



Sex determination in fish: *Odontesthes* spp. (Atherinopsidae) as experimental models

J.I. Fernandino¹, R.S. Hattori², C.A. Strüssmann³, Y. Yamamoto³, G.M. Somoza^{1,4}

¹Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (CONICET-UNSAM).
Chascomús, Buenos Aires, Argentina.

²Unidade de Pesquisa e Desenvolvimento de Campos do Jordão/APTA. Campos do Jordão, São Paulo, Brazil.

³Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan.

Abstract

The sex of the offspring is the result of a unique process leading, in a binary fate, to the development of male or female characteristics. In this context, the aim of the present work is to present atherinopsid fish as excellent models to study sex determination. Here we present two atherinopsid fish from South America; one species, *Odontesthes bonariensis*, has a strong temperature-dependent sex determination (TSD) and the other, *Odontesthes hatchery*, has a genotypic sex determination (GSD). However, the results obtained in the last years from our laboratories in both species show that the boundaries between these two sex determination mechanisms within Atherinopsidae are not as rigid as previously thought and support the notion that TSD and GSD are the extremes of a continuum.

Keywords: GSD, *Odontesthes*, sex determination, sex differentiation, TSD.

Introduction

Sex determination refers to the binary fate choice between becoming a male or a female that starts with the development of the gonads and sexual characteristics in a given organism. In vertebrates, the mechanisms that determine the sex of the offspring are grouped into two main categories, genotypic (GSD) and environmental (ESD) sex determination. In organisms with GSD, the primary sex of an individual is determined at fertilization by heritable genetic elements that differ between sexes and are usually located on the sex chromosomes; whereas in ESD sex is induced early during ontogeny by environmental factors (Devlin and Nagahama, 2002). Thanks to their diversity (Ravi and Venkatesh, 2008), fish are an interesting group of vertebrates because they exhibit a remarkable variety of sex-determining mechanisms (Kobayashi *et al.*, 2013). This group presents species with well-characterized GSD (Kikuchi and Hamaguchi, 2013), and species with TSD (Conover and Kynard, 1981; Strüssmann *et al.*, 1997; Kitano *et al.*, 1999; Pavlidis *et al.*, 2000). In this context, atherinopsid fish provide excellent models to study the interaction among GSD/TSD systems because

members of this family span from a classical TSD to a well-known GSD model, and even, with the coexistence of both systems.

Environmental sex determination in pejerrey (*Odontesthes bonariensis*)

The best-known environmental factor involved in ESD is temperature, and this particular sex-determining system is known as temperature-dependent sex determination (TSD). In this case, the differentiation of phenotypic sex is driven by temperature during a sensitive period early in larval development (Strüssmann and Patiño, 1999). In case of pejerrey, low temperatures (17-19°C) induce 100% females in the progeny whereas high temperatures produce 100% males (Strüssmann *et al.*, 1997). It is important to note that when pejerrey larvae are reared at high temperatures they eventually display signs of thermal stress, such as changes in their pigmentation pattern and progressive germ cell degeneration (Ito *et al.*, 2008). Furthermore it was then demonstrated that body levels of cortisol, the main stress hormone in vertebrates (Wendelaar Bonga, 1997), during the critical period of sex determination were positively correlated with rearing temperatures (Hattori *et al.*, 2009). Moreover, the supplementation of cortisol in the food given to pejerrey larvae reared at intermediate temperatures induced significant increases in the frequency of males. Similar results were observed in the Japanese flounder *Paralichthys olivaceus* (Yamaguchi *et al.*, 2010) and in the medaka *Oryzias latipes* (Hayashi *et al.*, 2010), indicating that cortisol also plays an important role(s) in the offspring sex in other fish species. This fact suggests that cortisol can affect sex determination in fishes.

