high *amha* expression during the estimated period of sex determination (37%) closely approximated the proportion of animals with low *cyp19a1a* during the same period (40%) and that of phenotypic males determined at 14 wah (38%). Also, when the gonads had clearly differentiated by 10 wah, gonads showing *amha* expression were testes whereas those without it were ovaries. Finally, all *amhy*$^{+/-}$ animals had high *amha* as well as low *cyp19a1a* transcription during the period of sex determination and all became males.

Taken together, these results strongly suggest that *amhy*$^{+/-}$ genotypes differentiate as males by expression of either *amhy* alone or *amhy* and *amha* together and that *amhy* may be implicated in the up regulation of *amha*. We also hypothesize that *amhy*$^{-/-}$ genotypes rely on *amha* expression for testis differentiation. Nevertheless, the actual processes underlying *amha* regulation in both genotypes remain to be elucidated. In this regard, it must be noted that the TGF-beta domain, the region that binds to the primary receptor AmhrII, is highly conserved in both *amhy* and *amha* genes of *O. bonariensis* as in *O. hatcheri* (Hattori et al., 2012). Thus, we suppose that Amha may activate the same AmhrII used by Amhy for the activation of downstream pathway of testis differentiation in *amhy*$^{-/-}$ genotypes. Ongoing studies are focusing on the thermal thresholds for mRNA expression, receptor binding, and the relative contributions of *amhy* and *amha* for masculinization.

The sex ratio in the controlled rearing experiment was significantly (about 70%) male-biased and only female-to-male sex-reversals were noted. This highlights the

importance of the discovery of *amhy* for unbiased and accurate screening of thermal effects on gonadal sex differentiation. Thus, the current results suggest that 25ºC might not be exactly neutral for pejerrey in terms of sex effects as previously assumed (Strüssmann et al., 1997a). Alternatively, other forms of stress may have caused elevation in cortisol levels, which is able to induce testicular differentiation (Hattori et al., 2009; Fernandino et al., 2012), and thus activated the male pathway leading to sex-reversal. Given the results obtained in this study, it could be argued that pejerrey possesses a genotypic sex determinant in spite of having a marked TSD. This finding underscores the difficulty in drawing a line between GSD and TSD and that these forms are likely part of a continuum (Strüssmann and Patiño, 1999; Barske and Capel, 2008). On the other hand, it is intriguing how *amhy* has been maintained in the course of evolution in a species whose sex is highly susceptible to temperature effects. The high thermal dependence of sex associated to the presence of a marker for genetic predisposition of gender makes *O. bonariensis* a very attractive model to study these issues as well as the molecular pathways of high temperature-induced masculinization and low temperature-induced feminization. Although in low frequency, both $amhy^{+/-}$ females and $amhy^{-/-}$ males were found in a wild population, raising concerns about its causes and the impact of temperature-dependent sex determination and sex-reversals on the population demographics (Strüssmann et al., 2010). The finding of *amhy* will make possible to monitor wild pejerrey populations for mismatches between genotypic and phenotypic sex and may prove instrumental for field studies addressing the effects of endocrine disruptors or abnormal temperatures on reproduction and the ecological relevance of TSD for this species.

In summary, this study demonstrated that the *amhy* gene is active in $amhy^{+/-}$ genotypes before, during, and after the critical time-window of TSD. Although some $amhy^{-}$

$^{/-}$ individuals developed as males, no *amhy*$^{+/-}$ females were found among fish reared at intermediate temperatures, suggesting that under similar conditions *amhy* is a strong determinant of testis differentiation. Taken together, the present results provide strong support for the coexistence of GSD and TSD in *O. bonariensis*.

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Tables and Figures

Table 1. Relationship between phenotypic (gonadal) sex and *amhy* genotype in wild pejerrey and laboratory-reared broodstock.

Source	Genotype	Phenotype		Total n (%)
		Female	Male	
Wild fish[1,2] (Lake Kasumigaura)	*amhy*$^{-/-}$	49	3	52 (57.8)
	amhy^{+}	1	37	38 (42.2)
	Total n (%)	50 (55.6) *	40 (44.4)	
Laboratory broodstock[1,2]	*amhy*$^{-/-}$	8	2	10 (41.7)
	amhy$^{+/-}$	2	12	14 (58.3)
	Total n (%)	10 (41.7)	14 (58.3)	

[1]No statistical significance difference in phenotypic sex ratio (Fisher's test, $p>0.05$).
[2]No statistical significance difference in *amhy* genotype ratio (Fisher's test, $p>0.05$).

Figures Legends

Figure 1. *amhy* gene structure, phylogenetic relationship, and broodstock genotyping. **A**: Structure of the *amhy* gene in *O. bonariensis*, size of exons, UTRs, and TGF-beta domain, and the respective identity values in relation to *O. bonariensis amha*. The third intron contains a 0.5 kb insertion in relation to *amha*. **B**: Phylogenetic tree (Neighbor-Joining method) for the amino acid sequences of *O. bonariensis* and *O. hatcheri* Amhy and Amha and the Amh of other teleosts. Numbers indicate *bootstrap* values based on *10000 replicates*. **C**: *amhy*-based sex genotyping in *O. bonariensis* broodstock using primers that amplify part of the 5' flanking region and part of the *amhy* gene (1896 bp); *amha* gene was used as positive control (2441 bp). The dotted-boxes indicate parents used in the rearing experiment and asterisks indicate disagreement between the *amhy*-based genotype and phenotypic sex. NC: negative control.

Figure 2. Expression of *amhy* and *amha* mRNAs in tissues and embryos. A: Tissue distribution of *amhy* and *amha* mRNAs in juvenile pejerrey (RT-PCR). **B**: Expression profile of *amhy* and *amha* during embryogenesis in $amhy^{+/-}$ and $amhy^{-/-}$ genotypes (RT-PCR). *β-actin* was used as endogenous control. NC: negative control.

Figure 3. **Quantification of *amhy, amha* and *cyp19a1a* mRNAs during sex differentiation**. **A** to **C**: Abundance of mRNA transcripts of *amhy* (A) and *amha* (B) in $amhy^{+/-}$ genotypes and of *amha* in $amhy^{-/-}$ genotypes (C) during larval development at 25ºC (n=3 to 6 per time point; qRT-PCR). **D**: Abundance of *amha* mRNA transcripts in relation

to *cyp19a1a* in *amhy*$^{+/-}$ and *amhy*$^{-/-}$ genotypes at 4 and 6 weeks after hatching (qRT-PCR); arrows indicate two arbitrarily-defined, opposing patterns of gene expression. *β-actin* was used as endogenous control. Values with different letters are statistically different from one another (One-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 4. Spatial expression of *amhy* and *amha* mRNAs in differentiating gonads. Localization *amhy* and/or *amha* transcripts by ISH (left panels) and light microscopic histology (right panels) of gonads in 4 and 10 week old larvae reared at 25ºC. Transcripts were detected in all *amhy*$^{+/-}$ genotypes (presumptive *amhy* and/or *amha* signals) and in about half of the *amhy*$^{-/-}$ genotypes (*amha* signals). At 10 wah, the expression was detected in developing testis but not in developing ovaries. Scale bars indicate 10 μm.

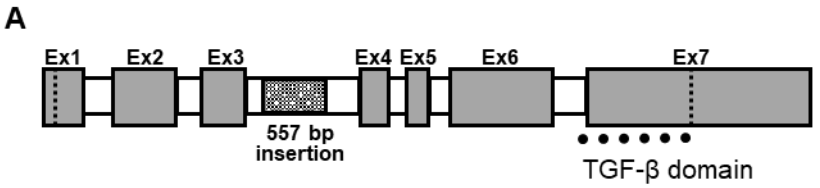
Table S1. Details of the primers used for *amhy* cloning, *amhy* genotyping and expression analysis with the respective PCR conditions.

Table S2. Proportion of *amhy*$^{+}$ and *amhy*$^{-/-}$ genotypes in the progenies produced by single-pair crosses using laboratory broodstock fish.

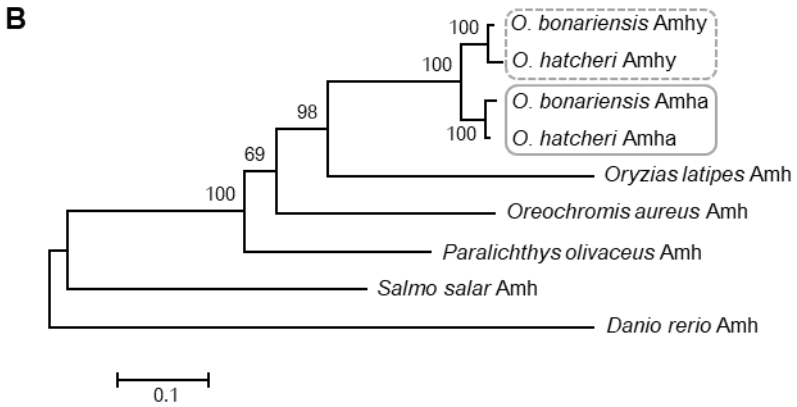
Figure S1. **Quantification of *β-actin* mRNA during larval development.** Abundance of *β-actin* mRNA transcripts in trunks of larvae reared from 1 to 10 weeks after hatching at 25ºC (qRT-PCR). Symbols and bars indicate the means and SEM, respectively. Values

with the same letter are not statistically different from one another (One-Way ANOVA with Bonferroni's post-test, $p>0.05$).

Figure 1



Region	Exon							5' UTR	3' UTR	TGF-beta domain
	I	II	III	IV	V	VI	VII			
Size (bp)	167	115	170	102	96	413	871	38	492	283
Identity (%)	86.2	95.5	97.0	96.0	100	96.6	95.3	71.0	94.5	96.1



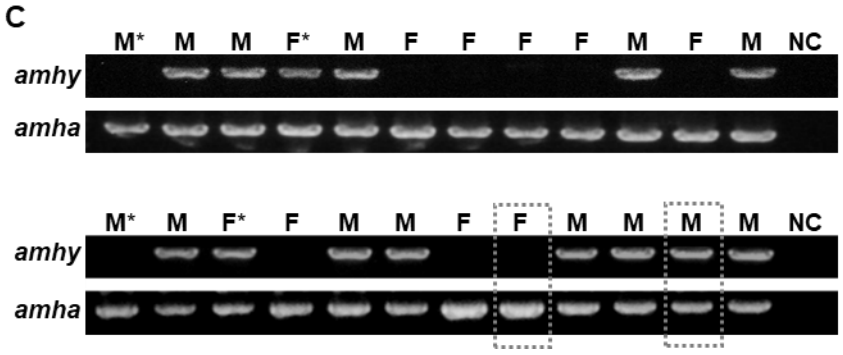


Figure 2

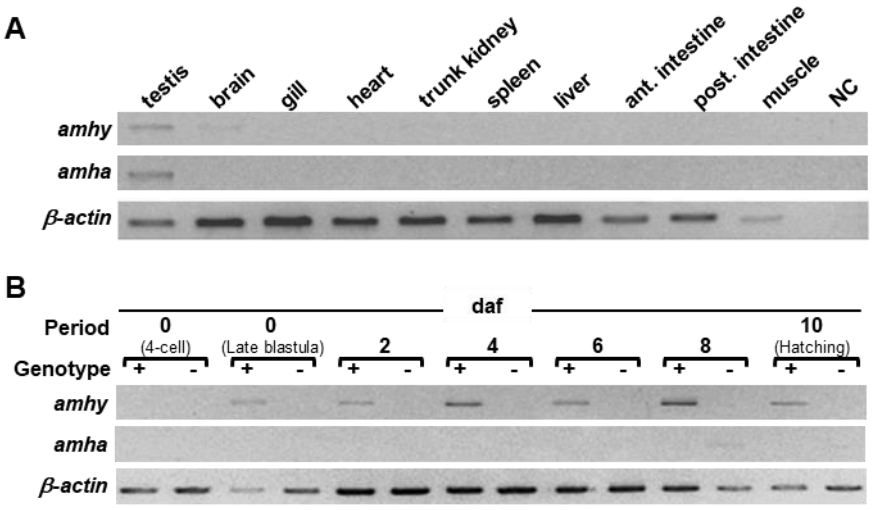

A
testis
brain
gill
heart
trunk kidney
spleen
liver
ant. intestine
post. intestine
muscle
NC
amhy
amha
β-actin
B
daf
Period
0
(4-cell)
0
(Late blastula)
2
4
6
8
10
(Hatching)
Genotype
+
-
amhy
amha
β-actin

