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Review

Genotypic sex determination in teleosts: Insights from the testis-determining *amhy* gene

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ABSTRACT

The master sex-determining genes identified so far in fishes are clearly not conserved, as evidenced by several unrelated genes reported to play critical roles in sex determination. In this study, we reviewed the molecular process of sex determination in the Patagonian pejerrey *Odontesthes hatcheri*, an emerging model due to the recent discovery that a Y-chromosome linked, duplicated copy of the anti-Müllerian hormone gene, *amhy* plays a pivotal role in sex determination. A comparative analysis with other newly found sex-determining genes of teleost fish, *DMY*/*dmrt1bY*, *sdY*, *amhr2*, and *gsdf*^{Y} is performed and alternative ideas are proposed to explain the mechanism involved in the rise of various types of non-homologous sex-determining genes.

1. Introduction

A key and conserved function in sexual reproduction of gonochoristic vertebrates is the fertilization of female gametes by male gametes produced by ovaries and testes, respectively. These homologous organs differentiate from a primordial gonad, whose fate is in principle programmed at the moment of fecundation by the combination of sex chromosomes. While eutherian mammals and avians have conserved mechanisms of chromosomal sex determination triggered by the genes *SRY* (Sinclair et al., 1990) and *DMRT1* (Smith et al., 2009), respectively, poikilothermic vertebrates, especially teleost fishes, show a wide variety not only in the genetic systems of sex determination but also in the kinds of extrinsic, environmental cues that can either trigger or modulate the pathway of sex differentiation (Devlin and Nagahama, 2002; Strüssmann and Patino, 1995; Strüssmann and Patino, 1998).

2. Sex determination in atherinopsid fishes

Atherinopsids, also known as Neotropical silversides (Dyer, 2006), inhabit freshwater, brackish, and coastal marine environments. They have no secondary sexual characters and are considered as differentiated gonochorists (Ito et al., 2003; Strüssmann et al., 1996a; Strüssmann et al., 1996b) but several species show temperature-dependent sex determination (TSD). The effects of water temperature on sex determination in atherinopsids have been investigated mainly in the genus *Menidia* (Conover and Kynard, 1981; Yamahira et al., 2003), from North America, and the genus *Odontesthes*, from South America (Strüssmann et al., 1996a; Strüssmann et al., 1996b; Strüssmann et al., 1997). Laboratory studies with *Odontesthes bonariensis*, *Odontesthes argentinensis*, and *Odontesthes hatcheri* revealed that the former has the highest variation among the three in the sex ratio response to temperature during the critical time of sex determination. Thus, sex ratios in this species range from 100% female to 100% male over a 10 °C range of environmentally relevant temperatures. At intermediate temperatures, sex ratios vary significantly among crosses without the existence of a clear thermal plateau associated with balanced sex ratios (Strüssmann et al., 1996a; Strüssmann et al., 1997). On the other hand, water temperature has no effect on sex ratios of *O. hatcheri* over a relatively broad range of temperatures (Strüssmann et al., 1996b). Thus, with the exception of low and high thermal extremes, which produce female- and male-skewed sex ratios, respectively, fairly consistent 1:1 sex proportions are obtained in this species at intermediate temperatures. *O. argentinensis* shows an intermediate pattern (Strüssmann et al., 1996b) between those of *O. bonariensis* and *O. hatcheri*, i.e., sex ratios are more susceptible to temperature in *O. argentinensis* than in *O. hatcheri* but they do not reach 100% of either females or males as in *O. bonariensis*. Moreover, although narrower than that of *O. hatcheri* a thermal plateau can be detected at intermediate temperatures. The similarities and differences in the response of sex ratio to temperature among these closely related species

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are likely the reflection of similarities and differences in their molecular processes of sex determination and gonadal differentiation, but the details are still unknown. Here we review the current knowledge on the molecular processes of sex determination and gonadal sex differentiation of the Patagonian pejerrey *O. hatcheri* and compare it with non-homologous sex-determining genes recently described in other teleosts.

