

Review

# Sex Determination and Male Differentiation in Southern Swordtail Fishes: Evaluation from an Evolutionary Perspective

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**Abstract:** Southern swordtail fishes, which belong to the viviparous teleosts called *Xiphophorus*, are unique models for studies of evolution of sex chromosomes. Monofactorial sex-determining systems, with either the male or the female being the heterogametic sex, as well as sex determination involving more than two sex chromosomes, are found in swordtails and related species. Some swordtail species seem to have originated by crossing between two closely related species. Although the sword has disappeared in many *Xiphophorus* species during evolution, females of non-sworded species still prefer sworded males, demonstrating a discrepancy between natural and sexual selection. Natural sex change has not been documented sufficiently convincingly in swordtails, but, at least in some subspecies, two or more male phenotypes exist. In a laboratory experiment performed for over 30 years, it has been observed that sex-determining genes may be translocated from one chromosome to another in hybrids of these evolutionary young species. While the factors suggested to play central roles in sex determination and differentiation, e.g., *Dmrt1* and AMH, are highly conserved during evolution, several master determining factors have been detected in teleosts. Endocrine-induced sex reversal has been demonstrated in the guppy *Poecilia reticulata*, another viviparous fish. In swordtails (*X. helleri*), endocrine disruptors such as nonylphenol and bisphenol A may cause testis cell degeneration and the inhibition of spermatogenesis. Furthermore, swordtails are very easy to breed in freshwater aquaria and, therefore, may be good models for studying the factors influencing sex determination and male differentiation.

**Keywords:** sex determination; male differentiation; sex chromosome; *Xiphophorus*; swordtail

**Key Contribution:** Swordtails are good candidates for studies of sex determination and possible evolutionary mechanisms. Experimental studies can easily be performed, since swordtails are live-bearing and easy to raise in aquaria.



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## 1. Introduction

Since a wide range of sex chromosome systems and a spectrum of male phenotypes are present in swordtails, and such fishes may be good models for sex determination and male differentiation. Since some southern swordtail species may have multifactorial sex determination and be affected by endocrine factors and pesticides [1], such models might also be useful for studies on the environmental effects on sex determination and differentiation. Furthermore, swordtails are viviparous and very easy to breed in freshwater aquaria. Therefore, I have found it relevant to present updated knowledge about the biology of southern swordtails, including the evolution of their sex chromosomes, suggested sex determination systems and variations in the development of male phenotypes. Southern swordtails might be a very good choice for further experiments.

Swordtail fishes belong to the genus of viviparous teleosts called *Xiphophorus*, which belongs to the family *Poeciliidae*. In addition to the 17 swordtail species, *Xiphophorus* includes 9 platy species. *Xiphophorus* species live in Central America, from Northern Mexico to Belize and Honduras (Figure 1). The name *Xiphophorus* has its roots in Greek,

where *xiphos* means “sword” and *pherin* means “to carry”. In swordtails, the ventral rays of the tail fin are elongated, forming an extended swordlike structure, while platies do not have a sword. However, there are a few exceptions: The small platy *X. xiphidium* has a small sword [2], while the males of the northern sheepshead swordtail *X. birchmanni* do not develop a sword.