Figure 3

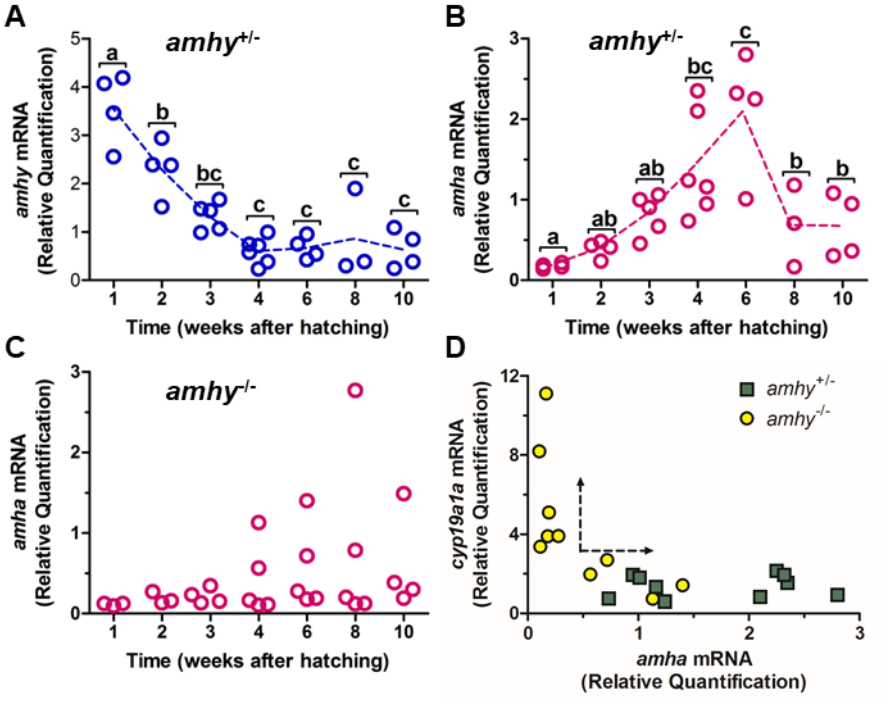

A
amhy+/-
amhy mRNA (Relative Quantification)
a
b
bc
c
c
c
c
Time (weeks after hatching)
B
amha+/-
amha mRNA (Relative Quantification)
a
ab
ab
bc
c
b
b
Time (weeks after hatching)
C
amhy-/-
amha mRNA (Relative Quantification)
Time (weeks after hatching)
D
amhy+/-
amhy-/-
cyp19a1a mRNA (Relative Quantification)
amha mRNA (Relative Quantification)

Figure 4

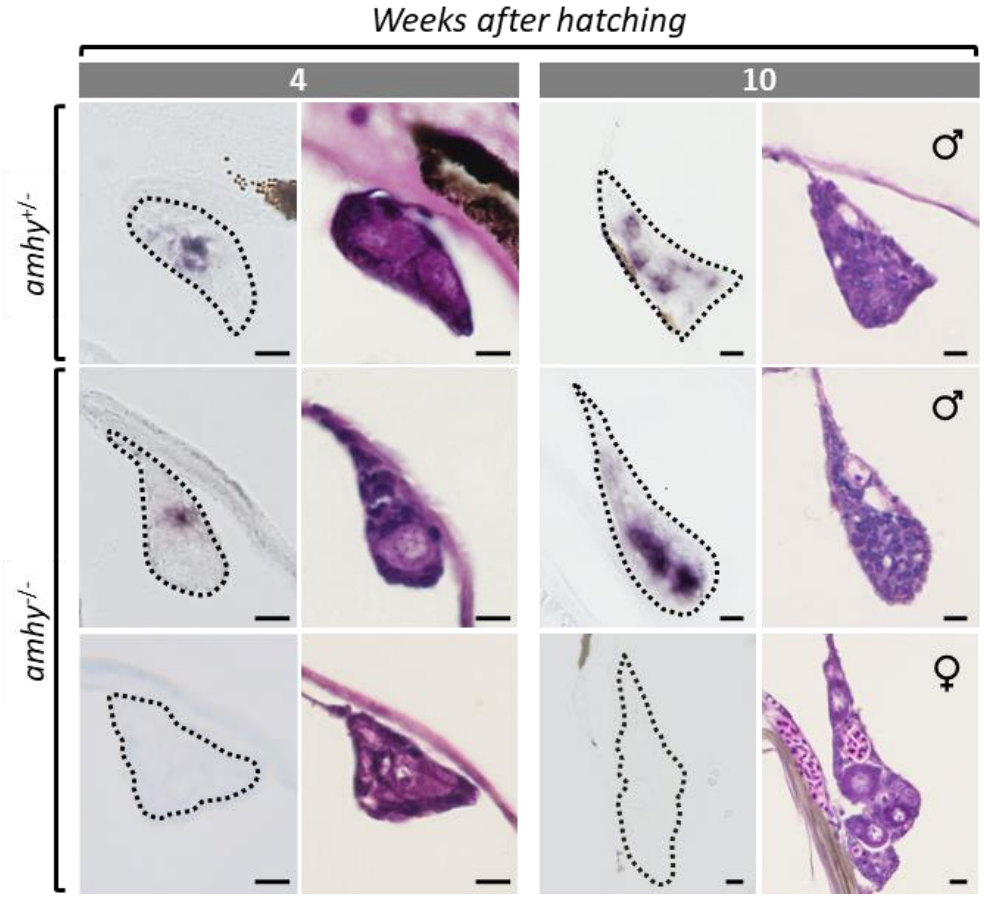


Table S1.

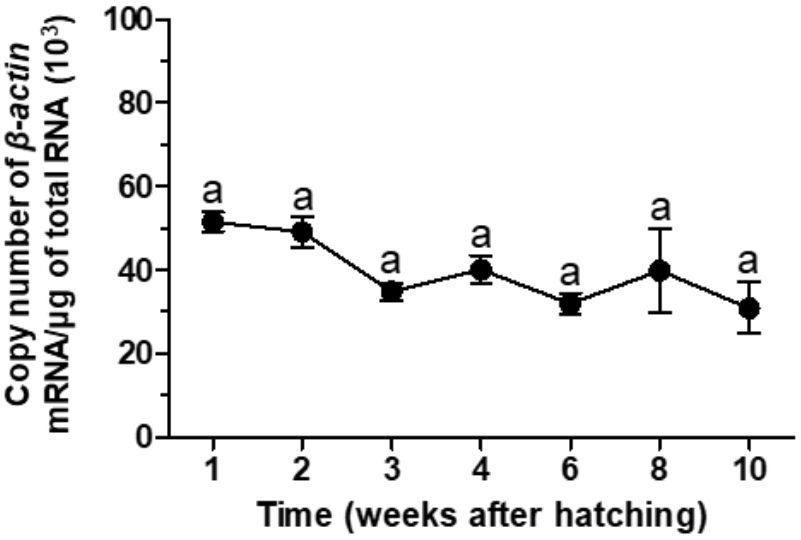
Purpose	Oligo name	Oligo sequence (’5 – ’3)	Primer binding sites (amplicon size)	PCR conditions
amhy UTRs amplification	Amhy 3RACE-1st	AGTCTCACCGTG TCCTTCGAAAAG	+1276 to +1299	1x 94°C 2 min; 35x 94°C 30 sec, 58°C 30 sec and 72°C 90 sec; 72°C 2 min
	Amhy 3RACE-2nd:	GGTGGTCATAGA CTGGAACGAGGA A	+1464 to +1488	1x 94°C 2 min; 35x 94°C 30 sec, 56°C 30 sec and 72°C 90 sec; 72°C 2 min
	amhy 5RACE-1st	CAGAAGACGCCA TATTGAGAC	+1905 to +1925	
	amhy 5RACE-2nd:	TCCACAAAGCAG GGTATGAG	+142 to +161	
amhy amplification (*amhy* genotyping)	OhaYPFw	AGTCAGCTCAGA TGCT	-1387	1x 94°C 5 min; 35x 95°C 30 sec, 60°C 30 sec, 72°C 150 sec; 1x 72°C 5 min
	OhaYPRv	AGCCGGATGCAA AACTTCCAGA	339	
			(1896 bp)	
amha amplification	OboAmhaFw	ACGCGGGTCACA CAGGCGTTTC	-38 to -17	1x 94°C 5 min; 35x 95°C 30 sec, 60°C 30 sec, 72°C 150 sec; 1x 72°C 5 min
	OboAmhaRv	CCGTCTGCATAA AACAAAC	+1194 to +2394	
			(2432 bp)	
amhy qRT-PCR (TaqMan)	qPCR-amhyFw	GCACGTCGGAGG TCGGA	-38 to -21	1x 50°C 2 min; 1x 95°C 20 sec; 40x 95°C 3 sec, 63°C 30 sec
	qPCR-amhyRv	GAGGTTATGAGG TGCTGAGGAAGT TA	+118 to +144	
	TaqMan Probe	TCGTGCATCGGC AGAG	+53 to +69	
			(182bp)	
amha qRT-PCR (Taqman)	qPCR-amhaFw	AAACAGCAGCAG GTGAGAGTCA	+1130 to +1151	1x 50°C 2 min; 1x 95°C 20 sec; 40x 95°C 3 sec, 60°C 30 sec
	qPCR-amhaRv	TGATGGAGAGAA AAGACTCTTCCG	+1511 to +1534	
	TaqMan Probe	CCAGTCCACGAC CTCCAGGGGGT	+1447 to +1469	
			(405bp)	
β-actin qRT-PCR	qPCR-actinFw	TCGTGCGCGACA TTAAGGA	+623 to +641	1x 50°C 2 min; 1x 95°C 20 sec;

(TaqMan)	qPCR-actinRv TaqMan Probe	GCAGCGGTCCCCATTTC CTGTGTTACGTTGCATTGGACTTTGAGCA	+676 to +692 +646 to +674 (70bp)	40x 95°C 3 sec, 60°C 30 sec
β-actin qRT-PCR (SYBR Green)	qPCR-obb actinFw qPCR-obb actinRv	GCTGTCCCTGTACGCCTCTGG GCTCGGCTGTGGTGGTGAAGC	+417 to +437 +596 to +616 (200bp)	1x 50°C 2 min; 1x 95°C 10 min; 40x 95°C 10 sec, 60°C 30 sec
cyp19a1a qRT-PCR (SYBR Green)	qPCR-AromGFw qPCR-AromGRv	GCGAGCTGTCTGGCTGAGAA AGGAGCAGCAGCATGAAGAAGA	+902 to +920 +980 to +1001 (200bp)	1x 50°C 2 min; 1x 95°C 10 min; 40x 95°C 10 sec, 60°C 30 sec

Table S2.

Crosses		Progeny (n)	Genotype Proportion	
Female (genotype)	**Male (genotype)**		***amhy*$^{+}$**	***amhy*$^{-/-}$**
F1 (*amhy*$^{-/-}$)	M1 (*amhy*$^{+}$)	36	47.2	52.8
	M2 (*amhy*$^{+}$)	50	50.0	50.0
	M3 (*amhy*$^{+}$)	94	48.7	51.3
	M4 (*amhy*$^{+}$)	81	47.5	52.5
	M5 (*amhy*$^{+}$)	98	53.1	46.9
	M6 (*amhy*$^{+}$)	24	33.3	66.7
	M7 (*amhy*$^{+}$)	56	51.8	48.2
	M8 (*amhy*$^{+}$)	49	48.9	51.1
	M9 (*amhy*$^{+}$)	30	53.3	46.7
F2 (*amhy*$^{+}$)	M10 (*amhy*$^{-/-}$)	35	40.0	60.0

Figure S1


Copy number of β-actin mRNA/μg of total RNA (10³)
100
80
60
40
20
0
a
a
a
a
a
a
a
1
2
3
4
6
8
10
Time (weeks after hatching)

Chapter 2

Differential expression and regulation of *amhy* and *amha* mRNA during temperature-dependent sex determination in pejerrey *O. bonariensis*

Abstract

Sex determination in pejerrey *Odontesthes bonariensis* is characterized by a strong temperature dependence (TSD). However, we recently identified a homologue of a testis determinant, *amhy*, and demonstrated that at an intermediate temperature its presence (XY/YY) or absence (XX) can favor the formation of males and females, respectively. In this study, we investigated the transcriptional profiles of *amhy* and the autosomal *amh*, *amha* at feminizing and masculinizing temperatures during early larval development with the aim to evaluate their relationship with TSD and testis formation. XY and XX larvae were reared at 17ºC and 29ºC (female- and male-promoting temperatures, respectively) during the critical period of thermolabile sex determination and used for transcriptional analyses of *amhy* and *amha* by qRT-PCR. The expression analyses showed that *amhy* mRNAs were highly expressed in XY larvae from both 17ºC and 29ºC groups at the beginning of sex determination period but declined thereafter. *amha* was upregulated during the sex determination period in a few XY larvae at 17ºC and in both genotypes at 29ºC and was highly correlated with maleness. As increased cortisol and subsequent increase of 11-ketotestosterone (11-KT) has been implicated in the temperature-induced masculinization in pejerrey, we performed a luciferase reporter assay with the presumptive promoters (~3kb 5' upstream fragment) of both *amh* paralogues to investigate their regulation by cortisol and 11-KT *in vitro*. The glucocorticoid receptor expression plasmid was co-transfected with luciferase reporter plasmids containing *amhy* or *amha* promoter into endothelial progenitor cells. Transcriptional activity was measured 48 hours post-transfection in cells exposed to different cortisol and 11-KT doses. Transcriptional activity analyses showed that the *amhy* promoter did not respond to any concentration of cortisol

and 11-KT, whereas *amha* transcription was activated by both cortisol and 11-KT in dose dependent manners. These results suggest that *amhy* is considered as a genotypic sex determinant and temperature-independent, but the *amha* regulated by cortisol and 11-KT might have key roles in a temperature-induced testicular formation in pejerrey.

Introduction

Sex determination (SD) in many teleost fish is considered a fine-tuned process driven by the balance between internal genotypic (genotypic sex determination or GSD) and external environmental (environmental sex determination or ESD) factors, particularly during a critical period of early gonadal development. Interaction of these internal and external factors can affect the ratio between androgens and estrogens, and thus ultimately determine the gonadal fate of an individual. In fish, environmental factors such as hypoxia, pH, background color, and temperature can significantly affect sex determination and differentiation (Cheung et al. 2014; Papoutsoglou et al. 2000; Rotllant et al. 2003; Merighe et al. 2004; Mankiewicz et al. 2013).

