

Genetics of Sexual Development: An Evolutionary Playground for Fish

Corina Heule, Walter Salzburger, and Astrid Böhne¹
 Zoological Institute, University of Basel, 4051 Basel, Switzerland

ABSTRACT Teleost fishes are the most species-rich clade of vertebrates and feature an overwhelming diversity of sex-determining mechanisms, classically grouped into environmental and genetic systems. Here, we review the recent findings in the field of sex determination in fish. In the past few years, several new master regulators of sex determination and other factors involved in sexual development have been discovered in teleosts. These data point toward a greater genetic plasticity in generating the male and female sex than previously appreciated and implicate novel gene pathways in the initial regulation of the sexual fate. Overall, it seems that sex determination in fish does not resort to a single genetic cascade but is rather regulated along a continuum of environmental and heritable factors.

IN contrast to mammals and birds, cold-blooded vertebrates, and among them teleost fishes in particular, show a variety of strategies for sexual reproduction (Figure 1), ranging from unisexuality (all-female species) to hermaphroditism (sequential, serial, and simultaneous, including outcrossing and selfing species) to gonochorism (two separate sexes at all life stages). The underlying phenotypes are regulated by a variety of sex determination (SD) mechanisms that have classically been divided into two main categories: genetic sex determination (GSD) and environmental sex determination (ESD) (Figure 2).

Environmental factors impacting sex determination in fish are water pH, oxygen concentration, growth rate, density, social state, and, most commonly, temperature (for a detailed review on ESD see, e.g., Baroiller *et al.* 2009b and Stelkens and Wedekind 2010). As indicated in Figure 2, GSD systems in fish compose a variety of different mechanisms and have been reviewed in detail elsewhere (e.g., Devlin and Nagahama 2002; Volff *et al.* 2007).

The GSD systems that have received the most scientific attention so far are those involving sex chromosomes, which either may be distinguishable cytologically (heteromorphic) or appear identical (homomorphic). In both cases, one sex is heterogametic (possessing two different sex chromosomes and hence producing two types of gametes) and the other

one homogametic (a genotype with two copies of the same sex chromosome, producing only one type of gamete). A male-heterogametic system is called an XX-XY system, and female-heterogametic systems are denoted as ZZ-ZW. Both types of heterogamety exist in teleosts and are even found side by side in closely related species [e.g., tilapias (Cnaani *et al.* 2008), ricefishes (Takehana *et al.* 2008), or sticklebacks (Ross *et al.* 2009)]; for more details on the phylogenetic distribution of GSD mechanisms in teleost fish, see Mank *et al.* (2006). Note that sex chromosomes in fish are mostly homomorphic and not differentiated (Ohno 1974), which is in contrast to the degenerated Y and W chromosomes in mammals (Graves 2006) and birds (Takagi and Sasaki 1974), respectively. This is one possible explanation for the viable combination of different sex chromosomal systems within a single species or population of fish (Parnell and Streelman 2013) and could be a mechanistic reason why sex chromosome turnovers occur easily and frequently in this group (Mank and Avise 2009). Additionally, fish can have more complex sex chromosomal systems involving more than one chromosome pair (see Figure 2). Even within a single fish species, more than two sex chromosomes may occur at the same time, or more than two types of sex chromosomes may co-exist in the same species (Schultheis *et al.* 2006; Cioffi *et al.* 2013), which can sometimes be due to chromosome fusions (Kitano and Peichel 2012).

Detailed insights on the gene level for GSD/sex chromosomal systems are currently available for only a limited number of fish species, and all but one of these cases involve

Reproductive strategies in fish

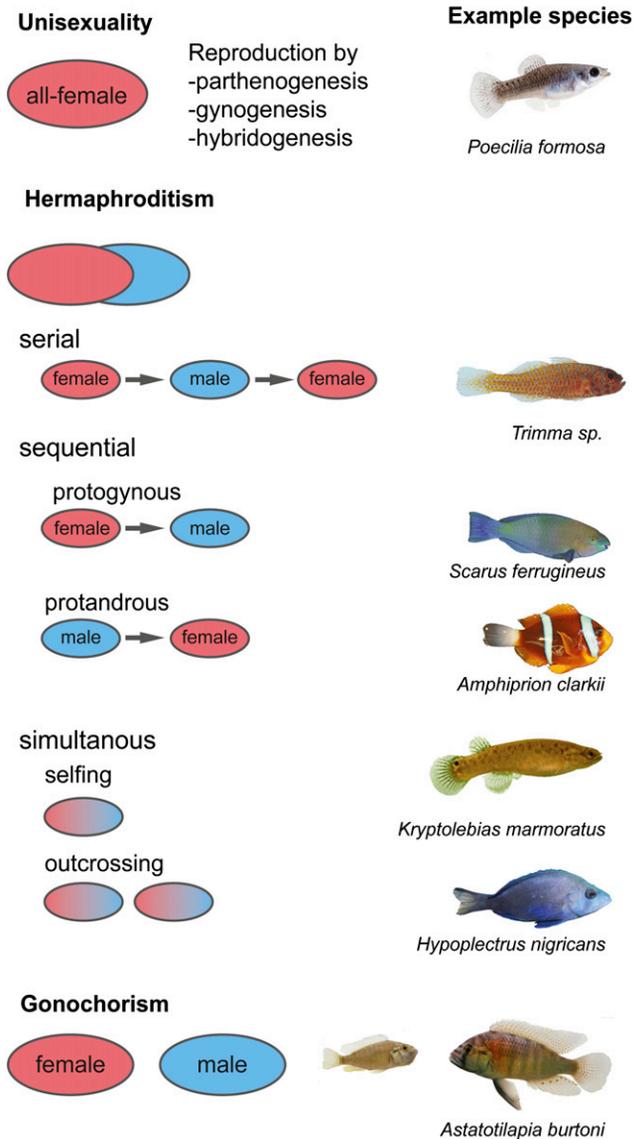


Figure 1 Reproductive strategies in fish. Fish can be grouped according to their reproductive strategy into unisexuales, hermaphrodites, and gonochorists. Further subdivisions of these three categories are shown with pictures of species exemplifying the strategies. Fish images: *Amphiprion clarkii* courtesy of Sara Mae Stieb; *Hypoplectrus nigricans* courtesy of Oscar Puebla; *Scarus ferrugineus* courtesy of Moritz Muschick; *Astatotilapia burtoni* courtesy of Anya Theis; *Poecilia formosa* and *Kryptolebias marmoratus* courtesy of Manfred Schartl; *Trimma sp.* courtesy of Rick Winterbottom [serial hermaphroditism has been described in several species of the genus *Trimma* (Kuwamura and Nakashima 1998; Sakurai *et al.* 2009; and references therein)].

a rather simple genetic system with male heterogamety and one major sex determiner (see below). The only exception is the widely used model species zebrafish (*Danio rerio*), which has a polyfactorial SD system implicating four different chromosomes (chromosomes 3, 4, 5, and 16) (Bradley *et al.* 2011; Anderson *et al.* 2012) and also environmental cues (Shang *et al.* 2006).