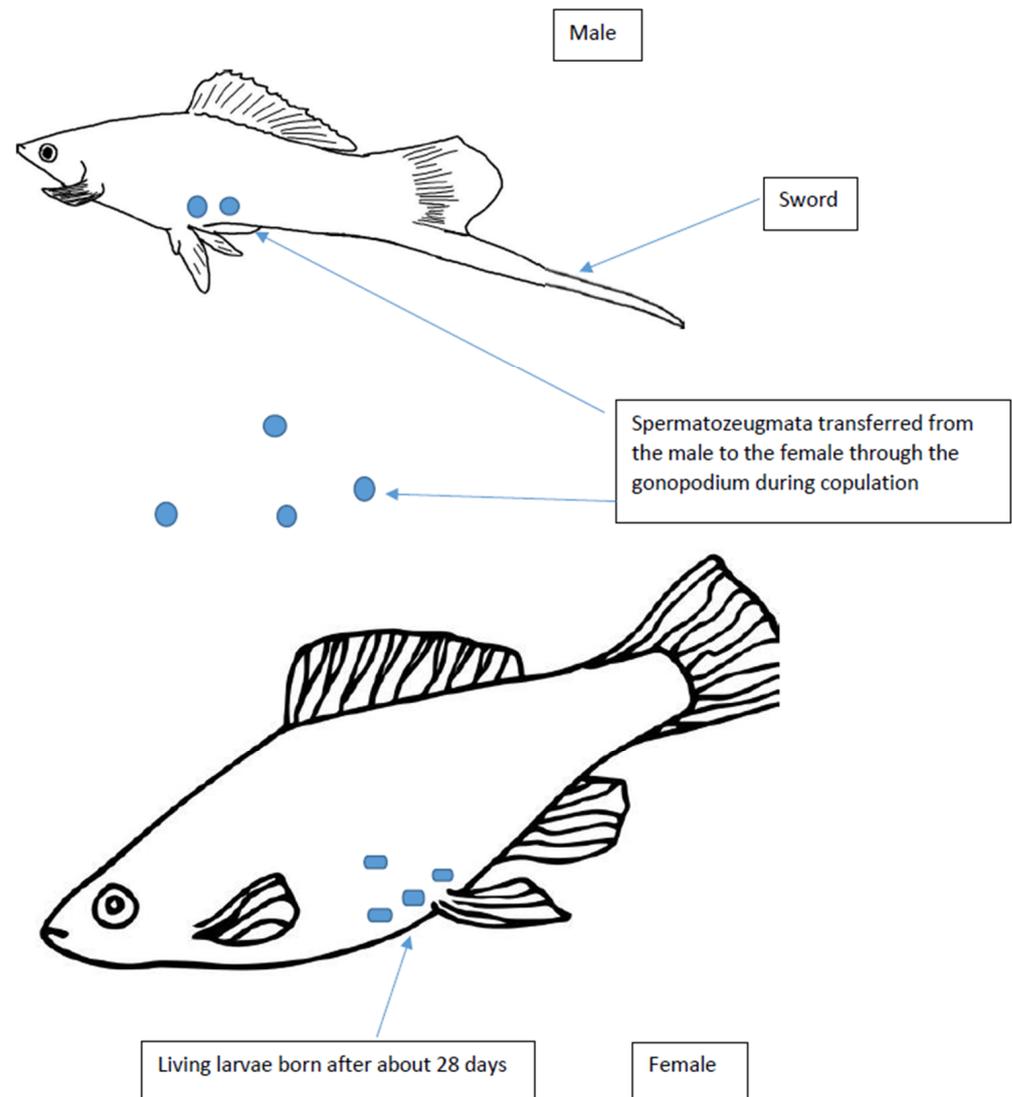
**Figure 1.** Geographical distribution of the eight southern swordtail species known to date. The black area surrounded by the black line shows the distribution of *X. helleri* (“green swordtail”) stretching from the middle of Mexico to Guatemala, Belize and Honduras. *X. alvarezii* (“blue swordtail”) is located in the light blue area, *X. signum* in the red area, *X. mayae* in the orange area and *X. kallmani* in the lilac area, while *X. clemenciae* (“yellow swordtail”), *X. monticolus* and *X. mixei* are located in the yellow area. The horizontal blue bar represents 1000 km, and the blue arrows show the direction north.

It is popular knowledge that there are small, as well as big, male *X. helleri* swordtails, and people sometimes think that the bigger ones are developed from females due to the shortage of male fish. Since sex reversal is well known in fish [3], this does not seem quite impossible. We know that the two kinds of males rather represent small fast-developing males and bigger males, which show male phenotypic characteristics much later.