Sex determination in pejerrey *Odontesthes bonariensis* is characterized by a strong temperature dependence (TSD). In this species, sex ratios reach 100% female or 100% male at the environmentally relevant temperatures of 17°C (female producing temperature, FPT) and 29°C (male producing temperature, MPT) respectively, when the thermal exposure occurs between hatching and the onset of histological differentiation of the gonads (around 5 weeks post hatch). Recently, we identified homologue of a testis-determining gene *amhy* (Y-linked anti-Müllerian hormone), which is thought to be a duplicated copy of autosomal *amh* (*amha*), and was demonstrated that its presence (XY/YY) or absence (XX) can favor the formation of males and females, respectively at an intermediate temperature (Yamamoto et al., 2014). This report represents the first clear evidences that genotypic and environmental sex determinants can coexist in species with marked TSD (Yamamoto et al., 2014). However, precise function and regulation of *amhy*

and *amha* genes during sex determination/differentiation in relation to temperatures remains unknown.

A number of studies have shown that stress hormone cortisol is involved in masculinization. For instance, blue background color and high temperature are able to yield male-biased populations, which seems to be mediated by increased levels of cortisol (Yamaguchi et al., 2010; Hayashi et al., 2010; Hattori et al., 2009). In medaka (*Oryzias latipes*), high temperature induced masculinization of genetically females by elevation of cortisol levels, which in turn suppressed both the expression of *follicle-stimulating hormone receptor* (*fshr*) mRNA and the female-type proliferation of germ cells during sexual differentiation (Hayashi et al., 2010). In flounder (*Paralichthys olivaceus*), Yamaguchi et al. (2010) proposed that cortisol induces masculinization by direct suppression of *cyp19a1a* mRNA expression via interference with cyclic adenosine monophosphate (cAMP)-mediated activation. The suppression of *cyp19a1a* transcription by cortisol was also found in pejerrey (Hattori et al., 2009).

The other important gene in thermal stress-induced masculinization in pejerrey is considered to be 11β-hydroxysteroid dehydrogenase (11β-HSD), which encodes an enzyme involved in both glucocorticoid and androgen syntheses (Fernandino et al., 2012; 2013). In pejerrey, cortisol treatment produced significant increases in *hsd11b2* mRNA expression and 11-ketotestosterone (11-KT) levels *in vivo* (Fernandino et al., 2012). For this reason, it has been proposed that the masculinization induced by thermal stress in pejerrey occurs by means of cortisol inactivation and the concomitant synthesis of 11-KT, which acts as an inducer of masculinization (Fernandino et al., 2013). In spite of such a progress made in the aspect of masculinization under thermal stress, how thermal stress

and androgen interact with sex-related genes and ultimately lead to testicular formation remains elusive.

To gain a better understanding of the mechanism of coexistence of GSD and TSD in pejerrey, in this study, we investigated the transcriptional profiles of *amhy* and *amha* at feminizing (17ºC) and a masculinizing (29ºC) temperatures during the critical period of sex determination/differentiation. In this experiment, we also examined expression profiles of AMH type II receptor (*amhrII*) and ovarian aromatase (*cyp19a1a*). Then, we investigated the *in vitro* regulation of *amhy* and *amha* by cortisol and 11-KT by a luciferase reporter assay.

Material and Methods

Rearing procedures and sampling for mRNA expression and histological analyses

An XX female and an XY male were selected and allowed to breed naturally in a 650-liter recirculated water rearing tank under controlled temperature (20°C), photoperiod (14L/10D), and salinity (0.2–0.5% NaCl in dechlorinated tap water). Fertilized eggs were collected, cleaned of chorionic filaments, and transferred to incubators with flowing brackish water (salinity of 0.2–0.5%) at 19°C. After hatching (about 9 days after fertilization), approximately 800 to 1000 newly-hatched larvae were stocked in each of two 60-liter tanks and reared at 17°C and 29°C for up to 14 weeks. Other rearing and sampling procedures followed the previous study (Yamamoto et al., 2014).

amhy genotyping of sampled larvae

Genomic DNA was extracted following the protocol described by Aljanabi and Martinez. Primers (YYFw 1548 5’ - AGTAAATTTGCCGGAGGCTTG - 3’ and Amhy 182R 5’- GAGGTTATGAGGTGCTGAGGAAGTTA -3’) designed within the 5’ flanking region of *amhy* gene of pejerrey were used for genotyping. PCR reaction conditions were as follows: 3 min at 94°C, 35 cycles of 15 sec at 94°C, 30 sec at 60°C and 2 min 30 sec at 72°C, final elongation for 5 min at 72°C. *amha* amplification was analyzed as a positive control based on previous study (Yamamoto et al., 2014)

Transcriptional analyses of amhy, amha, cyp19a1a and amhrII mRNAs

For the transcriptional analyses of *amhy, amha, cyp19a1a* and *amhrII* mRNAs, trunks of larvae were stored in RNAlater (Sigma-Aldrich, St. Louis, MO) at -80°C until use. Trizol Reagent (Life Technologies) was used for total RNA extraction. All procedures followed the reagent manufacturer's protocol. Synthesis of cDNA and transcription analyses of *amhy, amha*, and *β-actin* in larvae were performed by qRT-PCR following protocols described in the previous study (Hattori et al., 2012; Fernandino et al., 2008a; Yamamoto et al., 2014). Partial sequence of *amhrII* was isolated and primers for expression analysis were designed (Fig. 11, unpublished). The primers were: Obo qRT amhrII Fw2 5'- CCAACTCCTATTTTGCAGCTG - 3' and Obo qRT amhrII Rv3 5'- GGCTGTAATCATGACAAGAGG - 3'.

Isolation and sequencing of amhy and amha presumptive promoters

The 5'- flanking regions of pejerrey *amhy* and *amha* were isolated from the pejerrey genomic DNA of an XY adult male. Primers used for *amhy* promoter amplification were OhaYpro1Fw 5'- GTGGTCCGATGGAAAATTAAGTACTG - 3', designed from the Patagonian pejerrey *Odontesthes hatcheri amhy* promoter region and Amhy 182R 5'- GAGGTTATGAGGTGCTGAGGAAGTTA - 3', designed from the pejerrey *amhy* open reading frame (ORF). Pejerrey *amha* promoter was amplified using OboApro 22Fw 5' - CTAAGAAGGAGGCTCACTGTCCCTTGTC - 3', designed based on the Patagonian pejerrey *Odontesthes hatcheri* promoter sequence and Amha 201R 5' -

CCACAAAGCAGGGTGCG - 3' designed based on the pejerrey *amha* ORF (see Hattori et al., 2012 for more promoter information). Amplifications were done according to the following conditions: 3 min at 94°C, 35 cycles of 15 sec at 94°C, 45 sec at 60°C and 4 min at 72°C, 7 min at 72°C. Amplicons were then cloned in to pGEM®-T Easy Vector (Promega) following the manufacturer's instructions and sequenced in an ABI PRISM 3100 capillary sequencer (Life Technologies, Carlsbad, CA) using the BigDye Terminator method. Sequences were analyzed with GENETYX version 11.0 (GENETYX, Tokyo, Japan).

Isolation and sequencing of pejerrey glucocoticoid receptor1, gr1and androgen receptor α, arα cDNAs

1ug of total mRNAs extracted from adult testis (for *gr1*) and ovary (for *arα*) was reverse-transcribed following the protocol in previous study (Yamamoto et al., 2014). RT-PCR using adult testis cDNA in the mixture [0.2mM dNTPs, 1 x PrimeSTAR Buffer (Mg^{2+} plus), 0.25 unit of PrimeSTAR® HS DNA Polymerase, Takara, Japan] was performed to amplify the ORF of pejerrey *gr1* and *arα* . OboGr1 Fw1 5' - ACTGCCACTTTCAACCAAAACAATG - 3', degenerated from the 5'UTR of *Dicentrarchus labrax* (Genbank accession code AY619996) and *Oryzias dancena* (Genbank accession code HM598069) glucocorticoid receptors and OboGr1 90Rv 5'-GCTGTTGCTGAGGCCGTTAG - 3' designed on the basis of the pejerray *gr1* partial mRNA sequence (Genbank accession code HQ843506) were used to obtain the translation start site of pejerrey *gr1*. Thermal conditions of PCR amplifications were as follows: 10 sec at 98°C, 30 cycles of 10 sec at 98°C, 5 sec at 56°C and 30 sec at 72°C, then followed

by a final elongation for 3 min at 72°C. Primers used for amplifying pejerrey *arα* ORF (pjARalphaORF-Fw 5' - ATGGCCTTTCTCTCGAGCTTG - 3' and pjARalphaORF-Rv 5' - CTAGGCTCTATCGTGGAAAAGG - 3') were designed based on the whole genome sequence of pejerrey (unpublished). Thermal conditions for amplifications of *arα* were10 sec at 98°C, 30 cycles of 10 sec at 98°C, 5 sec at 56°C and 2 min 45 sec at 72°C, following a final elongation for 5 min at 72°C. PCR products were electrophoresed in 1% agarose gel, purified, and sequenced as described above.

Plasmid construction

The pejerrey *amhy* and *amha* presumptive promoters were ligated into the pGL4.10[*luc2*] reporter vector (Promega) to construct *amhy*- and *amha*- luciferase reporter plasmids. The GR1- and ARα-expression plasmids were constructed by ligating the *gr1* and *arα* ORF into pcDNA3.1 (Invitrogen) according to the manufacturer's protocol.

Transient transfection assay

EPCs (endothelial progenitor cells) generated from fathead minnow *Pimephales promelas*, was kindly gifted from Dr. Kunihiko Futami's lab, were cultured in D-MEM (Wako) supplemented with 5% charcoal- stripped fetal bovine serum (biowest) at 25°C. The cells were plated in 48-well plates 24 hours before transfection. 120 ng of the *amhy*- or *amha*- luciferase reporter, 120 ng of Gr1- or Arα- expression plasmid, and 30ng of the pRL-SV40 (Promega) normalization plasmid were then co-transfected in triplicate into the

cells following the protocol provided by TransIT-LT1 Transfection Reagent (Mirus). Cortisol (Sigma) and 11-KT (Sigma) were dissolved into DMSO and 100% ethanol, respectively. Cells were treated with or without cortisol (0 uM, 1 uM, 10 uM, 100 uM) or 11-KT (0 uM, 0.1 uM, 1 uM, 10 uM, 100 uM) 24 hours after transfection and another 24 hours later, luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) and measured the fluorescent by a luminometer Gene Light 55 (MICROTEC) according to the manufacturer's protocol.

Statistics analyses

Data for *amhy, amha, cyp19a1a* and *amhrII* expression were analyzed by one-way ANOVA with Bonferroni's post-test at different time point within treatments and Two-Way ANOVA with Bonferroni's post-tes between treatments at the same time point. Data for luciferase assay were analyzed by one-way ANOVA with Bonferroni's post-test. All the data analyses were performed using GraphPad Prism (v.5.00; GraphPad Software, San Diego, CA, USA).

Results

Phenotypic sex ratios at 17ºC and 29ºC reared larvae

Analysis of the phenotypic sex and genotype at 14 wah showed that all XX fish (n=38) at 17ºC were females whereas the XY were either males or females (n=16 and 11, respectively). 29ºC yielded a single-sex population with all larvae (n=50) developed as males regardless of *amhy* genotype (Table 1).

Transcriptional analyses of amhy, amha, cyp19a1a and amhrII at 17ºC, 25ºC and 29ºC reared larvae

amhy expression showed a transient peak at 2 wah at 17ºC and at 1 wah at 29ºC, followed by a decrease at both temperatures (Fig. 1). *amha* expression in XY fish was low from 1 wah to 6 wah and increased in a few XY larvae slightly but significantly after 8 wah in XY animals at 17ºC, whereas at 29ºC it was up-regulated between 3 and 8 wah (Fig. 2). All XX individuals at 17ºC had low *amha* expression throughout the experiment whereas at 29ºC a notable increase was observed between 3 and 8 wah (Fig. 3), showing a high correlation with maleness. *cyp19a1a* expression was inversely proportional to temperature in XX fish (Fig. 6B). XY fish had higher *cyp19a1a* at 17ºC compared to that at 25 and 29ºC (Fig. 6A). Dimorphic distribution of *cyp19a1a* in XX genotype at 25ºC and in XY genotype at 17ºC was detected. *amhrII* expression did not differ between XX and XY genotypes, but much more pronounced *amhrII* expression at 25ºC and 29ºC than that at 17ºC was observed in both genotypes (Fig. 7).

Isolation and sequencing of amhy, amha presumptive promoters and gr1, arα cDNA

amhy presumptive promoter 3522 bp and *amha* promoter 3341 bp upstream of translation start site were isolated (Fig. 8 and Fig. 9). A half GRE/ARE half site in *amhy* and 2 half GRE/AREs (5' – TGTTCT – 3') in *amha* promoter region were identified using ALGGEN-PROMO online free software (http://alggen.lsi.upc.edu/). The pejerrey *gr1* cDNA encoding a 782 amino acids (Fig. 10) and Androgen receptor α, *ar*α encoding 690 amino acids (Fig. 11) were isolated.

Cortisol and 11-KT activate amha but not amhy gene transcription via GR1and ARα *in vitro*

To investigate whether cortisol mediates high temperature-induced masculinization by activating *amhy* or *amha* transcription, a Dual-luciferase reporter assay was performed using *gr1*, *ar*α and the presumptive promoters (~3kb 5' upstream fragment) of both *amh* paralogues. Transcriptional activity analyses showed that while *amhy* promoter did not respond to any cortisol and 11-KT doses (Fig. 4A, Fig. 5A), *amha* transcription was distinctly activated by both cortisol and 11-KT (Fig. 4B, Fig. 5B). Transcriptional activity of *amha* promoter revealed a cortisol dose-dependent manner (Fig. 4B).