In this review, we focus on newly described genetic sex-determining systems and possible mechanisms allowing their emergence in fishes, which are the most successful group of vertebrates with ~30,000 species.

Genetic Basis of Sex Determination

Classic view on sexual development

The conventional definition of GSD states that the sex of an individual is fixed upon fertilization by inherited genetic factors. The establishment of the corresponding sexual phenotype is subsequently achieved through sexual development. The process of sexual development has classically been divided into sex determination and sex differentiation. Determination is understood as the “master” switch (initial inherited factor) that causes the first steps of the SD cascade, which then activate further downstream genes of sexual differentiation, which themselves regulate steroid hormone production, eventually leading to one functional gonad type with the corresponding sexual phenotype (Figure 3, top). In this scenario, the master switch functions as a presence-absence signal, in which the presence initiates the cascade to actively produce one sex, whereas the absence causes the other sexual differentiation program, which has long been considered as the default sex. However, this view has recently been challenged in, for example, mammals, where female development also needs active gene regulation and feedback loops (Munger and Capel 2012).

In the classic view, the SD cascade is considered to have evolved stepwise, in a retrograde fashion, with the downstream genetic network evolving first (and remaining conserved over time and between species), and new genes subsequently and independently added to the top of the hierarchy in different species/lineages (bottom-up theory) (Wilkins 1995, 2005) (Figure 3, top). That major changes occur frequently at the top of the cascade are substantiated by the observation that even closely related fish species (*e.g.*, of the same genus/family) do not share the same master SD gene (*e.g.*, the ricefishes, genus *Oryzias*) (Kondo *et al.* 2004).

However, the hypothesis of the existence of a conserved genetic cascade of SD common to all vertebrates governed by different lineage or species-specific genes [“masters change, slaves remain (Graham *et al.* 2003)] is largely based on sequence conservation in the downstream genes and conserved expression patterns for a handful of genes such as the testis-specific *dmrt1* (Smith *et al.* 1999).

Sex as a threshold phenotype based on a sex-determining network

More and more data reveal that, especially in teleost fishes, sex seems to be a rather plastic phenotype with natural and inducible sex reversal even after the genetic fixation of one sex at fertilization (Paul-Prasanth *et al.* 2013). These results thus question the scenario of a stepwise, hierarchical cascade with a fixed outcome. Instead, it has been suggested that sex should be interpreted as a threshold trait with

Described sex-determining mechanisms in fish

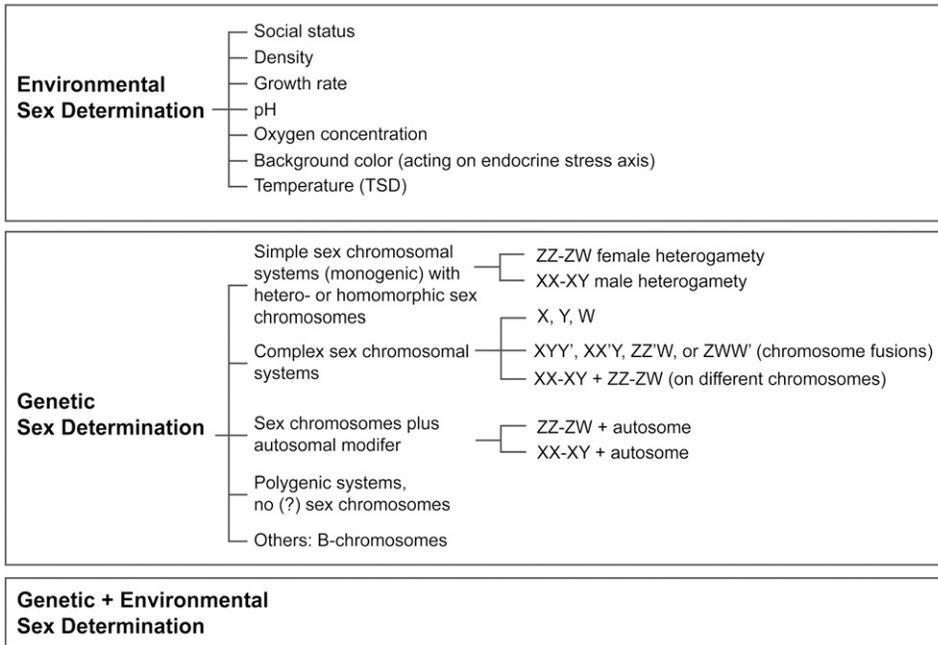


Figure 2 Sex-determining mechanisms in fish. Sex-determining systems in fish have been broadly classified into environmental and genetic sex determination. For both classes, the currently described subsystems are shown.

triggers (genetic, parental, or environmental) acting on a “parliament of interacting gene networks” (Crews and Bull 2009) and hormones (Uller and Helanterä 2011; Schwanz *et al.* 2013), resulting in a rather complex interplay between different signals (Figure 3, bottom). Importantly, gene networks are not necessarily hierarchical but rather modular (for review and further extension of this idea, see Salazar-Ciudad 2009 and Barabasi and Oltvai 2004). Assuming an interconnected network of genes and other factors impacting sex, the emergence of new sex determiners should be viewed from a developmental perspective (Figure 3, bottom), which allows for the emergence of major effect loci at different levels/nodes of the network.

In the next section, we present the current models for the evolution of such major-effect loci and our extension of these to a network concept of sex determination.