*Xiphophorus* species have internal fertilization [4]. The two ovaries are surrounded by muscular and connective tissue [5]. The anal fin of the males develops into the so-called *gonopodium*, a tube through which the male can transfer the sperm into a urogenital aperture of the female. After fertilization of the oocytes, the embryos develop inside the ovarian follicles, and when distended with embryos or larvae, the ovarian structure occupies the majority of the peritoneal cavity [5]. The ovaries are well vascularised, and the anal area, therefore, appears dark in the females (Figure 2).

During spermatogenesis, cysts termed *spermatozeugmata* are formed [6,7]. As sperm precursors in mammals, for example, develop inside tubules attached to Sertoli cells [8], sperm precursors in fish, e.g., swordtails, also develop in close association with Sertoli cells [6]. Mammalian sperm end up in the lumen of the seminiferous tubules after spermatogenesis in the tubular wall [8]. In swordtails, spermatogenesis occurs in a compact spherical structure, where all sperm precursors are at a similar developmental step [9]. After spermatogenesis, sperm in the mature *spermatozeugmata* have their heads embedded in the outer matrix, with the tail extending into the lumen of the *spermatozeugmata* [4].

During copulation, *spermatozeugmata* are delivered into the female swordtails. Sperm are released in the females, which makes them pregnant for about 28 days before they give birth to living larvae [10]. After copulation, sperm may persist in the female for up to 8 months [11], giving rise to several broods (Figure 2).



**Figure 2.** Illustration of transfer of spermatozeugmata with male gametes to the female giving birth to living offspring, which is characteristic for viviparous fishes.

It is a big mystery why the males of some *Xiphophorus* species have developed a sword. Based on the forward and backward movements of the males during courtship, some authors have suggested that the males use the sword to force the female into the right position during mating. Several studies have shown that female swordtails (*X. helleri*), as well as female platies (*X. maculatus*), prefer male partners with a sword [12,13]. This evidence was obtained based on a model using aquaria divided into three compartments using plexiglass plates. The female to be tested was placed in the middle compartment, while the test males [12] or a monitor showing monochrome images [14] were placed in the outer compartments.

It may be a mystery why females of the *Xiphophorus* species prefer males with a sword, even before the males of the species developed their sword. However, recent phylogenetic studies on nuclear and mitochondrial DNA seem to show that early ancestral fish from which the *Xiphophorus* species originated had a sword. This sword later disappeared in several species, particularly the platies [15,16]. The reason might be that it could be more difficult hiding from and escaping predators when sworded [17]. However, although the sword disappeared, the females might retain a preference for sworded males. These two conversely directed phenomena thus demonstrate the discrepancy between the benefits of sexual selection and the disadvantages of natural selection.

Further studies on the importance of the length, shape and colour of the sword are needed, and ideally, such studies should be evaluated in relation to the clarity and flow velocity of the water in which the fish live.

The fact that different sex chromosome systems are present in swordtails and crosses can be obtained between species with probably different sex chromosome systems make swordtails a unique model for studying the evolution of sex chromosomes.

## 2. Sex Chromosomes in Swordtails

A monofactorial sex-determining system based on a XX or XY sex chromosome system, where the male is the heterogametic sex, is found in mammals [18], while, in birds, a ZZ or ZW sex chromosome system, with the female being the heterogametic sex, is found [19].

The XY/XX sex chromosome system, where the male is the heterogametic sex, is found in placental mammals and marsupials, both of which originated from Laurasia [20], but also in the small group of mammals termed monotremes (the platypus and spiny anteater/echidna, although the platypus has chains with 10 sex chromosomes [21]), which originated from the other big land mass termed Gondwana [18,20]. That means that the XY/XX sex-determining model may have originated more than 160 mya. The ZW/ZZ sex chromosome system found in birds may have originated more than 100 mya [22]. Although the ratite birds were some of the earliest to be separated from other birds, their sex chromosomes are more homomorphic than usually seen in birds [22,23], showing that the homomorphic development of sex chromosomes is not only a question of time.