Discussion

In the present study, we examined the transcriptional profiles of the sex determinant *amhy* and its paralogue *amha* in pejerrey *Odontesthes bonariensis* to assess their relation to the temperature-dependent sex determination and testicular formation. The pejerrey larvae were reared at 29ºC for male promoting temperature and 17ºC for female promoting temperature during early larval development. Both of the temperatures showed a high at very early development and subsequent down regulation of *amhy* during the critical period for sex determination (1-5 wah). The similar expression profile of *amhy* is also observed at 25ºC, a mixed sex-producing temperature (Yamamoto et al., 2014), suggested that expression of *amhy* during sex determination/differentiation might be temperature-independent.

In contrast to *amhy*, clear differences in *amha* expressions were observed in different temperatures. At masculinizing temperature (29ºC), *amha* was up-regulated during sex determination/differentiation periods in both XY and XX genotypes and all the larvae developed as males. In contrast, at feminizing temperature (17ºC), *amha* expression was relatively low compared to those of at masculinizing temperature. Levels of *amha* mRNA maintained low during sex determination/differentiation periods in XX individuals and all of them developed as females. However, in XY individuals, *amha* expressions at 8 and 10 wah showed a bimodal pattern and 41% and 59% larvae developed as females and males, respectively. In our previous study revealed that high and low *amha* expressions were associated with maleness and femaleness, respectively at an intermediate temperature (Yamamoto et al., 2014). Therefore, XY individuals showed high *amha* expression

overlapping to *amhy* expression may differentiate as male and those without it may differentiate as female. Thus, expression profiles of *amha* at 17ºC and 29ºC suggested that *amha* seemed to be regulated by temperature and involved in the testicular differentiation of pejerrey. Moreover, high *amha* expression observed only in XY genotype at 17ºC indicated that *amhy* is a strong genotypic determinant and may act as a trigger of masculinization, which may in turn up-regulate *amha* during early larval development.

Interestingly, expression profile of *amhy* during sex determination/differentiation in pejerrey was different from those of in Patagonian pejerrey. In Patagonian pejerrey, species with marked GSD, expression of *amhy* mRNA maintained high during sex determination period and no overlapped expression of *amha* was observed at this period (Hattori et al., 2012). In contrast, in pejerrey, species with marked TSD, expression of *amhy* mRNA was high only at the beginning of sex determination period but declined thereafter. However, *amha* complementary expressed during sex determination period. Generally, sex-determining genes found in other species, such as *sdY* in rainbow trout and *dmy/dmrt1bY* in Japanese medaka, maintained high during the sex determination/differentiation period (Yano et al., 2012; Nanda et al., 2002). Pejerrey possesses strong TSD system and temperature modulates fate of sex after genotypic sex determination. Thus, this unique decline of genotypic sex determinant *amhy* may be linked to the mechanism of TSD in this species.

As introduced above, the stress hormone cortisol has been implicated in the high temperature-induced masculinization of many species including pejerrey (Hattori et al., 2009; Fernandino et al., 2012; Fernandino et al., 2013; Hayashi et al., 2010; Yamaguchi et al., 2010). In medaka (*Oryzias latipes*), high temperature induced masculinization of

genetically females by elevation of cortisol levels, which in turn suppressed both the expression of *follicle-stimulating hormone receptor* (*fshr*) mRNA and the female-type proliferation of germ cells during sexual differentiation (Hayashi et al., 2010). In flounder (*Paralichthys olivaceus*), Yamaguchi et al. (2010) demonstrated that cortisol induces masculinization by direct suppression of *cyp19a1a* mRNA expression via interference with cyclic adenosine monophosphate (cAMP)-mediated activation. In pejerrey, the other key gene in thermal stress-induced masculinization is considered to be the 11β-hydroxysteroid dehydrogenase (11β-HSD), one of the enzymes shared by the glucocorticoid and androgen pathways (Fernandino et al., 2012; 2013). Cortisol treatment in pejerrey produced significant increases in *hsd11b2* mRNA expression and 11-ketotestosterone (11-KT) levels *in vivo* (Fernandino et al., 2012), before the suppression of *cyp19a1a* transcription. For this reason, it has been proposed that the masculinization induced by thermal stress in pejerrey occurs by means of cortisol inactivation and the concomitant synthesis of 11-KT, which acts as an inducer of masculinization (Fernandino et al., 2013). The key role of cortisol during gonadal sex change has also been reported in several hermaphroditic fish species (Nozu and Nagahama, 2015; Solomon-Lane et al., 2013; Godwin and Thomas, 1993). In the protogynous bluehead wrasse *Thalassoma bifasciatum*, dimorphic expression of *hsd11b2* and glucocorticoid receptor in gonad was observed and local cortisol production was suggested to be important in sex differences (Liu et al., 2015).

In this study, we investigated whether cortisol and 11-KT can affect *amhy* or *amha* transcription *in vitro*. The presumptive promoters (~3kb 5' upstream region) of both *amh* paralogues were isolated. Sequencing of presumptive *amhy* promoter contained one downstream-half sequence (TGTTCT) of putative ARE/GREs (AGAACANNNTGTTCT), while presumptive *amha* promoter contained two downstream-half sequences of putative

ARE/GREs. Transcriptional activity of presumptive promoters of *amhy* and *amha* were analyzed in the presence of cortisol and 11-KT using a dual-luciferase reporter assay (DLR) system. Luciferase activity showed that *amhy* promoter with one ARE/GRE did not respond to any cortisol and 11-KT doses. On the contrary, *amha* transcription was distinctly activated by both cortisol and 11-KT and transcriptional activity of *amha* promoter increased with cortisol in a dose-dependent manner. As demonstrated in a number of previous studies, ARE/GRE-like sequence, particularly the downstream half site is capable of binding the relative receptors in mammals and fish (Del Monaco et al., 1997; Hayashi et al., 2012; Schiller et al., 2014). In this study, co-activation of *amha* promoter by both cortisol and 11-KT suggested a successful binding of the GRE/ARE-half sites in pejerrey *amha* promoter to the glucocorticoid and androgen receptors and thus revealed an important role of cortisol and 11-KT on *amha* transcription. Cortisol and 11-KT may work synergistically and act respectively as a first and second trigger during thermal stress-induced masculinization of pejerrey. Ongoing studies focus on confirmation of the function of the half GRE/ARE sites by producing ARE/GRE mutant in *amha* promoters. On the other hand, impervious transcription activity of *amhy* promoter by cortisol and 11-KT may due to less number of GRE/ARE-half site compared to *amha* promoter region. In this study, the expressions of ovarian aromatase *cyp19a1a* (critical for female development) and *amh* type II receptor *amhrII* (critical for male development) were examined at the FPT and MPT, respectively. *cyp19a1a* expression was found to be inversely proportional to temperature in XX fish whereas in XY genotypes a dimorphic distribution of *cyp19a1a* was observed at the 17ºC. *amhrII* expression did not differ between XX and XY fish although it was higher at 25ºC and 29ºC than that at 17ºC in both genotypes. These results suggest that *amhy* expression is temperature-independent while *amha* and *amhrII*

expression were temperature-dependent. In pejerrey, thermal stress may modulate *amha* expression though *amh* receptors which then induce masculinization in pejerrey. High water temperature induces *amha* expression by elevated cortisol and androgen levels in pejerrey

In conclusion, the results obtained in this study suggested that *amhy* is a genotypic sex determinant in pejerrey and regulated in temperature independent manner. In contrast, *amha* is upregulated in response to high water temperature and its overlapped expression with *amhy* is regulated via cortisol and 11-KT. Thus, overlapping expression of *amhy* and *amha*, early decrease of *amhy* expression, and *amha* regulation by temperature may be keys for the coexistence of genotypic and environmental sex determinants in this species.

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Tables and Figures

Table 1. Sex ratios at 17ºC and 29ºC in relation to *amhy* genotype

Temperature	Genotype	Phenotype		Total n (%)
		Female	Male	
17ºC	XX	38	0	38 (58.5)
	XY	11	16	27 (41.5)
	Total n (%)	49 (75.4)	16 (24.6)	
29ºC	XX	0	27	27 (54.0)
	XY	0	23	23 (46.0)
	Total n (%)	0 (0.00)	50 (100)	

Figure Legends

Figure 1. **Quantification of *amhy* mRNAs during sex differentiation by qRT-PCR**. Abundance of mRNA transcripts of *amhy* in XY genotypes during larval development at 17 and 29ºC (n=3 to 8 per time point; qRT-PCR). *β-actin* was used as endogenous control. Values with different letters are statistically different from one another (lower case letter for 17ºC and upper case letter for 29ºC respectively, One-Way ANOVA with Bonferroni's post-test, $p<0.05$). *Significant difference of expression between treatments at the same time point (Two-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 2. **Quantification of *amha* mRNAs during sex differentiation by qRT-PCR**. Abundance of mRNA transcripts of *amha* in XY genotypes during larval development at 17 and 29ºC (n=3 to 8 per time point; qRT-PCR). *β-actin* was used as endogenous control. Values with different letters are statistically different from one another (lower case letter for 17ºC and upper case letter for 29ºC respectively, One-Way ANOVA with Bonferroni's post-test, $p<0.05$). *Significant difference of expression between treatments at the same time point (two-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 3. **Quantification of *amha* mRNAs during sex differentiation by qRT-PCR**. Abundance of mRNA transcripts of *amha* in XX genotypes during larval development at 17 and 29ºC (n=3 to 8 per time point; qRT-PCR). *β-actin* was used as endogenous control. Values with different letters are statistically different from one another (lower case letter for 17ºC and upper case letter for 29ºC respectively, One-Way ANOVA with Bonferroni's

post-test, $p<0.05$). *Significant difference of expression between treatments at the same time point (two-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 4. **Effects of cortisol on *amhy* (A) and *amha* (B) promoter *in vitro***. Transcriptional activity of the pejerrey *amhy* and *amha* promoter in EPCs (endothelial progenitor cells). Relative luciferase activity was calculated based on the value of the control. Vertical bars indicate means (±s.e.m.). Values with different letters are statistically different from each treatment (One-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 5. **Effects of 11-KT on *amhy* (A) and *amha* (B) promoter *in vitro***. Transcriptional activity of the pejerrey *amhy* and *amha* promoter in EPCs (endothelial progenitor cells). Relative luciferase activity was calculated based on the value of the control. Vertical bars indicate means (±s.e.m.). Values with different letters are statistically different from each treatment (One-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 6. **Quantification of *cyp19a1a* mRNAs during sex differentiation by qRT-PCR**. Abundance of mRNA transcripts of *cyp19a1a* in XY (A) and XX (B) genotypes during larval development at 17, 25 and 29ºC (n=3 to 8 per time point; qRT-PCR). *β-actin* was used as endogenous control. Values with different letters are statistically different from one another (One-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 7. **Quantification of *amhrII* mRNAs during sex differentiation by qRT-PCR**.

Abundance of mRNA transcripts of *amhrII* in XY (A) and XX (B) genotypes during larval development at 17, 25 and 29ºC (n=3 to 8 per time point; qRT-PCR). *β-actin* was used as endogenous control. Values with different letters are statistically different from one another (One-Way ANOVA with Bonferroni's post-test, $p<0.05$).

Figure 8. **Partial sequence of *amhy* promoter**. Red colored base pairs represent the transcription start site (start codon).

Figure 9. **Partial sequence of *amhy* promoter**. Red colored base pairs represent the transcription start site (start codon).

Figure 10. **Complete CDs and the encoded amino acid of pejerrey glucocorticoid receptor 1**. Red colored base pairs represent the primer sequences used in RT-PCR amplification.

Figure 11. **Complete CDs and the encoded amino acid of pejerrey androgen receptor alpha**. Red colored base pairs represent the primer sequences used in RT-PCR amplification.

Figure 12. **Partial CDs of pejerrey amh type II receptor gene**. Green colored base pairs represent the primer sequences used in qRT-PCR analysis.