Suggested Mechanisms for the Emergence of New Master Sex Determination Genes

Based on the view of sex determination as a hierarchical cascade, the model of Schartl (2004) suggests that a new master SD gene can arise via a combination of gene duplication, sequence change (coding or regulatory), and (up-)recruitment [Figure 4, scenario 1 after (Schartl 2004)]. However, similar mechanisms of genetic changes, based mainly on duplication and subsequent mutation and especially without the loss of one of the duplicates, could also work with sex determination relying on a network of genes and must not necessarily occur only at the top of a cascade (Figure 4, scenario 2). These two ideas are rather mechanistic. More generally, the observation that similar/related genes, which are implicated in sexual development in several

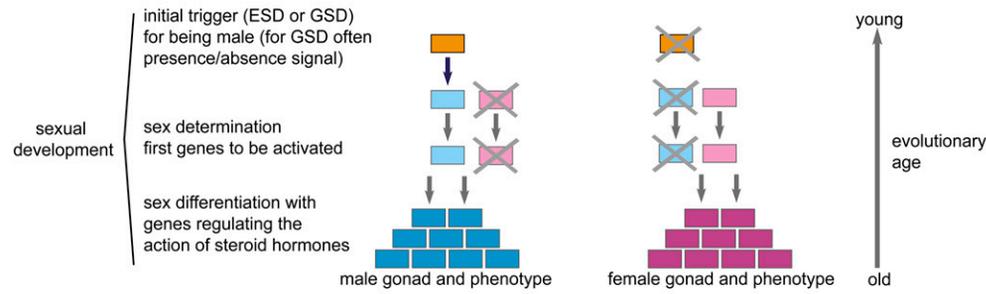
species, are recruited as master regulators (mainly dm-domain and HMG-domain transcription factors) led Marshall Graves and Peichel (2010) to put forward the “limited options” hypothesis. They suggested that a pool of genes/chromosomes “good at doing the job” might exist and be preferentially reused repeatedly in the vertebrate lineage (although in different combinations) rather than that “any” gene could be recruited as a master SD gene. As indicated in Figure 2, teleost fish are a particularly attractive system to study these models for the evolution of SD networks and their master genes.

Currently, detailed genetic information on male SD genes is available for six teleost species that we will discuss in detail in the next paragraph.

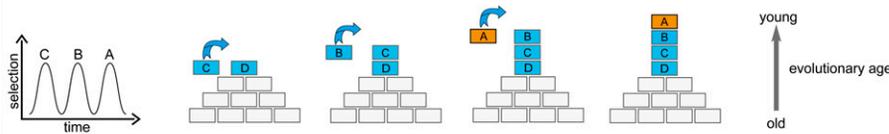
New master sex-determining genes in teleosts: *dmY* and *gsdf* in the *Oryzias* genus:

The best-studied SD gene in fish is the second vertebrate master SD gene that was described after the discovery of the mammalian *Sry* gene, namely *dmrt1by/dmy*, *DM-domain gene on the Y chromosome*, which acts as the master sex determiner in two species of the genus *Oryzias* [the medaka, *Oryzias latipes*, and the Malabar ricefish, *O. curvinotus* (Matsuda *et al.* 2002; Nanda *et al.* 2002)]. *Dmy* is not present in any other fish species studied so far, not even in the closely related *Oryzias* species (Kondo *et al.* 2003). *Dmy* arose in the common ancestor of *O. latipes* and its sister species *O. curvinotus* and *O. luzonensis* between 10 ± 2 and 18 ± 2 million years ago through a segmental duplication of a small autosomal region containing its precursor gene *dmrt1* [a gene with a well-described function in testis in vertebrates (Matson and Zarkower 2012) and some neighboring genes (Kondo *et al.* 2004) (see Figure 5A for illustration)]. This was

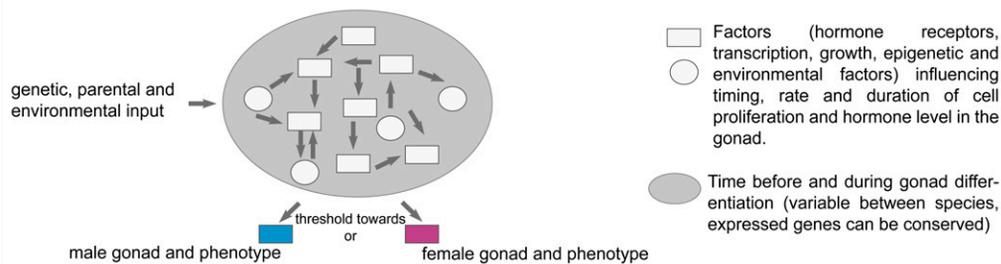
Classic view of the sex-determining cascade



Bottom-up scenario fitting the classic view: retrograde evolution after Wilkins (1995, 2005)



Sex determination with male and female state as threshold phenotype



Developmental perspective on the evolution of the SD network after (Crews and Bull 2009) and (Uller and Helanterä 2011)

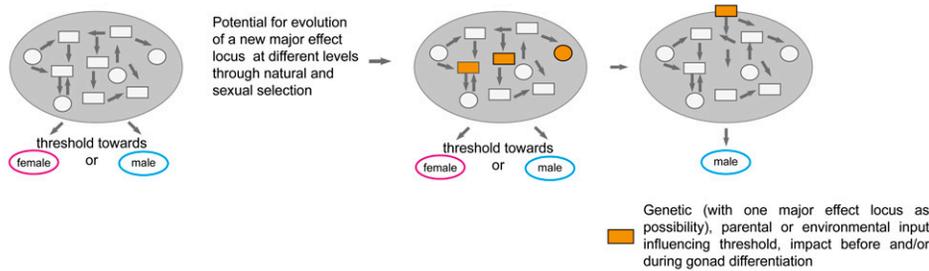


Figure 3 Two views on the sex-determining cascade. Classic view on the sex-determining cascade: The prevailing view on sexual development is the one of an initial trigger (environmental or genetic, mostly a presence/absence signal) initiating the sex determination cascade that activates sex differentiation, finally causing the establishment of one gonad type and the corresponding sexual phenotype. In this scenario, the presence of the initial trigger (here exemplified for a male master determiner) activates one cascade, whereas its absence leads to the other sex. Based on this cascade assumption, genes are added stepwise to the existing cascade. The cascade thus evolves in a retrograde fashion. The last (*i.e.*, the most downstream) step is the first one to be selected for. This evolution leads to the genetic network of sexual development divided into two steps: sex determination and differentiation (Wilkins 1995, 2005). Sex determination with male and female state as threshold phenotype: Based on a developmental perspective (Crews and Bull 2009; Uller and Helanterä 2011), sexual development is not split into determination and differentiation but rather controlled by a combination of different heritable and external factors influencing cell proliferation and hormone levels with a male and female threshold. Determining regulators evolve via canalization toward major-effect loci influencing the male/female threshold. Note that under this model without a strict hierarchical cascade, major-effect loci could emerge at all levels and are not imposed at the very top of the cascade.