3. The mechanism of sex determination in Patagonian pejerrey

3.1. Establishment of a sex-linked SNP marker

The Patagonian pejerrey *O. hatcheri* does not have heteromorphic sex chromosomes (Sola et al., 1998) but the stable occurrence of balanced sex ratios over a wide range of intermediate temperatures (Strüssmann et al., 1996b) strongly suggested that genotypic sex determining (GSD) factors might be well established in this species. Subsequently, a search for sex-specific markers based on the construction of a linkage map by AFLP analysis allowed the successful identification of the sex-linked DNA marker ACG/CAA-217 in the linkage group 13 of males (Koshimizu et al., 2010), which consisted of a single nucleotide length polymorphism (SNP). However, the analysis of sequences up- and downstream this SNP (about 5 kbp) showed no homology to any sequence in the GenBank database and no difference between sexes. Therefore, although relatively close, this marker was not part of the testis-determining gene or in its regulatory region, but was probably in the pseudoautosomal boundary of the Y chromosome. Further, it was demonstrated that this marker was neither applicable to other species of *Odontesthes* nor to other strains of *O. hatcheri*. In fact, it was specific to only one stock of *O. hatcheri* called Ehi-M13 (Hattori et al., 2010). Nevertheless, this marker proved useful in developmental studies on gonadal differentiation of *O. hatcheri* as it allowed the identification of sex-reversed females and males and a supermale (YY). These animals were used in crosses and backcrosses that provided clear evidenced that this species has an XX-XY, male heterogametic, sex-determining system (Hattori et al., 2010).

3.2. Transcriptome analysis during embryonic and larval development in Patagonian pejerrey

With the aid of the sex-linked SNP marker, transcriptome analysis of sex-linked genes during early developmental stages in XX and XY embryos and larvae of *O. hatcheri* revealed very unusual patterns in *dmrt1* (e.g. an absence of sexual dimorphism) and *amh* (very early onset) gene expression profiles (Fig. 1). The sex-related *dmrt1* gene shows a conserved role in testis differentiation of vertebrates (Volff et al., 2007; Koopmann, 2009) and in some taxa its homologues have acquired a critical role in sex determination (e.g. Z-linked *DMRT1 (*Smith et al., 2009), Y-linked *DMY/dmrt1bY (*Matsuda et al., 2002; Nanda et al., 2002), and W-linked *DM-W* (Yoshimoto et al., 2008) in birds, Japanese medaka, and African clawed frog, respectively). Sex-specific *dmrt1* expression profiles during sex differentiation have been described in all groups examined, including the congeneric species *O. bonariensis* (Fernandino et al., 2008), but surprisingly not in *O. hatcheri*. The teleost *amh* was initially described in the Japanese eel with the name *eSRS21* as a TGF-beta superfamily homologue of the mammalian *amh* gene (Miura et al., 2002). Similar functions as in mammals were attributed to this gene in fish, except for the regression of Müllerian ducts, structures which are absent in teleosts (Miura et al., 2002). *Amh* was shown to be important during male sex differentiation in Nile tilapia (Ijiri et al., 2008) and rainbow trout (Vizziano et al., 2007). Interestingly, while the sexually dimorphic expression of *amh* is preceded by that of gonadal aromatase in most teleosts, in *O. hatcheri* it showed sexual dimorphism before that of gonadal aromatase. These results suggested the presence of a distinct molecular pathway of male differentiation in *O. hatcheri* compared to other species.

3.3. The male–specific, duplicated amhy gene and its critical role on testis differentiation in Patagonian pejerrey

After an in-depth sequence analysis of transcripts at different developmental stages, it was found that the unusual pattern of premature *amh* expression in *O. hatcheri* was due to the presence of a Y chromosome-specific duplicated copy of the *amh* gene, termed *amhy* gene, in addition to the autosomal one (*amha*) Hattori et al., 2012. Besides the difference in amino acid sequences (92.2% and 91.4% for the entire protein and TGF-beta domain, respectively), *amhy* showed a 0.5 kbp insertion within the third intron and significant differences in the 5′ UTRs. Specific primers for each *loci* revealed that *amhy* expression started before hatching and was sustained during gonad differentiation. The *amha* gene expression, on the other hand, began to increase concomitantly with a decrease in *amhy* transcription and was consistently expressed in adult testis. In sub-adult females and males, the gonads have already completed the differentiation process and gamete maturation is actively under course. These profiles support the assumption that *amhy* and *amha* are in charge of testicular differentiation and gametogenesis, respectively. It is interesting to note that *amhy* and *amha* mRNA expression are comparable to that of the duplicated Y-linked *DMY/dmrt1bY* and the autosomal *dmrt1a*, respectively, of the Japanese medaka *Oryzias latipes* (Matsuda et al., 2002; Nanda et al., 2002). This suggests a conserved subfunctionalization of the Y- and autosomal-linked genes that probably appeared independently by an initial autosomal gene duplication followed by translocation onto a proto Y-chromosome until the diversification in a non-recombinant region (Schartl, 2004).