Figure 1

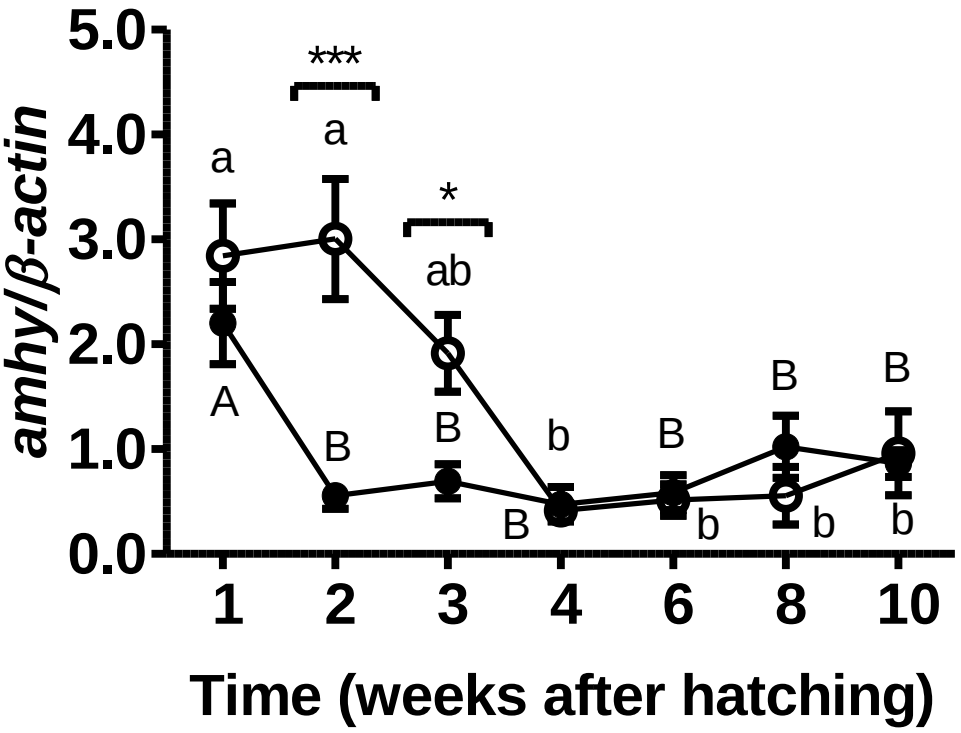
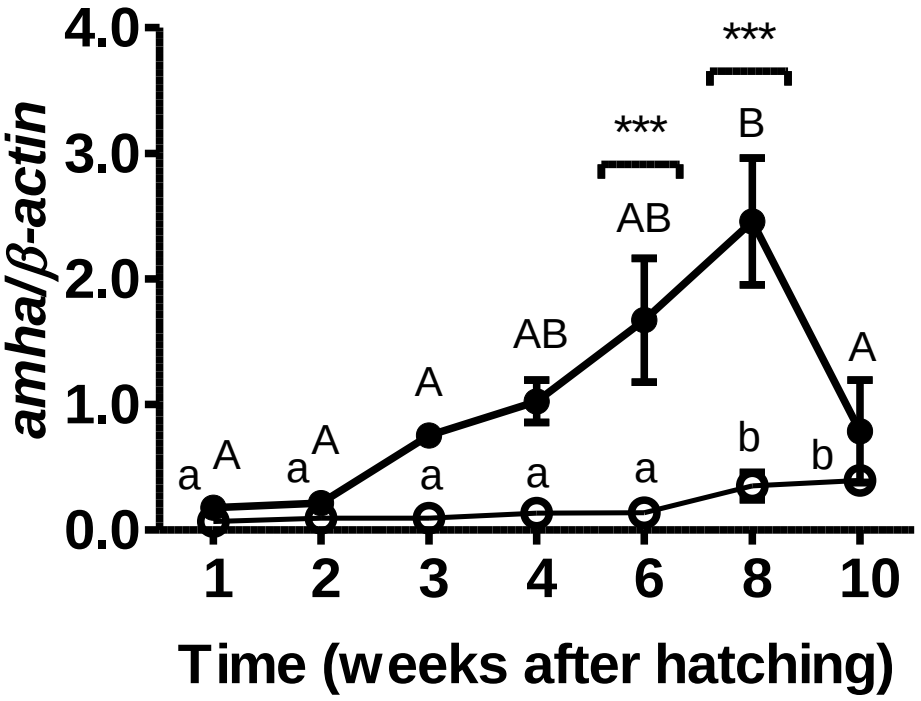

5.0
4.0
3.0
2.0
1.0
0.0
amhy/β-actin
***
*
a
a
ab
A
B
B
B
b
B
B
B
b
b
b
1
2
3
4
6
8
10
Time (weeks after hatching)

Figure 2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3.0
2.0
1.0
0.0
amha/β-actin
***
***
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AB
AB
A
A
A
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a
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a
a
a
b
b
1
2
3
4
6
8
10
Time (weeks after hatching)

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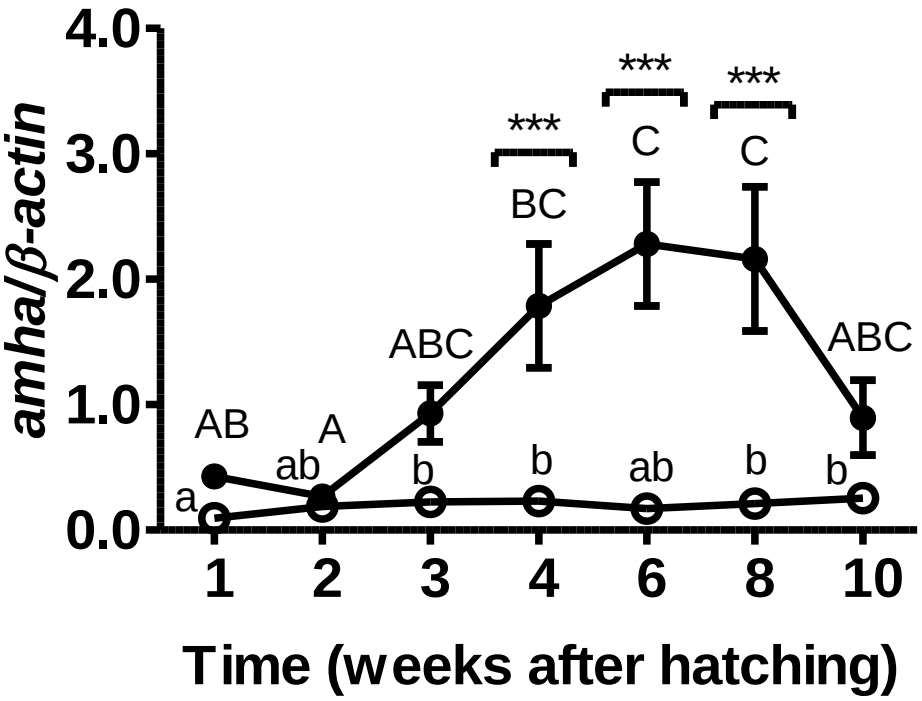
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3.0
2.0
1.0
0.0
amha/β-actin
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***
***
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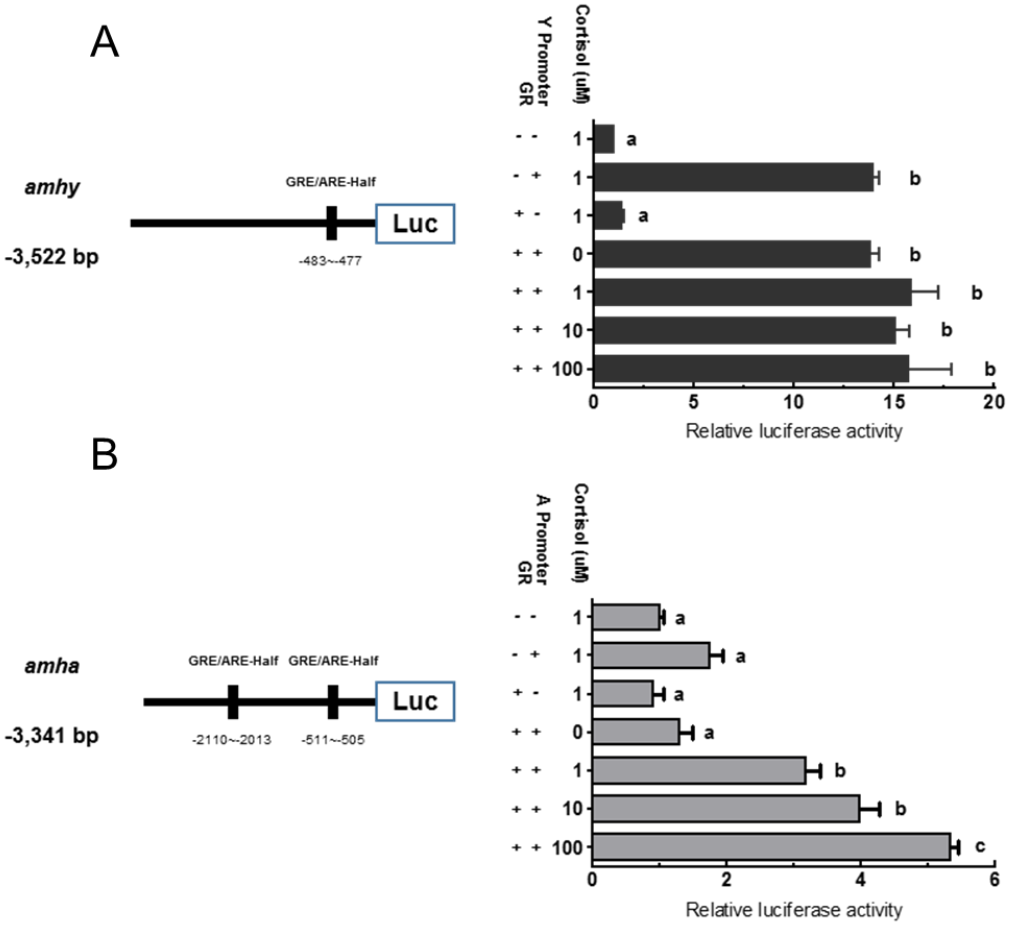
C
BC
ABC
ABC
AB
A
ab
a
b
b
ab
b
b
1
2
3
4
6
8
10
Time (weeks after hatching)

Figure 4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amhy
GRE/ARE-Half
Luc
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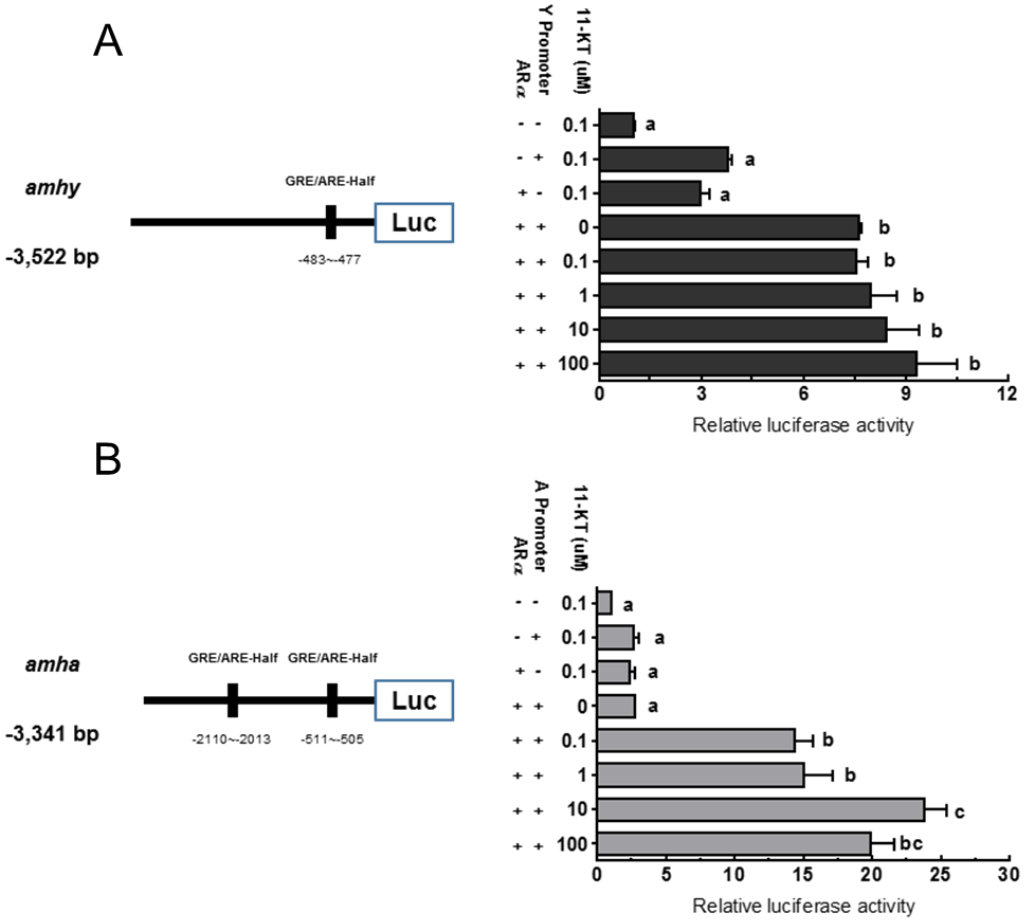
-483~-477
Y Promoter
GR
Cortisol (uM)
- - 1 a
- + 1 b
+ - 1 a
+ + 0 b
+ + 1 b
+ + 10 b
+ + 100 b
0 5 10 15 20
Relative luciferase activity
B
amha
GRE/ARE-Half GRE/ARE-Half
Luc
-3,341 bp
-2110~-2013
-511~-505
A Promoter
GR
Cortisol (uM)
- - 1 a
- + 1 a
+ - 1 a
+ + 0 a
+ + 1 b
+ + 10 b
+ + 100 c
0 2 4 6
Relative luciferase activity