In other vertebrate clades such as teleosts, reptiles and amphibians, several sex determination systems and mechanisms are in play. Some teleost species have stable genetic sex determination, with either the male or the female being the heterogametic sex. However, external influences may affect sex determination or cause sex changes in some teleosts [24], and even unisexuality is found in some teleost species [25].

In fish, a wide range of sex chromosome constitutions are found (Table 1). Thus, in some teleosts, more than one variant of a sex chromosome may be found, e.g., Y or Y' in *X. nezahualcoyotl* [26]. In the stickleback fish *Gasterosteus wheatlandi*, a  $X_1X_2Y$  sex determination system can be observed after an autosomal chromosome fuses with the Y chromosome [27]. The background of such unusual systems may be the result of structural chromosome rearrangements, i.e., inversions, translocations or duplications [18]. Furthermore, in many teleosts, genetic sex determination is so evolutionarily young that it is much more unstable compared to mammals and birds.

Live-bearing poeciliid fish, including the *Xiphophorus* species, show a lot of variations and are very easy to raise in aquaria. More sex chromosome systems are represented in these very closely associated species, thus indicating a biological crossroad with XX-XY systems and ZZ-ZW systems (Table 2) [26].

**Table 1.** Characteristics of *Xiphophorus* species compared to other teleosts according to sex determination, sex chromosomes, male phenotype and reproduction.

Fish Group	Sex Determination Mechanisms/Sex Chromosomes	Male Phenotype	Reproduction
<i>Xiphophorus</i> species	Different systems with heterogamety in males and females, respectively. Also polyfactorial systems suggested.	Swordtails usually have elongation of the ventral rays of the tail. Males smaller or equal to female size.	Internal fertilization. Living larvae born.
<i>Poecilia</i> species not belonging to <i>Xiphophorus</i>	Male heterogamety (XX-XY) detected in several species, incl. guppies and mollies. The Y chromosome degenerated in some species.	Males often smaller than females, and in fx. guppies males show more colours.	Internal fertilization. Living larvae born.
Teleosts not belonging to <i>Poecilia</i>	Huge variations including genetic (GSD) and environmental sex determination (ESD). Sex chromosomes may be homomorphic or heteromorphic, and species with multiple sex chromosomes occur.	Huge variations. Sequential hermaphroditism found in several species.	The majority of species produce eggs, which are fertilized outside the body.

**Table 2.** Complete overview of all known *Xiphophorus* species and survey of established evidence about sex chromosome systems in *Xiphophorus* species and a few other teleost species included in the review.

Fish group	Species	Sex Chromosome Systems	References
Southern swordtails	* <i>X. helleri</i> ** <i>X. alvarezi</i> <i>X. signum</i> <i>X. mayae</i> <i>X. kallmani</i> <i>X. clemenciae</i> <i>X. monticolus</i> <i>X. mixei</i>	* Female ZZ-ZW heterogamety or a polyfactorial sex determination system ** Female ZZ-ZW heterogamety (studies on sword colour)	* [26] * [28] ** [29]
Northern swordtails	<i>X. birchmani</i> <sup>1</sup> * <i>X. cortezi</i> <sup>1</sup> <i>X. malinche</i> <sup>1</sup> <i>X. continens</i> <sup>2</sup> <i>X. montezumae</i> <sup>2</sup> ** <i>X. nezahualcoyotl</i> <sup>2</sup> <i>X. multilineatus</i> <sup>3</sup> *** <i>X. nigrensis</i> <sup>3</sup> <i>X. pygmaeus</i> <sup>3</sup>	* XX-XY system. Pure XX males occur due to autosomal modifiers. ** XX-XY system. Two Y chromosomes: Y and Y'. XY females may occur due to an autosomal modifier *** XX-XY system. XY are males. XX usually are females but may be males due to autosomal modifiers.	* [30] ** [26] *** [31] *** [32]
Platies	<i>X. couchianus</i> <i>X. gordonii</i> <i>X. meyeri</i> <i>X. andersi</i> <i>X. evelynae</i> * <i>X. maculatus</i> <i>X. milleri</i> ** <i>X. variatus</i> <i>X. Xiphidium</i>	* ♀:XX, XW or YW. ♂: XY or YY. ** XX-XY system. Male heterogamety suggested from inheritance of sex-linked pigment phenotypes.	* [33] ** [34]
Non- <i>Xiphophorus</i> species included in this paper	* <i>Gasterosteus wheatlandi</i> ** <i>Poecilia reticulata</i> (guppy)	* X <sub>1</sub> X <sub>2</sub> Y sex determination system. ** XX-XY system, sensitive to hormones.	* [27] ** [35]