Until the discovery of *amhy* gene in Patagonian pejerrey (Hattori et al., 2012), all sex-determining genes identified in mammalian and non-mammalian vertebrates were restricted to either transcription factors with Zinc finger (Sinclair et al., 1990) or DM domains (Matsuda et al., 2002; Nanda et al., 2002; Smith et al., 2009; Yoshimoto et al., 2008), respectively. The *amhy* gene, in contrast, is a homologue of a well characterized hormone in mammals. It is a member of the TGF-beta superfamily and is generally assumed to be located downstream the cascade of testis differentiation. Thus, our findings demonstrated that switches of gonadal fate may not necessarily be confined to genes with DNA-binding motifs. This concept has got support from subsequent findings of new sex-determining genes in other teleosts. For example, Myosho et al. Myosho et al. (2012) showed that the sex-determining gene in *Oryzias luzonensis* (*gsdf*^{Y}) is also a TGF-beta superfamily member as it is *amhy*, in stark contrast to the *DMY/dmrt1bY* found in the closely related species *O. latipes* (Matsuda et al., 2002; Nanda et al., 2002). In fugu (*Takifugu rubripes*), Kamiya et al. Kamiya et al. (2012) localized a SNP associated with sex determination in the kinase domain of the anti-Müllerian receptor type II (*amhr2*) gene, the receptor which binds to the *Amh* protein. In rainbow trout and also in other salmonids, the immune-related *sdY* gene, derived from the interferon regulatory factor 9, has acquired the function of male sex-determining gene (Yano et al., 2012; Yano et al.,2013). These facts are strong evidence that sex-determining genes have arisen repeatedly and independently among the various taxonomic groups. They also suggest that other kinds of genes (steroidogenic enzymes?) or even the gene families already reported may evolve or have evolved as key genes in the sex differentiation cascade.