Figure 5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amhy
GRE/ARE-Half
Luc
-3,522 bp
-483~-477
Y Promoter
ARα
11-KT (uM)
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0.1
0.1
0
0.1
1
10
100
a
a
a
b
b
b
b
b
0
3
6
9
12
Relative luciferase activity
B
amha
GRE/ARE-Half
GRE/ARE-Half
Luc
-3,341 bp
-2110~-2013
-511~-505
A Promoter
ARα
11-KT (uM)
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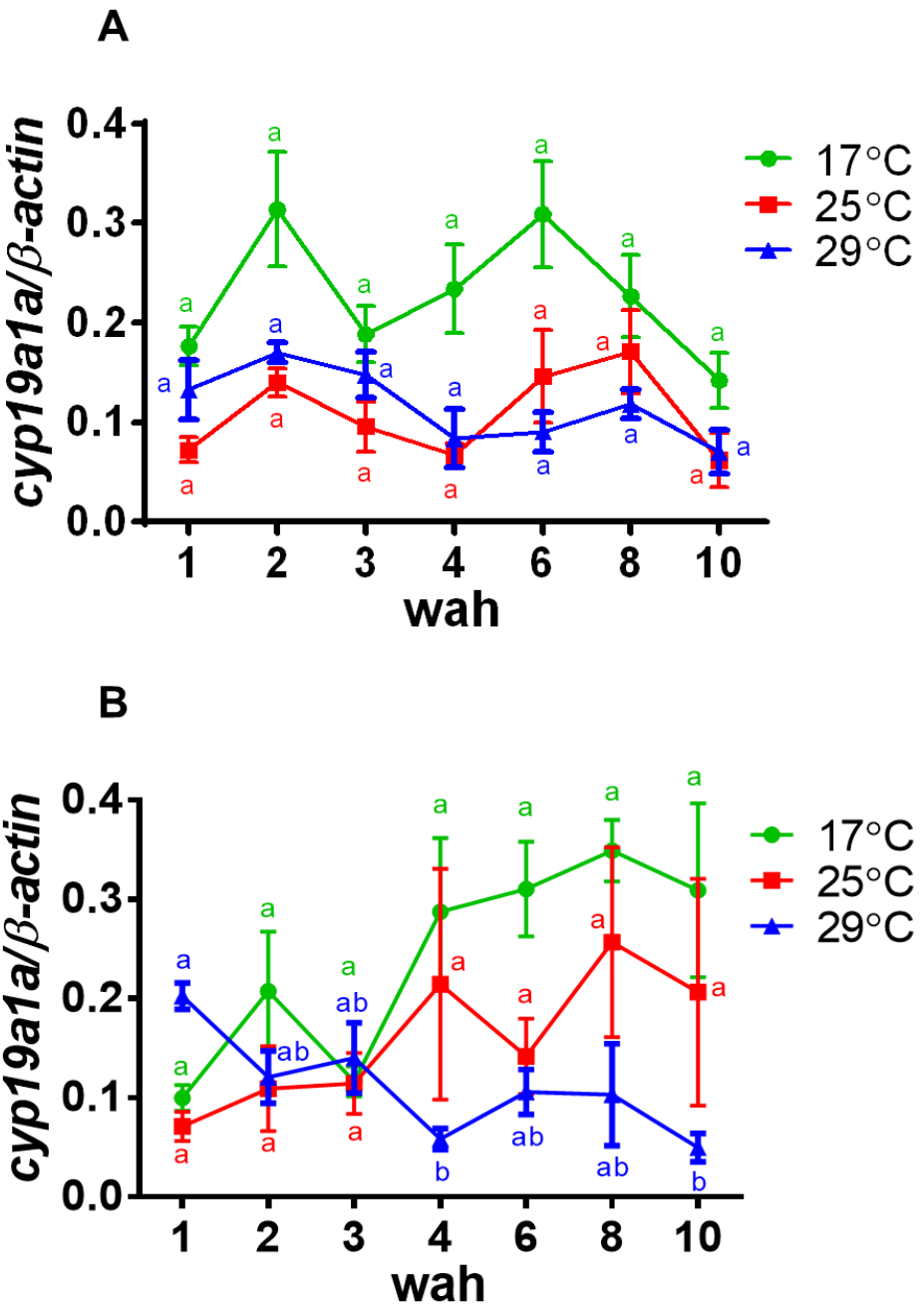
a
a
b
b
c
bc
0
5
10
15
20
25
30
Relative luciferase activity

Figure 6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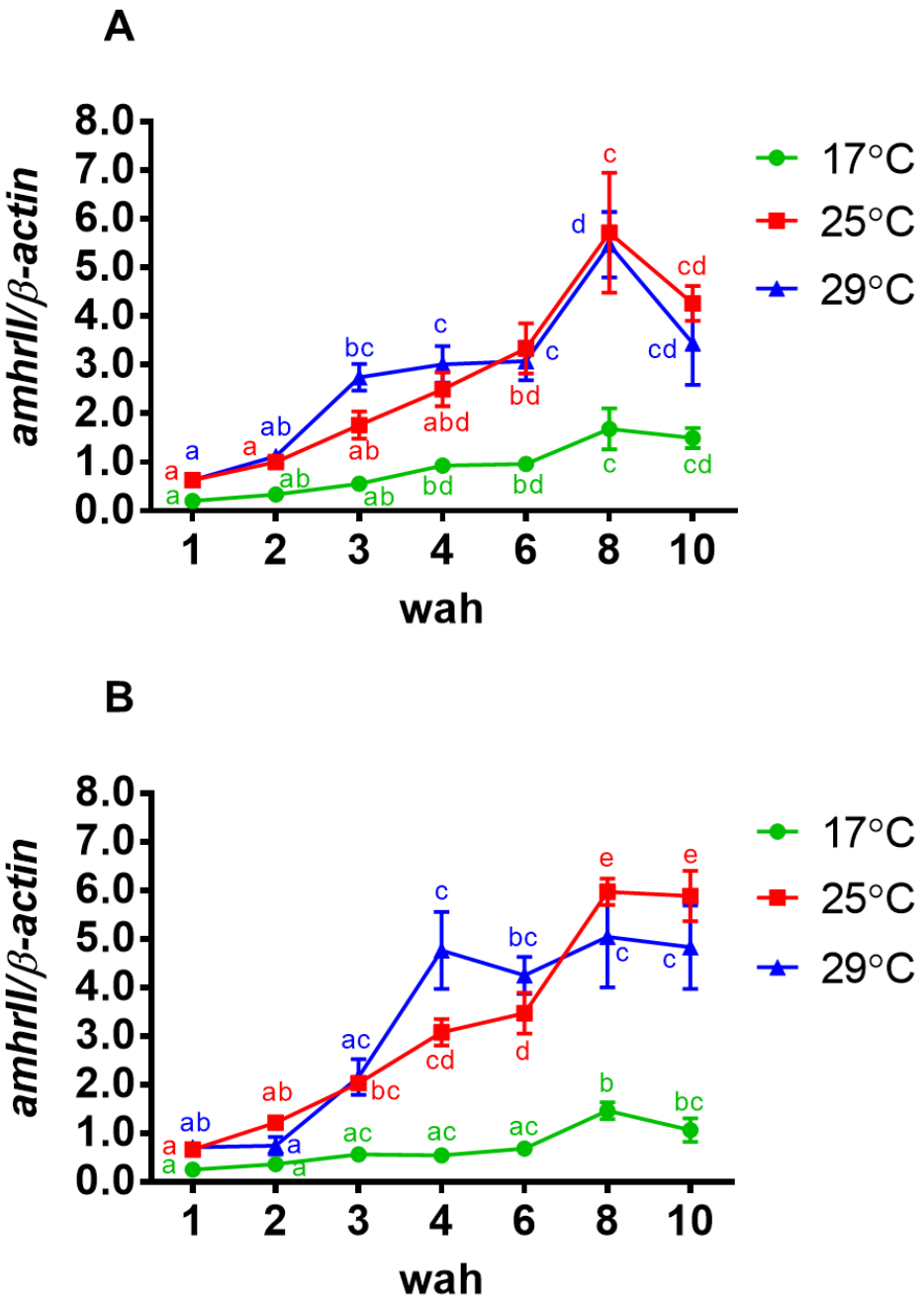
0.3
0.2
0.1
0.0
1
2
3
4
6
8
10
wah
17°C
25°C
29°C
B
cyp19a1a/β-actin
0.4
0.3
0.2
0.1
0.0
1
2
3
4
6
8
10
wah
17°C
25°C
29°C

Figure 7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amhrII/β-actin
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7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1
2
3
4
6
8
10
wah
17°C
25°C
29°C
B
amhrII/β-actin
wah
17°C
25°C
29°C

Figure 8

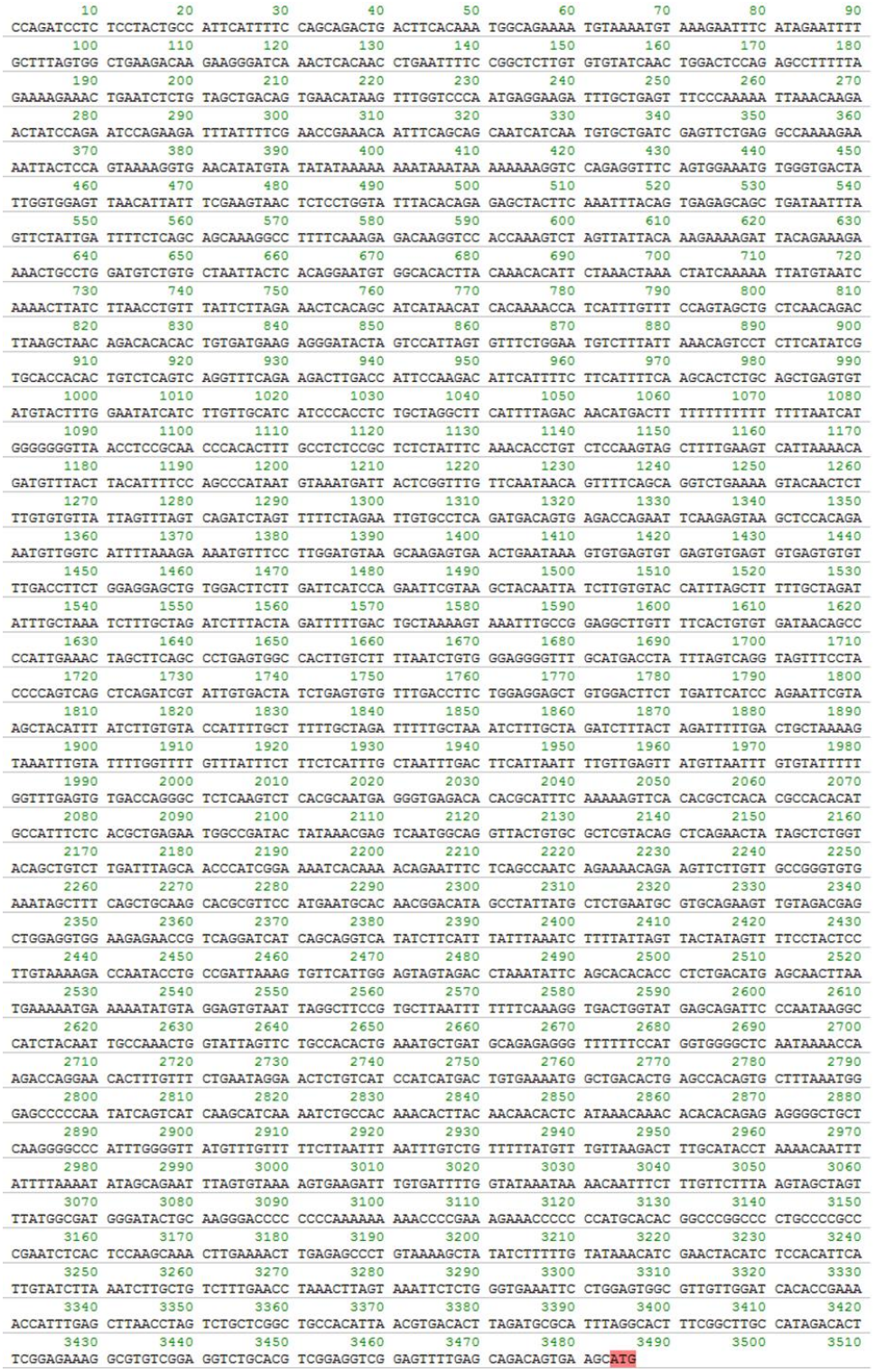
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Figure 9

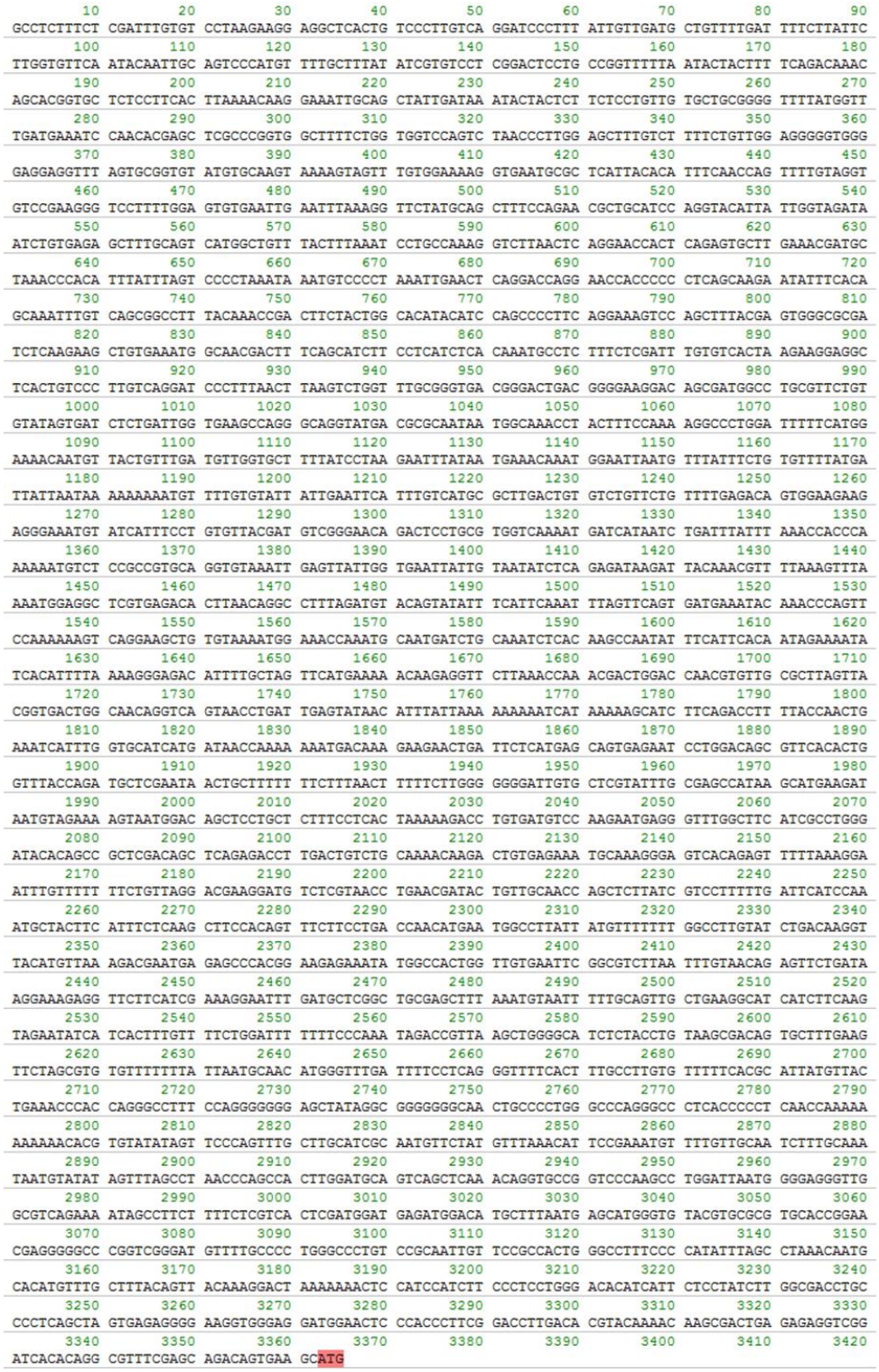
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Figure 10