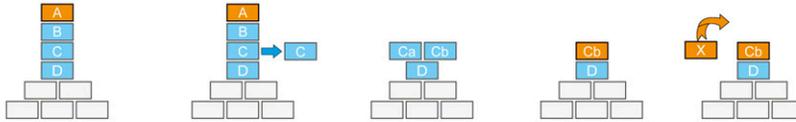
followed by the insertion of the duplicated region on the proto-Y chromosome, directly creating a region of suppressed recombination. Outside of the relatively small sex-determining region (258 kb), in which *dmy* remained the only functional gene, recombination is not suppressed between the medaka X and Y chromosomes (Brunner *et al.* 2001; Kondo *et al.* 2006). *Dmy* is often cited as a typical example of an up-recruited SD gene, as is *DM-W* in the frog *Xenopus laevis* and possibly *dmrt1* in birds. It is derived from a gene that has already been implicated in testis formation and male development, *dmrt1*, agreeing with the idea of limited options

for controlling sex determination. The mechanism of its evolution as a master SD gene likely followed the one described in Figure 4B.

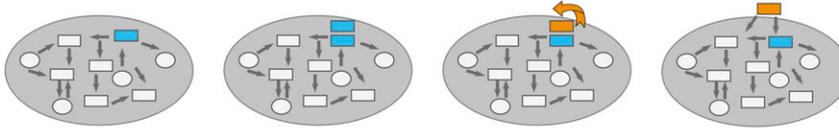
In a closely related *Oryzias* species, *O. luzonensis*, which has lost *dmY*, Myosho *et al.* (2012) identified a derived version of the *gonadal soma derived growth factor* (*gsdf*) as the male master sex determiner. The gene has a Y- and X-chromosome-specific allele, called *gsdf^Y* and *gsdf^X*. Gene expression experiments revealed an overexpression of *gsdf^Y* during the time period of sex determination, whereas expression levels of both alleles were similar later in development.

Changes at the top of the cascade: scenarios for the emergence of new master sex determinants

1. Evolution by duplication, deletion, mutation and recruitment of new genes after Scharl (2004)



2. Evolution by duplication and duplicate retention of the ancestral gene at its former position



3. Limited options/cycling after Marshall Graves and Peichel (2010) (note that A, B, C and D could also reflect entire chromosomes not only genes)

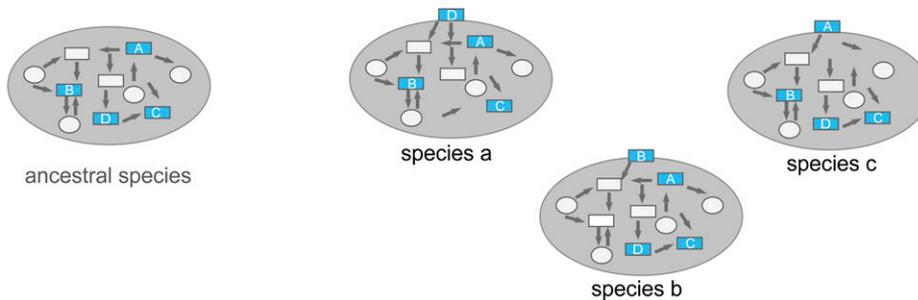


Figure 4 Models for the emergence of new genetic master sex determinants. Three mechanisms mainly influencing the evolution of master SD genes are shown. (1) Based on the classic cascade view, new genes formed by gene duplication or mutation in existing genes can be up-recruited to the top of the cascade (Scharl 2004). (2) Based on the view of SD as a genetic network, gene duplication and/or mutation of a member of the network could create potential material for the evolution of a new master gene without the loss of the ancestral gene. (3) The limited options theory after Marshall Graves and Peichel (2010) proposes that a pool of genes or entire chromosomes are reused in different species to become master determinants. Note that, as shown here, this model does not rely on a hierarchical cascade view of SD.

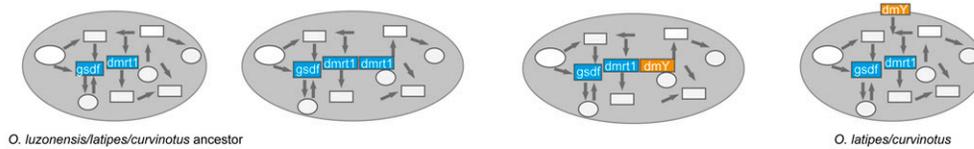
gsdf^Y and *gsdf^X* vary by 12 nucleotide substitutions, but all are silent. The male expression is thus likely caused by a mutation in a *cis*-regulatory region of *gsdf^Y*. Myosho *et al.* (2012) speculate that this mutation could involve a change in a putative binding site for steroidogenic factor 1 (Sf1), causing a loss of the binding site in *gsdf^Y* but not in *gsdf^X*. This change could lead to the observed higher expression during an earlier stage of sexual development compared to its ancestral counterpart. Transgenic transformation with *gsdf^Y* constructs led to XX males in *O. luzonensis* (as well as in its sister species *O. latipes* with a different SD gene), confirming a master sex-determining role for this gene. In *O. luzonensis*, *gsdf^Y* thus seems to have functionally replaced *dmY*. Note that the *O. luzonensis* genome harbors a pseudogenized copy of *dmrt1* called *Oludmrt1p*, in addition to the autosomal *dmrt1* gene (*Oludmrt1*). However, this pseudogene is located on a chromosome that is not syntenic to the Y chromosome of *O. latipes*. It is therefore likely that *O. luzonensis* has lost *dmY* from its genome and that *Oludmrt1p* is an independent pseudogene copy of *dmrt1* (Kondo *et al.* 2004; Tanaka *et al.* 2007).