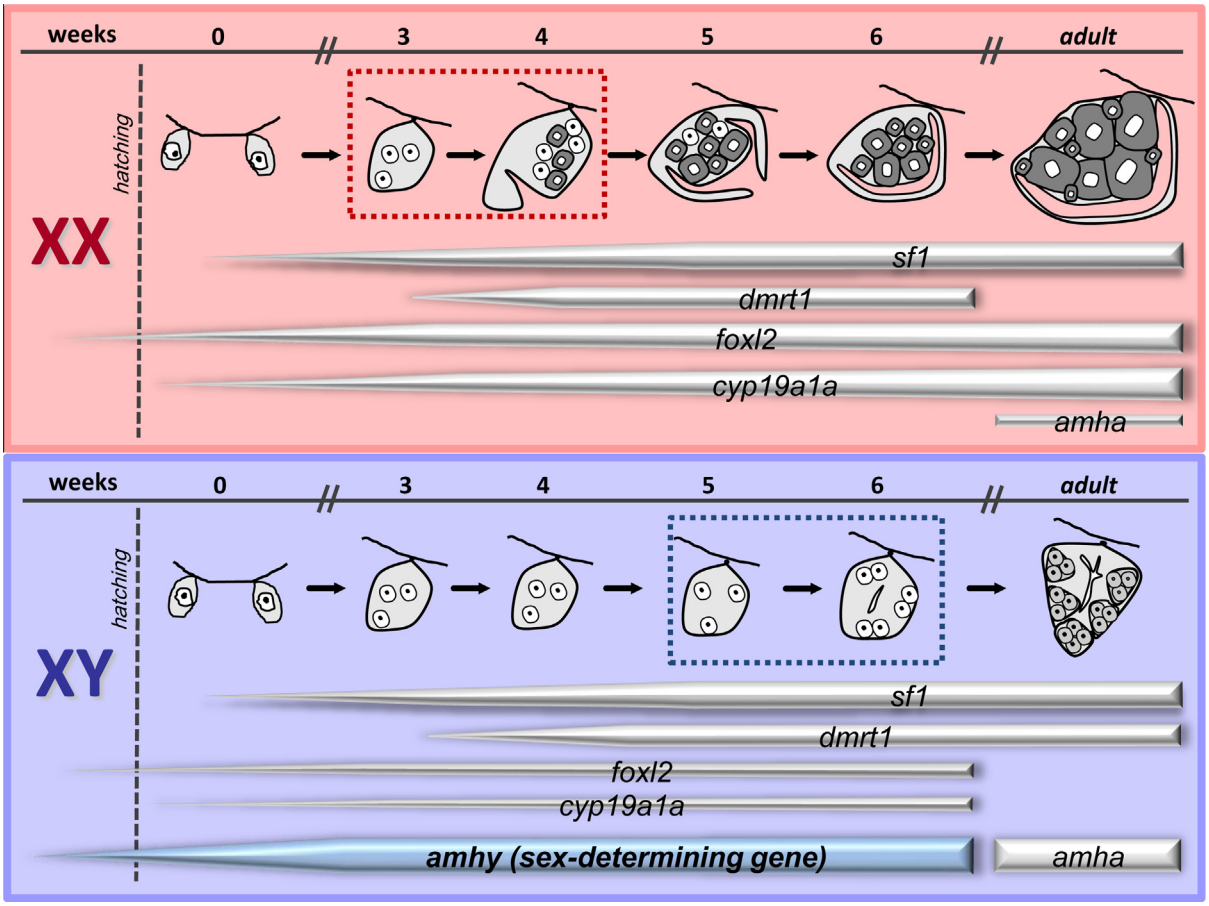

Fig. 1. Schematic representation of the expression profiles of some sex-related genes in relation to the timing of histological sex differentiation of the gonads (dotted boxes) in Patagonian pejerrey *O. hatcheri* XX and XY genotypes. Numbers indicate weeks after hatching. The thickness of the bars represents the levels of mRNA expression. Note that the transcription factor *dmrt1* does not display a sex-specific expression profile during the sex differentiation period.

4. *Amh/AmhrII* signaling and male fate in teleosts

The regression of Müllerian ducts induced by the AMH hormone in mammals is mediated by its specific primary receptor, known as AMHRII. Homologues of both genes have been reported in many teleost species (Morinaga et al., 2007; Wu et al., 2010). Interestingly, while all species analyzed present a single *locus* for both *amh* and its receptor, *O. hatcheri* possess two *amh loci*, the *amhy* and *amha*, but only a single *locus* for *amhrII*. Although we cannot rule out the existence of more than one *amhrII* in *O. hatcheri*, this possibility seems small because *amh* and *amhrII* are located in different gene clusters in vertebrates (Paibomesai et al., 2010) and it is unlikely that the duplication which gave rise to *amhy*/*amha* would have occurred also with *amhrII*. Nevertheless, the TGF-beta domain, which is the motif that binds to the receptor, is highly similar in the two *amh loci* with 92% of identity. Thus, it is plausible that both Amhs may share the same specific receptor AmhrII, rather than having specific receptors.

Regarding the role of Amh/AmhrII signaling on testis differentiation of teleost fish the information available is still incipient and mainly limited to Japanese medaka. In this model species, a mutation in *amhrII* leads to male-to-female sex reversal and an excessive proliferation of germ cells (Morinaga et al., 2007), suggesting that the *amh* signaling may control germ cell proliferation as proposed for the Japanese eel *amh* homologue (Miura et al., 2002). A similar function is also proposed for fugu (Kamiya et al., 2012) and for Patagonian pejerrey based on the localization of *amhy* transcripts in germ cell-supporting somatic cells (Hattori et al., 2012). Studies on the effects of germ cell depletion on sex differentiation suggest that germ cell numbers are crucial for gonadal fate in medaka (Kurokawa et al., 2007) and also in zebrafish (Siegfried and Nüsslein-Volhard, 2008). However, this may not be a general rule since in the loach (Fujimoto et al., 2010) and goldfish (Goto et al., 2012) germ-cell deficient animals developed either as females or males, with no effect in sex determination. Hence, it is necessary to clarify whether the action of *amh* signaling on testicular differentiation involves other molecular/cellular processes besides the control of germ cell proliferation.