<sup>1</sup> Fish from the Cortezi complex, <sup>2</sup> Fish from the Montezumae complex, and <sup>3</sup> Fish from the Pygmaeus complex. \*, \*\*, \*\*\* connect the references (column 4) and specific species (column 2).

The very well-examined southern platyfish (*X. maculatus*), which is closely related to southern swordtails, has three different sex chromosomes: X, Y and W. Females may have the sex chromosome constitutions XX, XW or YW and males XY or YY. These sex chromosome constitutions might be explained by a male-determining gene on the Y chromosome, which may be suppressed by genes on the W chromosome. Other possible explanations may be the dose-dependent expression of male-determining genes (two copies on the Y, one copy on the X and zero copies on W) or female-determining genes (two copies on W, one copy on X and zero copies on Y) [33].

While a ZZ-ZX sex-determining system seems to be dominant in the southern Chiapas swordtail *X. alvarezii* [29], a polyfactorial sex-determining system has, for many years, been suggested in *X. helleri* [28]. However, since *X. helleri* is geographically widely distributed in Southern Mexico and Guatemala (Figure 1), it cannot be excluded that different sex-determining mechanisms are predominant in different subspecies of *X. helleri* (Figure 3). In some *X. helleri* populations, a ZX/ZZ sex chromosome system, with the female being the heterogametic sex, as also found in *X. alvarezii*, is present [36].



**Figure 3.** *X. helleri* population caught in Rio Papaloapan in Veracruz, Mexico, in 2008. Both sexes of this strain have black spots. As in other *X. helleri* populations, the anal fin is changed to the gonopodium, and the sword is surrounded by a black edge. In dominating males, the dorsal fin develops a nice orange-yellow colour, while the sword remains green (photo from our own aquaria; Tyrevoldsdal, Skanderborg, Denmark).

In contrast, in northern swordtails, a XY/XX sex chromosome system, where the male is the heterogametic sex, has been detected. However, in, for example, *X. nezahualcoyotl*, two different Y chromosomes seem to exist, meaning that not all XY individuals are males, and in species like, for example, *X. nigrensis*, the genes on the autosomal chromosomes are suggested to modify the sex determination mechanisms [26].

It is interesting knowledge how sex chromosomes evolve and why the male is the heterogametic sex in some species, e.g., mammals, and the female is the heterogametic sex in other species, e.g., birds. In the early evolutionary age, chromosomes carrying male- or female-determining factors are very homomorphic with their sister chromosomes. Gradually, sexually agonistic or antagonistic ones may arise close to the sex-determining gene and also on the other half of the still homomorphic chromosome pair. The recombination ceases to an increased extent, followed by degeneration of the Y and W chromosomes [37]. During evolutionary development, the difference may increase [18]. However, the degeneration of sex chromosomes is not necessarily unidirectional. The processes can be reversible [37]. Sex chromosomes may fuse with autosomal chromosomes, giving rise to giant sex chromosomes [38], and the degree of sex chromosomal degeneration may show huge variations between closely related species [39]. In some species, even systems with multiple sex chromosomes may arise [40].