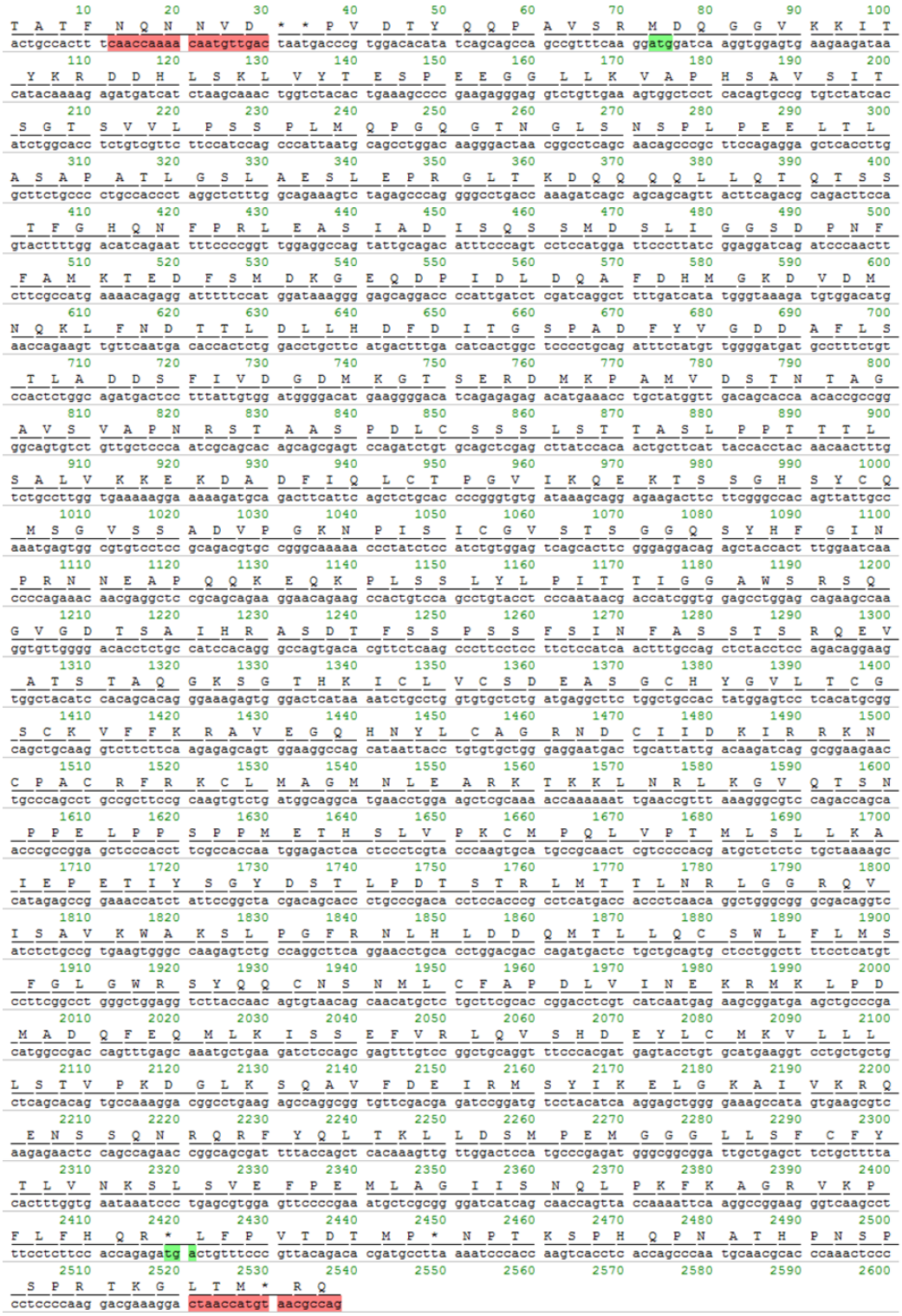
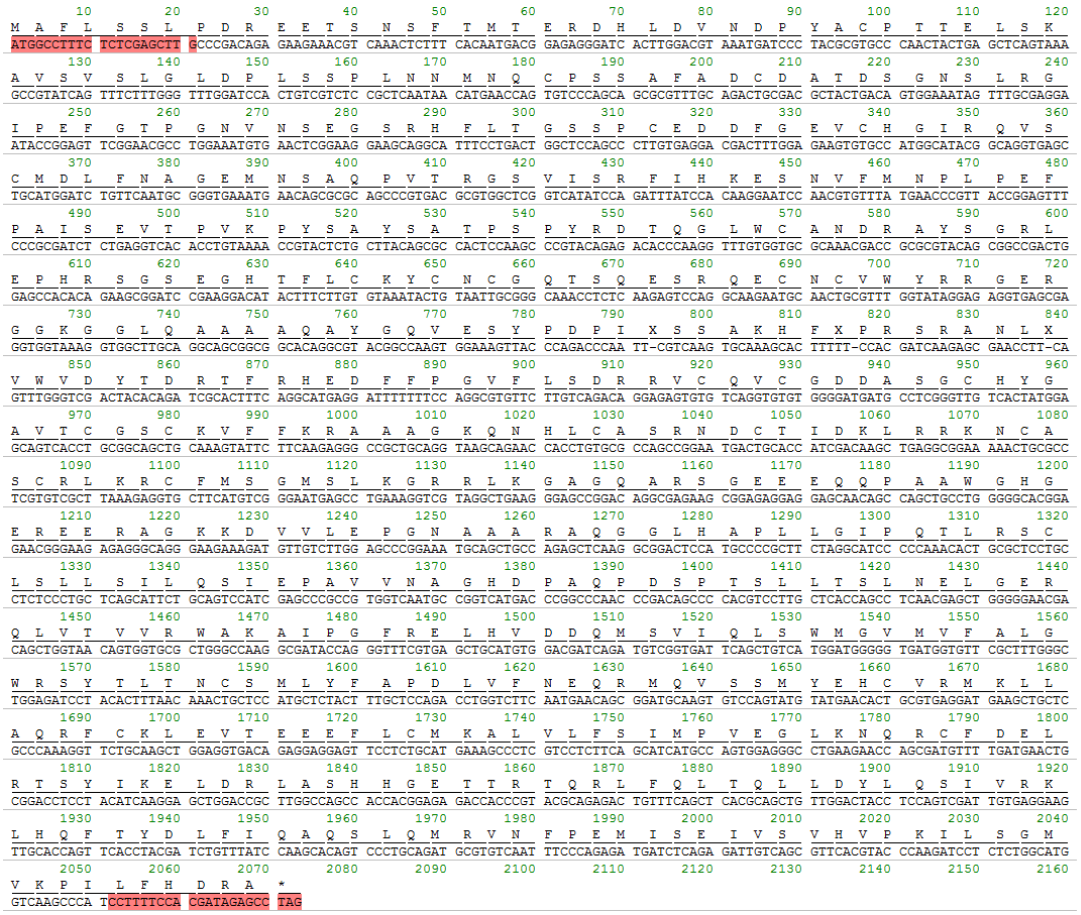


Figure 11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130 140 150 160 170 180 190 200 210 220 230 240
A V S V S L G L D P L S S P L N N M N Q C P S S A F A D C D A T D S G N S L R G
GCCGTATCAG TTTCTTTGGG TTTGGATCCA CTGTCGTCTC CGCTCAATAA CATGAACCAG TGTCCCAGCA GCGCGTTTGC AGACTGCGAC GCTACTGACA GTGGAAATAG TTTGCGAGGA
250 260 270 280 290 300 310 320 330 340 350 360
I P E F G T P G N V N S E G S R H F L T G S S P C E D D F G E V C H G I R Q V S
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370 380 390 400 410 420 430 440 450 460 470 480
C M D L F N A G E M N S A Q P V T R G S V I S R F I H K E S N V F M N P L P E F
TGCATGGATC TGTTCAATGC GGGTGAAATG AACAGCGCGC AGCCCGTGAC GCGTGGCTCG GTCATATCCA GATTTATCCA CAAGGAATCC AACGTGTTTA TGAACCCGTT ACCGGAGTTT
490 500 510 520 530 540 550 560 570 580 590 600
P A I S E V T P V K P Y S A Y S A T P S P Y R D T Q G L W C A N D R A Y S G R L
CCCGCGATCT CTGAGGTCAC ACCTGTAAAA CCGTACTCTG CTTACAGCGC CACTCCAAGC CCGTACAGAG ACACCCAAGG TTTGTGGTGC GCAAACGACC GCGCGTACAG CGGCCGACTG
610 620 630 640 650 660 670 680 690 700 710 720
E P H R S G S E G H T F L C K Y C N C G Q T S Q E S R Q E C N C V W Y R R G E R
GAGCCACACA GAAGCGGATC CGAAGGACAT ACTTTCTTGT GTAAATACTG TAATTGCGGG CAAACCTCTC AAGAGTCCAG GCAAGAATGC AACTGCGTTT GGTATAGGAG AGGTGAGCGA
730 740 750 760 770 780 790 800 810 820 830 840
G G K G G L Q A A A A Q A Y G Q V E S Y P D P I X S S A K H F X P R S R A N L X
GGTGGTAAAG GTGGCTTGCA GGCAGCGGCG GCACAGGCGT ACGGCCAAGT GGAAAGTTAC CCAGACCCAA TT-CGTCAAG TGCAAAGCAC TTTTT-CCAC GATCAAGAGC GAACCTT-CA
850 860 870 880 890 900 910 920 930 940 950 960
V W V D Y T D R T F R H E D F F P G V F L S D R R V C Q V C G D D A S G C H Y G
GTTTGGGTCG ACTACACAGA TCGCACTTTC AGGCATGAGG ATTTTTTTCC AGGCGTGTTC TTGTCAGACA GGAGAGTGTG TCAGGTGTGT GGGGATGATG CCTCGGGTTG TCACTATGGA
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
A V T C G S C K V F F K R A A A G K Q N H L C A S R N D C T I D K L R R K N C A
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1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
S C R L K R C F M S G M S L K G R R L K G A G Q A R S G E E E Q Q P A A W G H G
TCGTGTCGCT TAAAGAGGTG CTTCATGTCG GGAATGAGCC TGAAAGGTCG TAGGCTGAAG GGAGCCGGAC AGGCGAGAAG CGGAGAGGAG GAGCAACAGC CAGCTGCCTG GGGGCACGGA
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
E R E E R A G K K D V V L E P G N A A A R A Q G G L H A P L L G I P Q T L R S C
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1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
L S L L S I L Q S I E P A V V N A G H D P A Q P D S P T S L L T S L N E L G E R
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1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
Q L V T V V R W A K A I P G F R E L H V D D Q M S V I Q L S W M G V M V F A L G
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1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
W R S Y T L T N C S M L Y F A P D L V F N E Q R M Q V S S M Y E H C V R M K L L
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1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
A Q R F C K L E V T E E E F L C M K A L V L F S I M P V E G L K N Q R C F D E L
GCCCAAAGGT TCTGCAAGCT GGAGGTGACA GAGGAGGAGT TCCTCTGCAT GAAAGCCCTC GTCCTCTTCA GCATCATGCC AGTGGAGGGC CTGAAGAACC AGCGATGTTT TGATGAACTG
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
R T S Y I K E L D R L A S H H G E T T R T Q R L F Q L T Q L L D Y L Q S I V R K
CGGACCTCCT ACATCAAGGA GCTGGACCGC TTGGCCAGCC ACCACGGAGA GACCACCCGT ACGCAGAGAC TGTTTCAGCT CACGCAGCTG TTGGACTACC TCCAGTCGAT TGTGAGGAAG
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
L H Q F T Y D L F I Q A Q S L Q M R V N F P E M I S E I V S V H V P K I L S G M
TTGCACCAGT TCACCTACGA TCTGTTTATC CAAGCACAGT CCCTGCAGAT GCGTGTCAAT TTCCCAGAGA TGATCTCAGA GATTGTCAGC GTTCACGTAC CCAAGATCCT CTCTGGCATG
2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160
V K P I L F H D R A *
GTCAAGCCCA TCCTTTTCCA CGATAGAGCC TAG