5. Transposable elements-mediated gene duplication and the evolution of master sex-determining genes

Duplicated genes are common among teleosts due to the whole genome duplication that occurred during the evolution of this taxonomic group (Amores et al., 1998; Amores et al., 2004; Meyer and Schartl, 1999; Taylor et al., 2001a; Taylor et al., 2001b; Taylor et al., 2003). This is supported by the fact that several genes, including those involved in sex differentiation like the subtypes of the genes *sox9* in zebrafish and stickleback (Cresko et al., 2003) or *foxl2* in rainbow trout (Baron et al., 2005), are found in duplicate. Gene duplication also comprises an important mechanism for the evolution of sex-determining genes (Schartl, 2004; Volff et al., 2007), but since the evidences point out to relatively recent and independent evolutions in several groups, their

appearance might not be the result of ray-finned fish whole genome duplication.

Transposable elements are a class of repetitive sequences with the ability to undergo replicative transposition and with roles on genome restructuring and evolution. Interestingly, they have been detected in the *cis*-regulatory regions of sex determining genes. In medaka, *Izanagi* DNA transposons and *Rex1* elements have been found in the promoter regions of the *dmrt1bY* but not in the autosomal *dmrt1a (*Herpin et al., 2010). Yano and collaborators have also detected transposable elements few kilobases upstream salmonids' sex determining gene *sdY* (Yano et al., 2012, 2013). Thus, these elements are supposed to be responsible for carrying the duplicated genes from an autosome to a proto Y or W chromosome or for translocating potential regulatory regions into the promoter of a sex-related gene, inducing changes in the mechanisms of transcription activation, and occasionally resulting in the differentiation of a sex-determining gene.

6. Environmental sex determination as a possible reason for independent evolution of sex-determining genes

The process underlying the rise of a sex-determining gene seems to have a high plasticity since different mechanisms of chromosomal sex-determining systems and different kinds of sex-determining genes have been identified in many groups, even among very closely related species. A possible explanation lies in the fact that in many teleosts, even in those with established sex-determining genes, temperature can produce fully fertile sex-reversed organisms (Azuma et al., 2004; Strüssmann et al., 1996b; Strüssmann et al., 1997). This plasticity would hamper the establishment and maintenance of sex-determining genes compared to the case in homeotherm vertebrates. For example, in a hypothetical situation, high temperature, which has a masculinization effect in many species (Devlin and Nagahama, 2002; Hattori et al., 2007; Sato et al., 2005), could favor the formation of female-to-male sex reversal (e.g. XX males) by over expression of an autosomal sex-related gene. The sex-reversed male, without the testis-determining gene, would compete with the non-reversed XY male for the XX females, which may lead to a decrease in the proportion of XY fish and thus in the males with the sex-determining gene. If the period with high temperature persists during a large temporal scale, more XX males would be produced in the following generations, without the action of the sex-determining gene. In an extreme scenario, the proportion of Y-chromosome bearing males would gradually decrease until its complete extinction from the population (Hurley et al., 2004; Kanaiwa and Harada, 2002). Conversely, after the temperatures return to lower non-masculinizing levels, there could be an increased selection for genetic females with "mutated" sex-related genes that would allow the formation of males under such conditions. This assumption agrees with the finding that genotypic sex determination strength increases with increasing latitude (decreasing mean temperatures) in atherinopsids (Strüssmann and Patino, 1995; Strüssmann and Patino, 1998; Yamahira et al., 2003) as these environments would potentiate the appearance of strong sex determination mechanisms.

The identification of sex-determining genes in other taxonomic groups of teleosts by both genome and transcriptome analyses, which may advance faster from now on thanks to technologies like Next Generation Sequencing as well as by other newly emerging approaches, may help to clarify the processes behind the evolution of mechanisms of sex determination in fish and will also provide new tools for applied researches on reproductive ecology and aquaculture technology.

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