In contrast to *dmY*, *gsdf* is not a transcription factor but a member of the TGF- β superfamily and specific to fish [it is present in all available teleost genomes and in the sarcop-

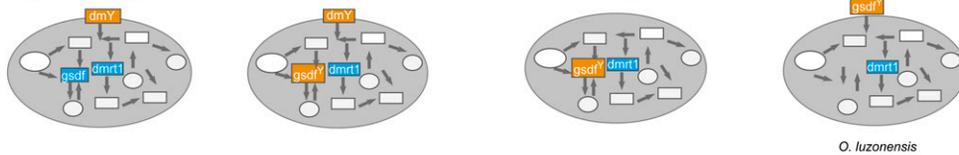
terygian *Latimeria* but not in tetrapods (Forconi *et al.* 2013)]. In *O. latipes* males, *gsdf* is transcribed at 6 days post fertilization in the primordial gonad and colocalized with *dmy* in the somatic cells (Sertoli cells) of the adult male gonad (Shibata *et al.* 2010). This suggests that *gsdf* acts rather early [indeed, it is expressed earlier than *dmrt1*] (Kobayashi *et al.* 2004)], which possibly facilitated its recruitment as a master SD gene. Functional data for *gsdf* are still limited, but it has been implicated with the proliferation of primordial germ cells (PGCs) and spermatogonia in the rainbow trout (Sawatari *et al.* 2007) and it possibly acts during female-to-male sex change, promoting proliferation of spermatogonia and spermatogenesis in the wrasse *Halichoeres trimaculatus* (Horiguchi *et al.* 2013). Furthermore, *gsdf* turns out to be a good candidate for the sex-determining locus in a member of another fish genus, the sablefish *Anoplopoma fimbria* (Rondeau *et al.* 2013), suggesting a central role for this gene in SD in fish and calling for further studies. The model of Uller and Helanterä (2011) (see Figure 3) suggests that factors important in the network of sex determination (and possibly driving the threshold toward one sex) are those influencing timing, rate, and duration of cell proliferation. With the information available for *gsdf*, this gene might be one such factor, and this could explain

Possible scenarios for the evolution of fish master SD genes

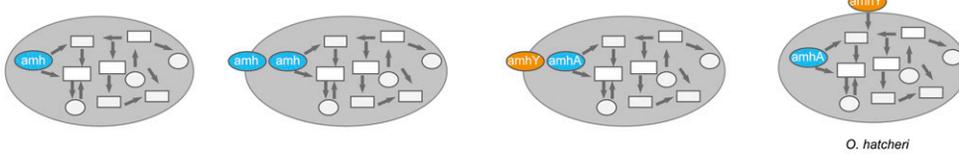
A *dmrt1bY/dmy* in *Oryzias latipes* and *curvinotus*, exemplifies models 2. and 3. in Figure 4



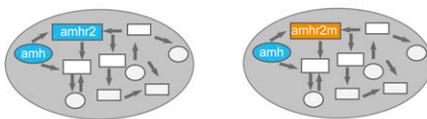
B *gsdfY* in *Oryzias luzonensis*, exemplifies models 1. and 3. in Figure 4



C *amhY* in *Odontesthes hatcheri*, exemplifies models 2. and 3. in Figure 4



D Sex-linked SNP in *amhr2* in the *Takifugu* genus, exemplifies model 3. in Figure 4



E *sdY* in the Salmonidae family: two hypothetical scenarios

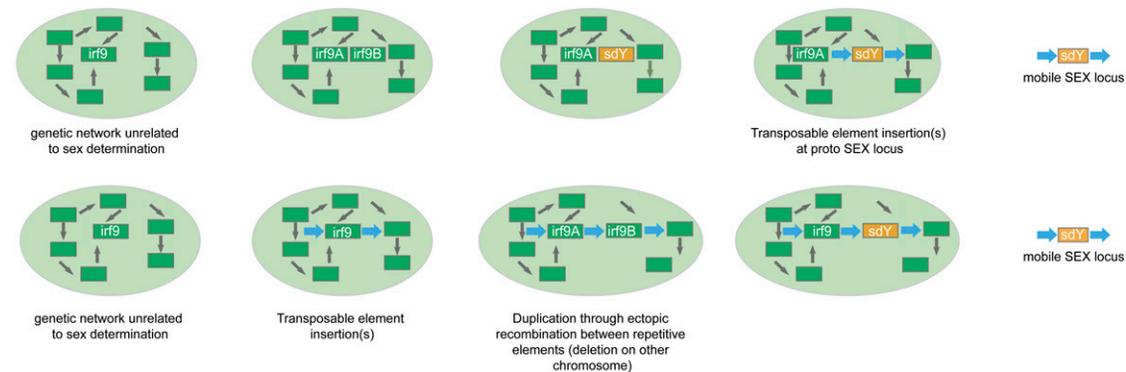


Figure 5 Possible scenarios for the evolution of fish SD genes. The figure illustrates possible evolutionary paths for the currently known master SD genes in teleost fish: (A) *dmrt1bY/dmy*, (B) *gsdfY*, (C) *amhY*, (D) *amhr2*, and (E) *sdY*. Horizontal arrows indicate gene duplications; color change of boxes to orange indicates acquisition of master-determining function via mutation (coding or regulatory). Green boxes in E indicate that the genetic network that *irf9* belongs to was not related to sex determination before the emergence of *sdY*.

why a gene that is not a transcription factor has been recruited as a master SD gene and could, at least in fish, represent support for the limited options hypothesis.