Figure 12


10 20 30 40 50 60
TGGTTCTGGC TGTGCAATGC GTCTTTACAT GCATCTCCCA GCAGTCTTTA CTTCAGAAGA
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Obo
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370 380 390 400 410 420
Obo qRT-amhrII Fw2
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Obo qRT-amhrII Rv3
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TGGTCAAAGC AGATGGCACC TGCGTTCTGT GTGATTTTGG ATGCTCCACC ATCCTGCGTT
1030 1040 1050 1060 1070 1080
CTTGCTCTGG GCGTGGCCTG TGGCAGCAAC ACACCACAAA CATGAAGGAT CATGCCCAGC
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1150 1160 1170 1180 1190 1200
GCTTATTTCT CATGCAGGGG GACATCTATG CCTTGGGTTT GCTATTGTGG GAGATCTGGA
1210 1220 1230 1240 1250 1260
TGCGCTGCTC AGATTTATTT GAGGGTGCCA TTGTTCCACA GCATCTATTG CCTTATGAAT
1270 1280 1290 1300 1310 1320
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1330 1340 1350 1360 1370 1380
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General Discussion and Final Conclusion

Pejerrey is a gonochoristic species with a striking temperature-dependent sex determination. Single sex population can be consistently obtained when the larvae are raised between hatching and on set of histological differentiation of gonads at 17ºC (female promoting temperature, FPT) and 29ºC (male promoting temperature, MPT). At intermediate temperatures (24~25ºC; mixed-sex producing temperature, MixPT), mixed-sex populations can be produced but a clear thermal plateau with balanced sex ratio (female: male 1:1) is absent. For these reasons, genotypic sex determinant has been considered as virtually inexistent in pejerrey. However, this is not a foregone conclusion. Large variation of sex ratios observed at 25ºC among different crosses suggested an implication of genetic components on gonadal fate (Strussmann et al., 1996a, 1997). This scenario has become more plausible after a recent study on the genotypic sex determination in the congeneric species Patagonian pejerrey *O. hatcheri*. In *O. hatcheri*, we identified a sex determining gene *amhy* (Y-linked anti-Mullerian hormone). Because Patagonian pejerrey and pejerrey are closely related and share a high genetic identity, it is conceivable that *amhy* could also exist in pejerrey.

In this study, I first probed the presence of a genotypic sex determinant *amhy* (high linkage with maleness, conserved gene structure, specific expression in testis and brain) in pejerrey and showed the first clear evidence of the coexistence of TSD and GSD in this species. I then investigated the transcriptional profiles of *amhy* and *amha* to unravel their participation in TSD process. The expression analyses of *amhy* and *amha* at FPT, MixPT and MPT during early larval development revealed that *amhy* is temperature-independent

genotypic sex determinant. The *amhy* mRNA expressed high at the beginning of sex determination/differentiation period but declined thereafter regardless of the temperatures. In contrast, *amha* is temperature-dependent and up-regulated in response to the existence of *amhy* or high water temperature. In individuals possesses *amhy* (XY) differentiate as males by overlapped expressions of *amhy* and *amha*. However, if *amha* expression is inhibited by an environmental factor such as low water temperature, these individuals cannot differentiate as males and developed as females. On the other hand, in individuals do not possess *amhy* (XX) normally differentiate as female without expression of *amha*. However, if *amha* expression is induced by an environmental factor such as high water temperature, these individuals differentiate as males without genotypic sex determinant *amhy*.

A number of studies have shown that the stress hormone cortisol was reported a mediator in the high temperature-induced masculinization in fish (Hayashi et al., 2010; Yamaguchi et al., 2010). In pejerrey, cortisol was suggested to promote the synthesis of the 11-KT, most potent androgen in fish, during high temperature-induced masculinization by modulation of *hsd11b2* mRNA expression (Hattori et al., 2009; Fernandino et al., 2012, 2013). Since both *amhy* and *amha* seems like to be involved in masculinization in pejerrey (Yamamoto et al., 2014 and present study), in this study, I also investigated the effects of cortisol and androgen on the *amhy* and *amha* transcription. These analyses revealed that both cortisol and 11-KT activated *amha* promoter in dose dependent manners, however, neither cortisol or 11-KT has effect on *amhy* promoter at any concentration. These results highlighted the importance of cortisol and androgen signaling in *amha* regulation but not in genotypic sex determinant *amhy* regulation. Such no impact of steroid hormone on sex determining gene is also reported in Japanese medaka (Scholz et al., 2003, Nagahama et al.,

2004; Nagahama, 2005).

Taken all together, my results suggested that *amhy* is a genotypic sex determinant in pejerrey and regulation of this gene is temperature independent. In contrast, *amha* is upregulated in response to high temperature and its expression is regulated via cortisol and 11-KT. Although whether *amhy* induces *amha* expression or *amhy* and *amha* are related to germ cell proliferation (Herpin et al., 2007) as the described in Japanese medaka (Herpin et al., 2010) still needs to be assessed, overlapping expression of *amhy* and *amha*, early decrease of *amhy* expression, and *amha* regulation by temperature may be keys for the coexistence of genotypic and environmental sex determinants in this species. Future studies will focus on the interactions between *amhy* and *amha* by producing transgenic pejerrey and estrogenic regulation of *amhy* and *amha* as to unravel the molecular mechanisms of low temperature-induced feminization.

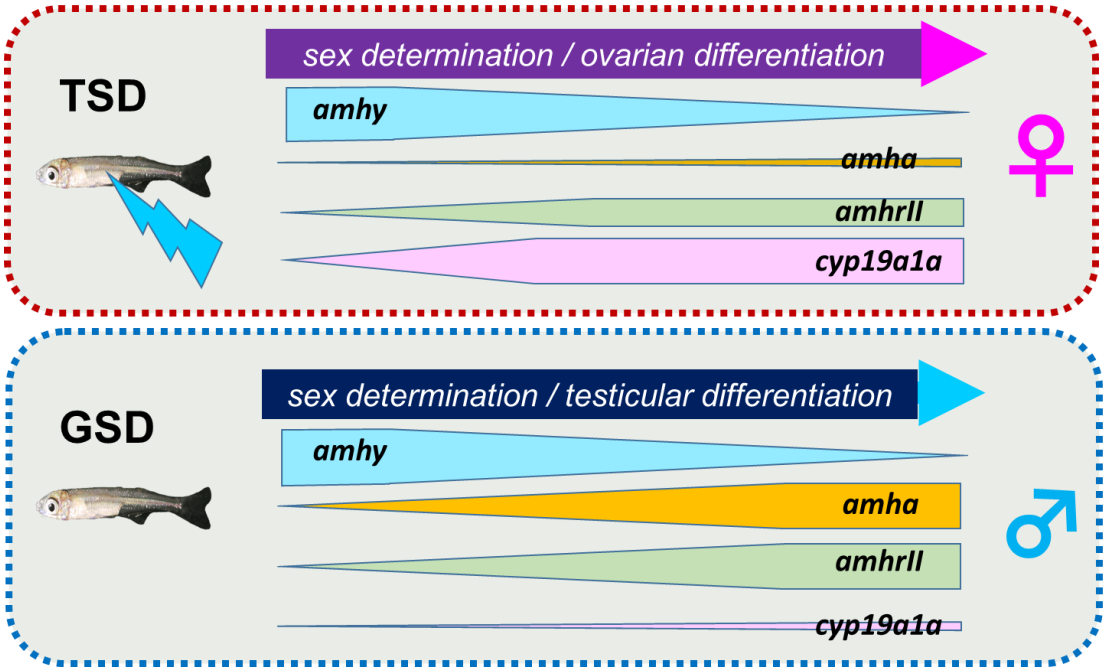

Figure 1. Schematic representation of the expression profiles of some sex-related genes during sex determination/gonadal differentiation in pejerrey XY genotypes. Boxes with blue and red dotted lines represent male and female development respectively. The thickness of the bars represents the levels of mRNA expression. Note that the sex determinant *amhy* does not display a temperature-independent expression profile during early sex differentiation period.

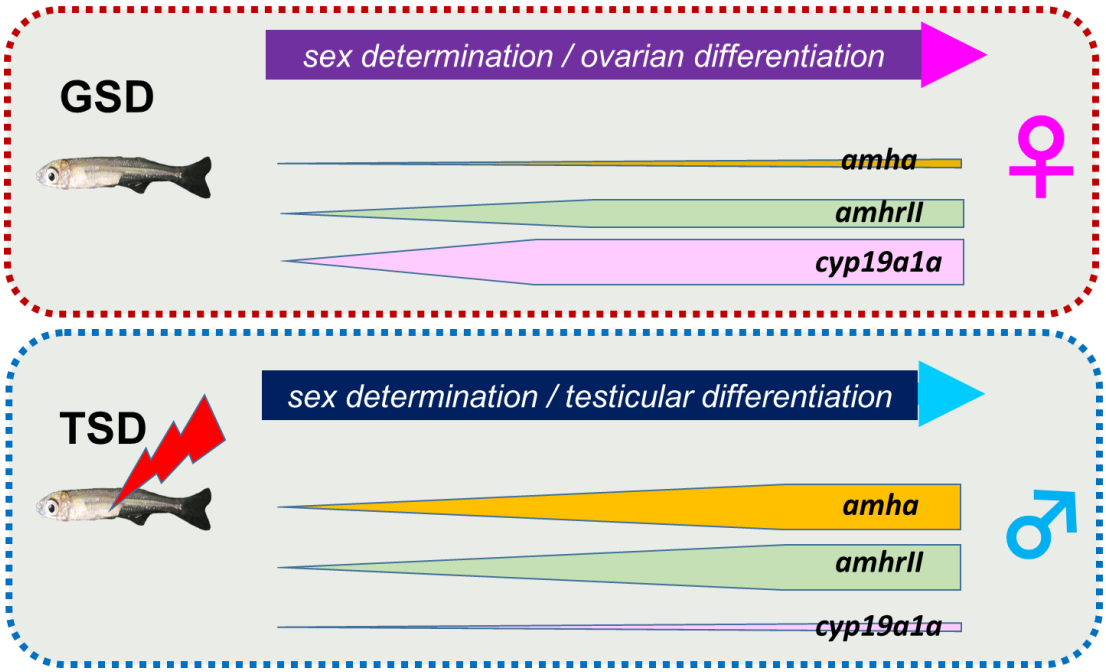

Figure 2. Schematic representation of the expression profiles of some sex-related genes during sex determination/gonadal differentiation in pejerrey XX genotypes. Boxes with blue and red dotted lines represent male and female development respectively. The thickness of the bars represents the levels of mRNA expression. *amha* expression is correlated with maleness.

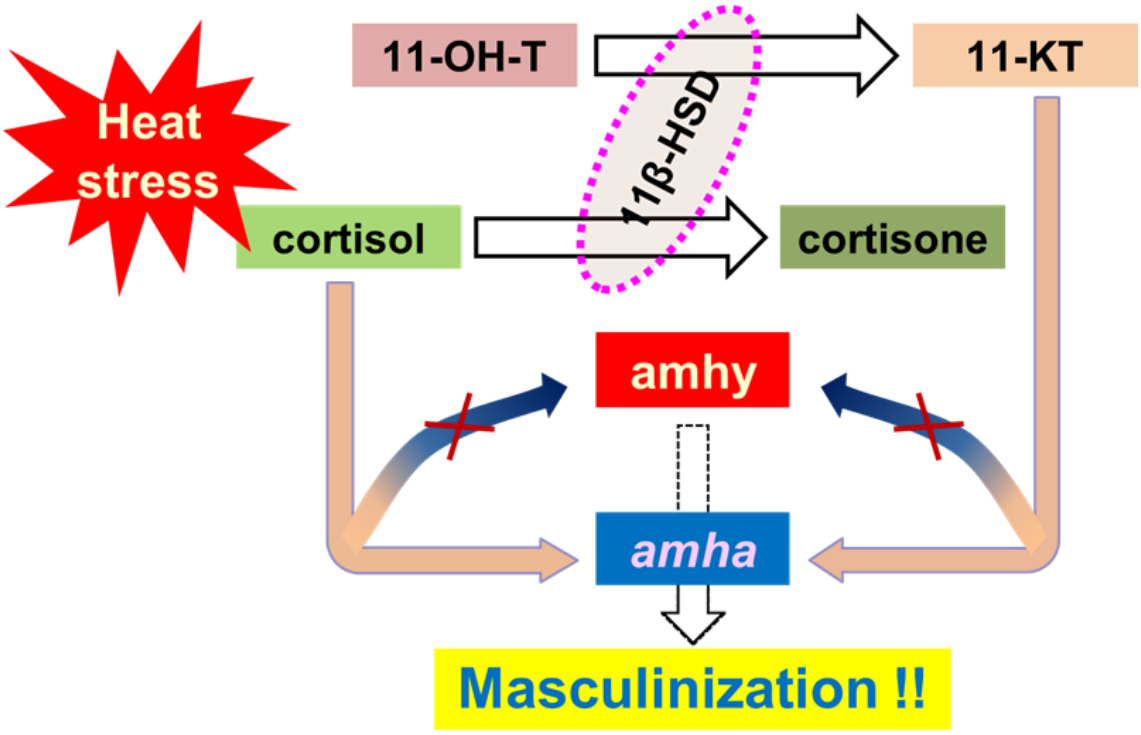

Figure 3. Schematic representation of *amhy* and *amha* regulation by cortisol and 11-KT in thermal stress-induced masculinization pathway in pejerrey.

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