The mode of action of cortisol during environmental masculinization has been recently elucidated. Actually, its action emerges as a sequence or combination of at least three effects: (a) cortisol causes reduction in the number of primordial germ cells (PGCs), (b) cortisol inhibits aromatase expression and, (c) cortisol modulates androgen synthesis. The relation between number of PGCs and somatic cells (SC) in the gonad seems to be critical to direct the sexual development (Tanaka *et al.*, 2008). Thus, depletion in

⁴Corresponding author: somoza@intech.gov.ar
Phone: +54(2241)43-0323; Fax: +54(2241)42-4048
Received: July 28, 2014
Accepted: September 2, 2014



the number of PGCs/SCs or a delay in the beginning of PGC proliferation produces a masculinization of the still undifferentiated gonad (Saito *et al.*, 2007; Nakamura *et al.*, 2012). In pejerrey larvae, cortisol triggers apoptosis among PGCs (Hattori *et al.*, 2009), as observed in animals reared at male promoting temperatures (Fernandino *et al.*, 2011; Yamamoto *et al.*, 2013). Second, it is also well known that the inhibition of estradiol synthesis induces a male-biased sex-ratio in pejerrey (Karube *et al.*, 2007; Fernandino *et al.*, 2008) and that during cortisol-induced masculinization, *cyp19a1a* (gonadal aromatase, the enzyme that converts testosterone into estradiol) expression is down-regulated, with the concomitant inhibition of estradiol synthesis (Hattori *et al.*, 2009). Also, in the Japanese flounder, cortisol acting on a glucocorticoid receptor, suppressed *cyp19a1a* transcription *in vitro* by binding to a glucocorticoid responsive element in its promoter region (Yamaguchi *et al.*, 2010). Finally, and probably the first cortisol effect during ontogeny, is the modulation of androgen synthesis. In *Odontesthes bonariensis*, cortisol levels were correlated with those of the androgens testosterone (T) and 11-ketotestosterone (11-KT; Hattori *et al.*, 2009), that have been shown to have masculinizing effects in several teleosts (Devlin and Nagahama, 2002). In pejerrey larvae, it was suggested that the rise of 11-KT can be explained through different mechanisms of action, the up-regulation of the *hsd11b2* transcript, the gene that codes for 11 β -HSD (Fernandino *et al.*, 2012), and/or through the hepatic catabolism of cortisol (Kime, 1978; Schulz, 1986). Thus, the rise of cortisol induced the increase of androgen biosynthesis with the concomitant masculinization of larvae (Fernandino *et al.*, 2013).

Genotypic sex determination in Patagonian pejerrey (*Odontesthes hatcheri*)

In contrast to the TSD of pejerrey, the congeneric species, the Patagonian pejerrey *O. hatcheri*, has a clear GSD system (Hattori *et al.*, 2013). The first attempts to look for a genetic marker of sex in this species led to the discovery of a sex-linked single nucleotide length polymorphism (SNP) marker (Koshimizu *et al.*, 2010); however, the analysis of sequences up- and down-stream of this SNP showed no difference between sexes. However, recently, a sex determining gene (*amhy*) was described in this species (Hattori *et al.*, 2012). The *amhy* gene is a Y chromosome-specific duplicated copy of the *amh* gene (hence the name *amhy*, in contrast to the autosomal *amh*; Hattori *et al.*, 2012). The Anti-Müllerian hormone is responsible for the regression of the Müllerian ducts during male fetal development in mammals, birds and reptiles but it is not considered as the major sex determinant gene (Josso *et al.*, 2001). Before our discovery, all sex-determining genes identified in mammalian and non-mammalian vertebrates were

restricted to either transcription factors with Zinc finger or DM domains (see references in Hattori *et al.* 2013), respectively. In contrast, the *amhy* gene is a member of the TGF-beta superfamily. Thus, our findings demonstrated that switches of gonadal fate may not necessarily be confined to genes with DNA-binding motifs. Similar autosomal paralogs, generated by gene duplication or element transposition of genes involved in gonadal differentiation, have been also characterized in other fish species (Matsuda *et al.*, 2002; Nanda *et al.*, 2002; Hattori *et al.*, 2012; Myosho *et al.*, 2012). Also, it should be noted that accumulations of mutations either on the promoter or in the transcriptional unit of other master-gene determining gene have been observed in other species (Kamiya *et al.*, 2012; Yano *et al.*, 2012; Takehana *et al.*, 2014). However, although it has been observed that *amhy* is necessary for testicular formation in Patagonian pejerrey (Hattori *et al.*, 2012), its mechanism of action is still unclear.