***amhY* and *amhr2*:** Two other newly described sex determiners, *amhy* and *amhr2*, also belong to the TGF- β superfamily. *amhy* is a gene duplicate of *amh* (*anti-Müllerian hormone*) found on the Y chromosome of the Patagonian pejerrey, *Odontesthes hatcheri*. This gene has a 557-bp long

insertion in the third intron and 92.2% sequence identity on the amino acid level compared to the autosomal copy (*amha* for *amh* autosomal) (Hattori *et al.* 2012). Hattori *et al.* (2012) showed that *amhy* is expressed during gonadal formation starting at 6 days after fertilization whereas *amha* is expressed later (12 weeks after fertilization). *amhy* transcripts are located in somatic cells (likely Sertoli cells) surrounding germ cells in the developing male gonad, a typical expression pattern for SD genes. Knockdown of *amhy* in XY

embryos led to an up-regulation of female factors and, consequently, to the development of ovaries (Hattori *et al.* 2012).

The *amhr2* (*amh receptor 2*) gene has been identified as possible third nontranscription factor master SD gene in the tiger pufferfish, *Takifugu rubripes* (Kamiya *et al.* 2012). Here, a single nucleotide polymorphism (SNP) in the kinase domain of *amhr2* has been associated with sex, with males being heterozygous and females homozygous. This sex-specific SNP, which changes an amino acid (His384 to Asp384), is also associated with sex in two other *Takifugu* species (*T. pardalis* and *T. poecilonotus*), which diverged around 5 million years ago from *T. rubripes*. The authors suggest that sex in the tiger pufferfish is determined by a combination of the two *amhr2* alleles, with the Y allele being dominant.

AMH is a secreted glycoprotein of the TGF- β superfamily best studied in tetrapods, where it is produced by the Sertoli cells and causes regression of Müllerian ducts during male sexual development (Josso *et al.* 2001; Rey *et al.* 2003; Johnson *et al.* 2008). AMH signaling functions through its primary receptor, AMHR2, which, upon AMH-binding, induces the assembly of a receptor complex eventually, activating expression of target genes (Josso *et al.* 2001) (see Figure 6 for details).

Although fish possess *amh* and *amhr2*, they lack Müllerian ducts. In fish, the AMH-signaling pathway has been implicated in PGC proliferation and spermatogenesis, similar to the function proposed for Gsdf (Morinaga *et al.* 2007). In general, AMH signaling in fish could decrease the number of germ cells (Morinaga *et al.* 2007; Lee *et al.* 2009), which in some species, such as the medaka or zebrafish, are crucial for gonadal fate determination (Kurokawa *et al.* 2007; Siegfried and Nüsslein-Volhard 2008) with more germ cells leading to a female gonad. However, this seems not to be a general mechanism in fish (Fujimoto *et al.* 2010; Goto *et al.* 2012). Another proposed mechanism of action for AMH/AMHR2 in fish is through the activation of the aromatase enzyme that synthesizes estrogens from androgens as has been described in mammals (Di Clemente *et al.* 1992). Higher estrogen levels result in ovary differentiation in fish (Devlin and Nagahama 2002). A suppression of the estrogen production by AMH/AMHR2 signaling on the aromatase could thus decrease estrogen levels and so promote testis formation.

amhy, like *dmY*, exemplifies the mechanism of gene duplication for the formation of new master SD genes. Based on the limited data available, members of the TGF- β superfamily (*gsdf*, *amh*, and *amhr2*) could be part of the limited options for master SD genes, at least in fish. However, the options for the evolution of new SD genes in fish could be less limited as the next example suggests.

***sdY* in the salmonid family:** The fourth newly discovered sex regulator in fish is *sdY* (*sexually dimorphic on the Y chromosome*) in the rainbow trout, *Oncorhynchus mykiss* (Yano *et al.* 2012). This gene is the first example of a SD gene not related to a gene that was already implicated with sexual development and, hence, could be an example for functional *de novo* evolution (Yano *et al.* 2012). *sdY* shows sequence similarity to *irf9* (*in-*

terferon regulatory factor 9), a transcription factor that acts in the immune system (Yano *et al.* 2012). The presence of *sdY* was confirmed in 15 salmonid species, and male sex linkage was found for 13 of these (Yano *et al.* 2013). Interestingly, the Y chromosomes in the investigated salmonid species are not syntenic, and hence the SD locus is located on different chromosomes. Before the discovery of *sdY*, the idea of a (likely small) conserved but mobile SD locus had been put forward for the salmonid family as a possible explanation for the fact that different chromosomes showed sex linkage in different species (Davidson *et al.* 2009) and even within species (Moghadam *et al.* 2007; Küttner *et al.* 2011; Eisbrenner *et al.* 2013).

To conclude this section, support for the limited-options theory comes certainly from *dmy* [duplication of *dmrt1*, a gene involved in development of testis in vertebrates and retention of *dmrt1* at its ancestral position (Kondo *et al.* 2006)], and possibly also *amhy* [duplication of *amh*, a gene that is responsible for the degeneration of the Müllerian ducts in male mammals (Klattig and Englert 2007) and retention of its ancestor *amha*], *gsdf^Y* [derived from *gsdf*, a gene involved in the proliferation and differentiation of germ cells in fish (Gautier *et al.* 2011a)], and *amhr2* [a receptor involved in the AMH pathway in vertebrates (Klattig and Englert 2007)]. By contrast, *irf9*, the next most closely related gene to the salmonid SD gene *sdY*, has not been implicated in SD before and so could have been added on top of a conserved genetic network at a later stage.

Further Flexibility in the Network: Variation in Downstream Gene Usage

Until recently, the steps following the initiation of sex determination were thought to follow a rather well-orchestrated and conserved cascade of genes (some of them candidates for the limited options) controlling sexual development mainly via the action of steroid hormones (Lange *et al.* 2002; Nakamura 2010; Angelopoulou *et al.* 2012; Morohashi *et al.* 2013). Among the most prominent examples of apparently conserved genes of sexual development are *dmrt1* (Matson and Zarkower 2012) and *sox9* (Morrish and Sinclair 2002; Kobayashi *et al.* 2005) for testis formation and *wnt4* (Smith *et al.* 2008) and the aromatase *cyp19a1* (Valenzuela *et al.* 2003; Diotel *et al.* 2010) for ovary development. However, recent data from various fish species suggest that, in addition to the flexibility at the top of the cascade, even these key elements are not as conserved as previously assumed. This is evidenced by varying and species-specific expression patterns between sexes and throughout development (see, e.g., Vizziano *et al.* 2007; Ijiri *et al.* 2008; Hale *et al.* 2011; Herpin *et al.* 2013; and Table 1). For example, *sox9* and the aromatase *cyp19a1* show expression patterns in East African cichlid fishes that are not consistent with conserved testis and ovary functions, respectively (Böhne *et al.* 2013).