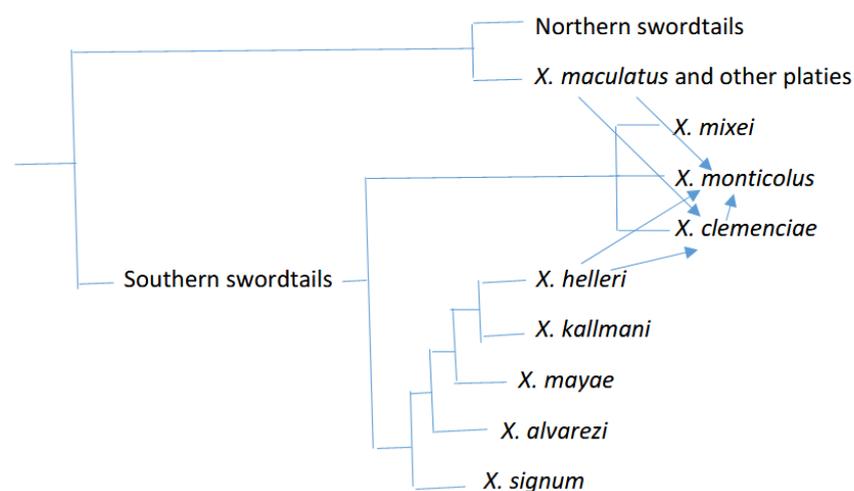
The origins of new sex chromosomes may be explained, to some extent, from the findings on the southern swordtail *X. clemenciae* (Figure 4), which has been suggested to have originated from the females of the southern platy *X. maculatus* crossing with *X. helleri* males living in the same geographical areas [41]. This is supported by the finding of *X. clemenciae* mitochondrial DNA (mtDNA) being almost identical to *X. maculatus* mtDNA (the divergence measured at 3.1%), while the nuclear DNA (nDNA) in *X. clemenciae* is more similar to *X. helleri* nDNA (the sequence divergence measured at only 1.0% in six selected nucleus markers). While mtDNA is inherited from the mother, nDNA is inherited from both parents, and the higher degree of similarity of *X. clemenciae* nDNA with *X. helleri* nDNA, compared to *X. maculatus* nDNA, may be due to backcrosses between hybrid females with *X. helleri* males [42].



**Figure 4.** *X. clemenciae* male of a population caught in Puente Chino Luis, 9 km from Sarabia, in 2000. It has a more yellow body stain and, therefore, is called the yellow swordtail. Furthermore, the two red lines along the body are characteristic of this strain. The sword is usually shorter compared to that of *X. helleri* (photo by Robert Knutsson, Hässelby, Sweden).

Also, the southern swordtail *X. monticolus* is suggested to be the result of hybridization. Geographically, it lives in a relatively small geographical area in Mexico, close to the areas where *X. clemenciae* and *X. mixei* are also found [15]. The wide geographic distribution of *X. maculatus* and similarities in the gonopodial structures between the two species may suggest *X. maculatus* to be the maternal origin, although other platy species are genetically more closely related to *X. monticolus*. A southern swordtail, such as *X. helleri* or *X. mixei* (or even *X. clemenciae*), is probably the paternal origin of *X. monticolus*, since *X. mixei* (and *X. clemenciae*) lives in the same area as the two species suggested to be the result of hybridization between two species [15]. Since *X. clemenciae* and *X. monticolus* are suggested to be the result of a non-sworded maternal lineage (platy) and a sworded paternal lineage (swordtail), it is not surprising that these two species have relatively short swords compared to, for example, *X. helleri*.