Coexistence of ESD and GSD in Atherinopsids

As discussed above, sex determination in pejerrey is characterized by a strong temperature dependence whereas in Patagonian pejerrey it is highly dependent on the genotype, hence the classification of the two species as TSD and GSD, respectively. However, it is now clear that the boundaries of these two sex determination mechanisms within Atherinopsidae are not as rigid as previously assumed (Fernandino *et al.*, 2013). In the Patagonian pejerrey, for example, extreme temperatures can override GSD in spite of the existence of *amhy* (Strüssmann *et al.*, 1997; Hattori *et al.*, 2012, 2013). Similar effects of extreme temperatures have been found also in the medaka (Sato *et al.*, 2005; Hattori *et al.*, 2007) and rainbow trout *Oncorhynchus mykiss* (Magerhans *et al.*, 2009; Magerhans and Hörstgen-Schwark, 2010) and several other species which have identified or presumed genotypic sex determinants. On the other hand, we have recently discovered a homolog of the aforementioned master sex-determining gene *amhy* of Patagonian pejerrey in the pejerrey (Yamamoto *et al.*, 2014). More importantly, our research shows that *amhy* plays a significant role in testis determination in the latter species within the intermediate range of temperature where individuals of both sexes are formed. Collectively, these phenomena suggest that the limit between TSD and GSD is blurred, and that both systems can, at least in part, stably and simultaneously exist (Mork *et al.*, 2014). These findings support the notion that TSD and GSD are the extremes of a continuum (Strüssmann and Patiño, 1999).

Acknowledgments

This work was supported by grants from: Consejo Nacional de Investigaciones Científicas y



Técnicas (#D731) to JIF; Japan Society for the Promotion of Science (#P12092 and #24-0292) to R.S.H.; Agencia Nacional de Promoción Científica y Tecnológica (#2012/0366) to JIF and (#00519, #01383 and #2619) to G.M.S., and Ministry of Education, Culture, Sports, Science and Technology of Japan (#22380109) to C.A.S.