The case of the aromatase *cyp19a1* is particularly striking. As already mentioned, this enzyme converts androgens

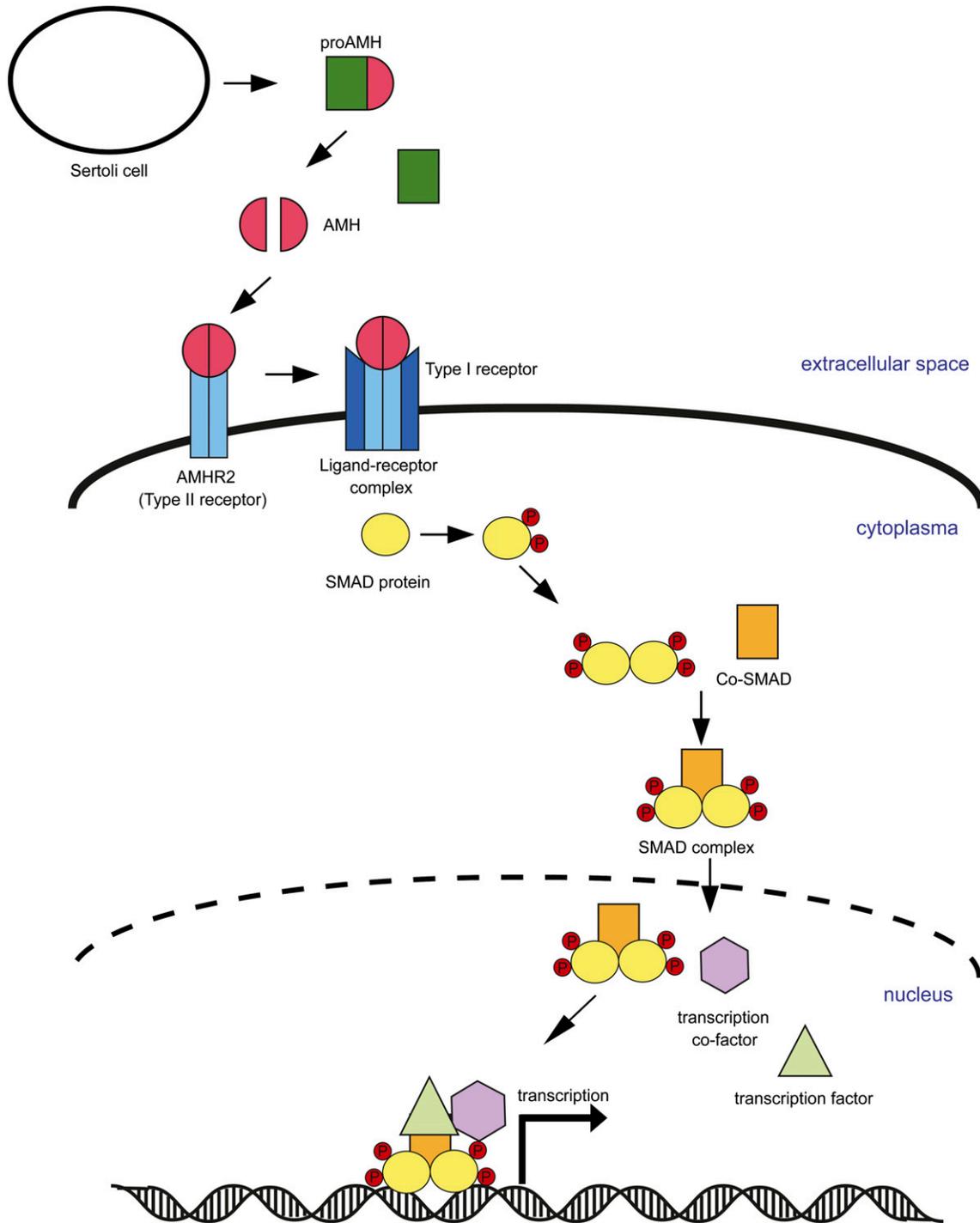


Figure 6 AMH/AMHR2-signaling pathway. Sertoli cells secrete a precursor form of AMH. After cleavage, an AMH dimer binds to AMHR2, which activates a type I receptor (currently not characterized; mechanism derived from comparisons with other receptors of the TGF- β superfamily). The ligand-receptor complex phosphorylates SMAD proteins, which then form a complex by incorporating Co-SMADs. This complex translocates into the nucleus and regulates gene expression together with transcription factors and cofactors. Figure modified after Fan *et al.* (2012) and Kikuchi and Hamaguchi (2013) and references therein.

into estrogens and was thought to control the female and male pathway by its presence or absence in expression, respectively (Guiguen *et al.* 2010). After the teleost-specific genome duplication, teleost fish retained two copies of this gene (*cyp19a1A* and *cyp19a1B*), which are active in ovary

and brain tissue, respectively. Some derived East African cichlid species express the otherwise ovary-specific gene *cyp19a1A* in testis (Böhne *et al.* 2013). This finding asks for a critical revision of the hormonal control of sexual differentiation.

Table 1 Variation in gene expression profiles of sexual development genes in major teleost model species

Gene	Nile tilapia (<i>O. niloticus</i>)	Medaka (<i>O. latipes</i>)	Patagonian pejerrey (<i>O. hatcheri</i>)	Rainbow trout (<i>O. mykiss</i>)	Zebrafish (<i>D. rerio</i>)	Burton's haplo (<i>A. burtoni</i>)
<i>amh</i>	♂	♀/♂	♂ (first <i>amhy</i> , then <i>amha</i>)	♂	♂	♂
<i>cyp19a1A</i>	♀	♀	♀	♀	♀	♀
<i>cyp19a1B</i>	♀	♀	NA	♂	♂/♀	♂
<i>dmrt1</i>	♂	♂	♀/♂	♂	♂	♂
<i>gsdf</i>	♂	♂	NA	♂	♂/♀	♀
<i>sf-1</i>	first ♀, then ♂	♀	♂/♀	♂	♂	♂
<i>sox9A</i>	♂	♀	NA	♂	♂	♂
<i>sox9B</i>	♂	♂	NA	♂	♀	♀