Southern swordtails are genetically very closely related to each other and to platies (Figure 5) [43]. These *Xiphophorus* species are thus able to mate across their specific species and produce fertile offspring (although they will not do that under natural conditions, according to the species definition). As already described, female fish prefer males with a sword, and that does not only include female swordtails but also female platyfish [41]. This forms the basis of a hypothesis that female platies (*X. maculatus*) might prefer and mate with male green swordtails (*X. helleri*) from the same area and that hybrid fish might prefer *X. helleri* males, which possess a longer sword than hybrid males [41]. The species *X. clemenciae*, which lives in a small geographical area, might have originated in this way, and the sex-determining genes located on a new chromosome became a new sex chromosome, though homomorphic with its sister chromosome. This “hybrid hypothesis” is in agreement with restriction site-associated DNA sequencing [43] and analyses of mitochondrial DNA [41]. Furthermore, the hypothesis is heavily supported by an evolutionary laboratory experiment performed for over 30 years that included more than 100 fish generations. *X. maculatus* females with a known XX sex chromosome constellation were mated with *X. helleri* males with a ZZ sex chromosome constellation. Next, female F<sub>1</sub> hybrids were mated with *X. helleri* ZZ males, giving rise to ZZ female platyfish. A sequence of genes, including a gene for female sex determination in one case, were translocated to a chromosome other than Z, and in this way, a new sex chromosome, “W”, seems to have originated [36].



**Figure 5.** Phylogenetic tree of the eight southern swordtail species and the other *Xiphophorus* species. The southern swordtail fishes may be divided into two clades termed the helleri and the clemenciae clades. *X. clemenciae* and *X. monticolus* from the clemenciae clade seem to origin from hybridization between platies and other southern swordtail species (illustrated by blue arrows).

### 3. H-Y Antigen in Swordtails

A few decades ago, histocompatibility-Y (H-Y antigen), which is very well conserved through evolution [44], was suggested to play a central role in sex determination in most animal species, including swordtails. Although it is not that simple, it seems relevant, from a historical perspective, to present a little about the H-Y antigen story.

In 1955, Eichwald and Silmsler detected that male skin transplants were rejected by female mice faster than female skin transplants by females, female skin transplants by males and male skin transplants by males [45]. This observation was considered to be due to the histocompatibility-Y (H-Y) antigen. Later, several serological techniques growing from cytotoxicity tests [46] to ELISA (enzyme-linked immunosorbent assay) [47] and RIA (radioimmunoassay) [48] were applied to detect male-specific antigens. These antigens have been detected in the heterogametic sex of a wide range of mammals and in the homogametic sex of birds and reptiles [44,49].

In mammals, levels of the serologically detectable H-Y antigen have been found to be positively associated with the number of Y chromosomes and negatively associated with the number of X chromosomes [50]. However, this does not exclude that a structural gene may be located on an autosomal chromosome [51].

The expression of the H-Y antigen was analysed in southern (*X. helleri*, *X. alvarezi*, *X. clemenciae* and *X. signum*) and northern (*X. montezumae*, *X. nigrensis* and *X. pygmaeus*) swordtail fishes, as well as in other *Xiphophorus* species [52], using a cytotoxicity test with epididymal mouse sperm [46]. In all cases, the males were H-Y-positive and females H-Y-negative.

The H-Y antigen, also called sex-specific or male-specific antigen, was suggested to play a key role in testicular differentiation [53–56] and found to be associated with deficient testicular differentiation [57]. Interestingly, we found a girl with the 46,XY karyotype to be positive for a serologically detectable H-Y antigen but negative for a H-Y antigen detected by transplantation [58,59]. At that time, it was difficult to convince the world that at least two different H-Y antigens might exist. However, more recently, it has been shown that male-specific antibodies suggested to be directed at the H-Y antigen in mammals may be directed at several specific proteins in the outer cell membranes, including male-enhanced antigen 1 and 2 (MEA1 and MEA2), SRY protein and a small Y-linked peptide (DBY) [60].

Some progress, albeit slow, has been made regarding some severe difficulties in the production of useful anti-H-Y antibodies. The reason is because there are only small differences in the antigens between sequences carrying the male-specific genes and their equivalents in females. Thus, only a few amino acids in this region encoded by the X and Y chromosomes in mammals seem to differ [61], and the role of H-Y antigens in sex determination now seems questionable.

### 4. Chromosomes Versus Sex-Determining Genes

In addition to the former empirical experiments suggesting the central role of a H-Y antigen in sex determination (SD), more than 20 primary or master sex-determining (MSD) factors have now been identified, and development is happening fast [62].