References

- Conover DA, Kynard BY.** 1981. Environmental sex determination: interaction of temperature and genotype in a fish. *Science*, 213:577-579.
- Devlin RH, Nagahama Y.** 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, 208:191-364.
- Fernandino JI, Hattori RS, Kimura H, Strussmann CA, Somoza GM.** 2008. Expression profile and estrogenic regulation of anti-Mullerian hormone during gonadal development in pejerrey *Odontesthes bonariensis*, a teleost fish with strong temperature-dependent sex determination. *Dev Dynam*, 237:3192-3199.
- Fernandino JI, Popescu J, Paul-Prasanth B, Xiong H, Hattori RS, Oura M, Strüssmann CA, Somoza GM, Matsuda M, Nagahama Y, Trudeau V.** 2011. Analysis of sexually dimorphic expression of genes at early gonadogenesis of pejerrey *Odontesthes bonariensis* using a heterologous microarray. *Sex Dev*, 5:89-101.
- Fernandino JI, Hattori RS, Kishi A, Strüssmann CA, Somoza GM.** 2012. The cortisol and androgen pathways cross talk in high-temperature induced masculinization: 11 β -hydroxysteroid dehydrogenase as a key enzyme. *Endocrinology*, 153:6003-6011.
- Fernandino JI, Hattori RS, Moreno Acosta OD, Strüssmann CA, Somoza GM.** 2013. Environmental stress-induced testis differentiation: androgen as a by-product of cortisol inactivation. *Gen Comp Endocrinol*, 192:36-44.
- Hattori RS, Gould RJ, Fujioka T, Saito T, Kurita J, Strüssmann CA, Yokota M, Watanabe S.** 2007. Temperature-dependent sex determination in Hd-rRmedaka *Oryzias latipes*: gender sensitivity, thermal threshold, critical period, and DMRT1 expression profile. *Sex Dev*, 1:138-146.
- Hattori RS, Fernandino JI, Kishii A, Kimura H, Kinno T, Oura M, Somoza GM, Yokota M, Strussmann CA, Watanabe S.** 2009. Cortisol-induced masculinization: does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? *PLoS One*, 4:e6548.
- Hattori RS, Murai Y, Oura M, Masuda S, Majhi SK, Sakamoto T, Fernandino JI, Somoza GM, Yokota M, Strussmann CA.** 2012. A Y-linked anti-Mullerian hormone duplication takes over a critical role in sex determination. *Proc Natl Acad Sci USA*, 109:2955-2959.
- Hattori RS, Strüssmann CA, Fernandino JI, Somoza GM.** 2013. Genotypic sex determination in teleosts: insights from the testis-determining amhy gene. *Gen Comp Endocrinol*, 192:55-59.
- Hayashi Y, Kobira H, Yamaguchi T, Shiraishi E, Yazawa T, Hirai T, Kamei Y, Kitano T.** 2010. High temperature causes masculinization of genetically female medaka by elevation of cortisol. *Mol Reprod Dev*, 77:679-686.
- Ito LS, Takahashi C, Yamashita M, Strussmann CA.** 2008. Warm water induces apoptosis, gonadal degeneration, and germ cell loss in subadult pejerrey *Odontesthes bonariensis* (Pisces, Atheriniformes). *Physiol Biochem Zool*, 81:762-774.
- Josso N, di Clemente N, Gouédard L.** 2001. Anti-Müllerian hormone and its receptors. *Mol Cell Endocrinol*, 20:25-32.
- Kamiya T, Kai W, Tasumi S, Oka A, Matsunaga T, Mizuno N, Fujita M, Suetake H, Suzuki S, Hosoya S, Tohari S, Brenner S, Miyadai T, Venkatesh B, Suzuki Y, Kikuchi K.** 2012. A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger Pufferfish, *Takifugu rubripes* (Fugu). *PLoS Genet*, 8:e1002798.
- Karube M, Fernandino JI, Strobl-Mazzulla P, Strüssmann CA, Yoshizaki G, Somoza GM, Patiño R.** 2007. Characterization and expression profile of the ovarian cytochrome P-450 aromatase (cyp19A1) gene during thermolabile sex determination in Pejerrey, *Odontesthes bonariensis*. *J Exp Zool A Ecol Genet Physiol*, 307:625-636.
- Kikuchi K, Hamaguchi S.** 2013. Novel sex-determining genes in fish and sex chromosome evolution. *Dev Dyn*, 242:339-353.
- Kime DE.** 1978. The hepatic catabolism of cortisol in teleost fish-Adrenal origin of 11-oxotestosterone precursors. *Gen Comp Endocrinol*, 35:322-328.
- Kitano T, Takamune K, Kobayashi T, Nagahama Y, Abe SI.** 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys solivaceus*). *J Mol Endocrinol*, 23:167-176.
- Kobayashi Y, Nagahama Y, Nakamura M.** 2013. Diversity and plasticity of sex determination and differentiation in fishes. *Sex Dev*, 7:115-125.
- Koshimizu E, Strussmann CA, Okamoto N, Fukuda H, Sakamoto T.** 2010. Construction of a genetic map and development of DNA markers linked to the sex-determining locus in the Patagonian pejerrey (*Odontesthes hatcheri*). *Mar Biotechnol (NY)*, 12:8-13.
- Magerhans A, Müller-Belecke A, Hörstgen-Schwark G.** 2009. Effect of rearing temperatures post hatching on sex ratios of rainbow trout (*Oncorhynchus mykiss*) populations. *Aquaculture*, 294:25-29.
- Magerhans A, Hörstgen-Schwark G.** 2010. Selection experiments to alter the sex ratio in rainbow trout (*Oncorhynchus mykiss*) by means of temperature treatment. *Aquaculture*, 306:63-67.



- Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M.** 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*, 417:559-563.
- Mork L, Czerwinski M, Capel B.** 2014. Predetermination of sexual fate in a turtle with temperature-dependent sex determination. *Dev Biol*, 386:264-271.
- Myosho T, Otake H, Masuyama H, Matsuda M, Kuroki Y, Fujiyama A, Naruse K, Hamaguchi S, Sakaizumi M.** 2012. Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*. *Genetics*, 191:163-170.
- Nakamura S, Watakabe I, Nishimura T, Toyoda A, Taniguchi Y, Tanaka M.** 2012. Analysis of medaka sox9 orthologue reveals a conserved role in germ cell maintenance. *PLoS One*, 7:e29982.
- Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Schartl M.** 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA*, 99:11778-11783.
- Pavlidis M, Koumoundouros G, Steriotti A, Somarakis S, Divanach P, Kentouri M.** 2000. Evidence of temperature-dependent sex determination in the european sea bass (*Dicentrarchus labrax* L.). *J Exp Zool*, 287:225-232.
- Ravi V, Venkatesh B.** 2008. Rapidly evolving fish genomes and teleost diversity. *Curr Opin Genet Dev*, 18:544-550.
- Saito D, Morinaga C, Aoki Y, Nakamura S, Mitani H, Furutani-Seiki M, Kondoh H, Tanaka M.** 2007. Proliferation of germ cells during gonadal sex differentiation in medaka: insights from germ cell-depleted mutant zenzai. *Dev Biol*, 310:280-290.
- Sato T, Endo T, Yamahira K, Hamaguchi S, Sakaizumi M.** 2005. Induction of female-to-male sex reversal by high temperature treatment in Medaka, *Oryzias latipes*. *Zool Sci*, 22:985-988.
- Schulz RW.** 1986. In vitro metabolism of steroid hormones in the liver and in blood cells of male rainbow trout (*Salmo gairdneri* Richardson). *Gen Comp Endocrinol*, 64:312-319.
- Strüssmann CA, Patiño R.** 1999. Sex determination, environmental. In: Knobil E, Neill JD (Ed.). *Encyclopedia of Reproduction*. San Diego, USA: Academic Press. vol. 4, pp. 402-409.
- Strüssmann CA, Saito T, Usui M, Yamada H, Takashima F.** 1997. Thermal thresholds and critical period of thermolabile sex determination in two atherinid fishes, *Odontesthes bonariensis* and *Patagonina hatcheri*. *J Exp Zool*, 278:167-177.
- Takehana Y, Matsuda M, Myosho T, Suster ML, Kawakami K, Shin-I T, Kohara Y, Kuroki Y, Toyoda A, Fujiyama A, Hamaguchi S, Sakaizumi M, Naruse K.** 2014. Co-option of Sox3 as the male-determining factor on the y chromosome in the fish *Oryzias dancena*. *Nat Commun*, 5:4157.
- Tanaka M, Saito D, Morinaga C, Kurokawa H.** 2008. Cross talk between germ cells and gonadal somatic cells is critical for sex differentiation of the gonads in the teleost fish, medaka (*Oryzias latipes*). *Dev Growth Differ*, 50:273-278.
- Wendelaar Bonga SE.** 1997. The stress response in fish. *Physiol Rev*, 77:591-625.
- Yamaguchi T, Yoshinaga N, Yazawa T, Gen K, Kitano T.** 2010. Cortisol is involved in temperature-dependent sex determination in the *Japanese flounder*. *Endocrinology*, 151:3900-3908.
- Yamamoto Y, Hattori RS, Kitahara A, Kimura H, Yamashita M, Strussmann CA.** 2013. Thermal and endocrine regulation of gonadal apoptosis during sex differentiation in pejerrey *Odontesthes bonariensis*. *Sex Dev*, 7:316-324.
- Yamamoto Y, Zhang Y, Sarida M, Hattori RS, Strüssmann CA.** 2014. Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *Plos One*, 9:e102574.
- Yano A, Guyomard R, Nicol B, Jouanno E, Quillet E, Klopp C, Cabau C, Bouchez O, Fostier A, Guiguen Y.** 2012. An immune-related gene evolved into the master sex-determining gene in rain-bow trout, *Oncorhynchus mykiss*. *Curr Biol*, 22:1423-1428.
-