Data: *Oreochromis niloticus* (Kobayashi *et al.* 2008; Ijiri *et al.* 2008; Baroiller *et al.* 2009a; Poonlaphdecha *et al.* 2011, 2013; Tao *et al.* 2013; <http://www.uniprot.org/uniprot/E9RGV7>), *Oryzias latipes* (Suzuki *et al.* 2004; Klüver *et al.* 2005; Nakamoto *et al.* 2007; Shibata *et al.* 2010; Siegfried 2010; Okubo *et al.* 2011; Nakamura *et al.* 2012), *Odontesthes hatcheri* (Hattori *et al.* 2013), *Oncorhynchus mykiss* (Baron *et al.* 2005; Sawatari *et al.* 2007; Gohin *et al.* 2011; Vizziano-Cantonnet *et al.* 2011), *D. rerio* (Chiang *et al.* 2001; Guo *et al.* 2005; Von Hofsten and Olsson 2005; Kallivretaki *et al.* 2007; Siegfried 2010; Gautier *et al.* 2011b; Nakamura *et al.* 2012), *Astatotilapia burtoni* (Böhne *et al.* 2013; A. Böhne and W. Salzburger, unpublished results). ♂, male specific or overexpressed; ♀, female specific or overexpressed; NA, not tested.

Interlinking GSD and ESD

Interestingly, steroid hormones, or the regulation of their production, are a putative link between ESD and GSD (Nakamura 2010; Angelopoulou *et al.* 2012), with *cyp19a1* being a prime candidate (Valenzuela *et al.* 2013). Navarro-Martín *et al.* (2011) have recently investigated the methylation levels of the aromatase (*cyp19a1A*) promoter in the European sea bass, *Dicentrarchus labrax*, a species with a polygenic SD system influenced by temperature (Vandeputte *et al.* 2007). They found that the *cyp19a1A* promoter in males features twice as many methylated sites compared to females, leading to an under-expression of this gene in males. The methylation rate is positively correlated with water temperature, and temperature-masculinized fish (*i.e.*, sex-reversed genetic females) have a higher methylation rate and lower levels of *cyp19a1A* expression.

In some species thought to have GSD, temperature has been shown to impact sex ratios (*e.g.*, the Nile tilapia) (Baroiller *et al.* 2009a). Furthermore, some species previously thought to have ESD were later found to have genetic mechanisms of sex determination (Ospina-Álvarez and Piferrer 2008). This has led to a controversial discussion about the occurrence of true or obligate temperature-dependent sex determination (TSD) in fish. A critical evaluation of the available data on the prevalence of TSD in fish (Ospina-Álvarez and Piferrer 2008) suggested two prerequisites to a true TSD system: (1) there must be the absence of sex chromosomes and (2) the temperature shifts influencing sex ratios must occur during decisive developmental steps. Based on these assumptions, TSD in fish appears to be much less widespread than previously thought with many observed cases involving (extreme) temperatures that lie outside the naturally occurring range (Ospina-Álvarez and Piferrer 2008). However, the two assumptions seem rather strict, given the fact that sex in fish is often not stable but can change even after juvenile stages. Baroiller *et al.* (2009b) suggested, in a less strict way, the classification of TSD fish species into three categories: (1) species with strong GSD and an absence of, or weak sensitivity to, temperature; (2) species that are highly sensitive to temperature or other environmental factors; and (3) species that are sensitive

to several environmental cues. This definition seems to better reflect the empirical data and offers another part of the framework toward a new understanding of the mechanisms driving sex determination systems and their evolution.

Conclusion

Considering the recent findings of a substantial flexibility in expression and timing in the downstream network of sexual differentiation, the diversity of master SD genes found in fish and the co-influence of genetic and environmental factors, we think that it is time to redefine our understanding of the SD cascade. An attempt into this direction has recently been made by proposing that one should not strictly distinguish between sex determination and sex differentiation (Uller and Helanterä 2011). Instead, the entire process should be seen as a continuum in which all factors (genetic, maternal, and environmental) work together in a nonhierarchical network to ultimately form a male or female phenotype. This process would be canalized toward the male or female fate above a certain sex-specific threshold of genetic and environmental interactions influencing cell proliferation and hormonal levels (Figure 3, top). An extreme canalization of this process can result in the evolution of one or more major-effect locus/loci, such as the master SD genes described earlier. However, this does not have to happen, as reflected by the polygenic SD system in the zebrafish.

Interestingly, this view also offers a developmental explanation for how factors that, at the first glance, seemed unlikely master regulators, such as the members of the TGF- β family *gsdf*, *amh*, and *amhr2*, could become important in sex determination. The evolution of the TGF- β family members as master SD genes, although not acting as transcription factors, might simply reflect their previous involvement in the regulation of timing, rate, and duration of cell proliferation (in the developing gonad) and interaction with steroidogenesis, as described for their ancestral genes (Teixeira *et al.* 1999; Yamamoto *et al.* 2002; Sawatari *et al.* 2007; Shiraishi *et al.* 2008). For example, *amha* and *amhy* are spatially colocalized but not temporally (Hattori *et al.* 2012), providing

one example of how a change in timing of expression could lead to the up-recruitment of a seemingly downstream factor.

To understand the evolution of sex-determining networks—and possibly there is not only one cascade of SD but rather several network modules that are slightly rewired to ever-new combinations—we have to understand the different steps of sexual development and their interconnections (see Table 1 for a first approach to understanding shifts in gene expression in fish). In this context, the network modules could also be considered as limited options; however, we are far away from understanding where the limits really are, especially in fish.

We suggest a critical rethinking of the current models for the evolution of sex-determining genes with a stronger focus on the interactions further downstream in the network. All currently proposed models aim to explain switches in the master control genes without considering the dynamics of the subsequent networks. Hence, much effort has been put toward the identification of new “masters,” especially in fish. Why SD systems are so extremely divergent and why turnovers of sex-determining systems are so seemingly frequent in fish remains to be answered. We propose regarding the SD pathway as a whole and not considering only its master switches as an isolated phenomenon when investigating these systems.

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