The SRY gene on the Y chromosome in mammals was detected in 1990 [63] and seems, with a few exceptions, to be the MSD gene in all placental mammals and marsupials. The explanation for women with a 46,XY karyotype [64,65], as well as men with a 46,XX karyotype [66], is usually that the presence of sex-determining genes is not in accordance with the sex chromosome system. Thus, the majority of 46,XX men have an SRY gene (usually found on the Y) crossed over to the short arm of the X chromosome [66]. In 46,XY women, gonadal dysgenesis may be due to the absence or mutation of the SRY gene, but in other cases, 46,XY shows a male gonad sex but a female external phenotype due to absent or defective androgen receptors encoded by a gene on the X chromosome [64].

Likewise, the Z-linked transcription factor *Dmrt1* serves as a MSD gene in male birds. It is suggested that *Dmrt1* in birds acts in a dose-dependent way, but the fact that ZZW birds are females, while Z0 birds are males, may support a hypothesis about the presence of an ovary-inducing gene on the W chromosome [67]. *Dmrt1* found in birds and mammals (on chromosome 9 in humans) also have equivalents in fish [68].

An increasing number of MSD genes are being described, most of those in teleosts (Table 3) [62]. The huge amount of factors involved in sex determination and testis differentiation is generally found across the spectrum of vertebrates. However, due to the reduced recombination of the area around the MSD gene, the sizes of the sex chromosomes gradually decrease until they finally completely disappear. Then, the MSD gene may be replaced by a new MSD gene switching with the ancient MSD gene to play a role downstream the cascade of events determining the sex and initiating gonadal differentiation [37]. Therefore, factors such as fx *DMRT1* and AMH seem to consistently play central roles in this event in most vertebrates, though being the MSD factor in much fewer species.

The many MSD genes identified in fish usually code for either transcription factors related to MSD transcription factors detected in birds and mammals or, more often, factors related to TGF- $\beta$  (Table 3). The TGF- $\beta$ -related AMH, which induces the regression of Müllerian structures in male mammals [69] and male birds [70], has an equivalent in fish [71] and seems to be the MSD factor in many teleosts. However, since studies of such MSD factors are still missing for southern swordtails, and for *Xiphophorus* species in general, I will not go into further detail here, but it will absolutely be a focus area in *Poecilia* research in the near future.

**Table 3.** The most important master sex determining (MSD) factors found in teleosts.

Main Group	MSD Factor	Description	Fish in Which the MSD Factor Is Detected	Reference
Transcription factors	<i>Dmy</i>	<i>Dmy</i> (MSD in medaka) arose from duplication of the autosomal <i>Dmrt1</i> ( <i>doublesex and mab-3 related transcription factor 1</i> ) gene. It acts through activation of <i>Gsdf</i> (see below) during early development of the gonads. <i>Dmrt1</i> is well conserved during evolution and plays central roles in testis differentiation in animals ranging from mammals to insects. In birds it seems to be the MSD factor.	<i>Oryzias latipes</i> (medaka) <i>Cynoglossus semilaevis</i> (chinese tongue sole)	[72–74]
	<i>Sox3</i>	<i>Sox3</i> shares homology with SRY, which is the MSD factor in most mammals. In fish it acts through activation of <i>Gsdf</i> as found for <i>Dmy</i> in the medaka (see above).	<i>Oryzias dancena</i>	[75]
	<i>Sdy</i>	<i>Sdy</i> ( <i>sexually dimorphic on the Y chromosome</i> ) seems to be the MSD in the rainbow trout and other salmonids.	<i>Oncorhynchus mykiss</i> (rainbow trout)	[76,77]

Table 3. Cont.

Main Group	MSD Factor	Description	Fish in Which the MSD Factor Is Detected	Reference
TGF- $\beta$ related factors	<i>Gsdf</i>	<i>Gsdf</i> (gonadal soma derived growth factor) seems to be the MSD factor and has shown to be necessary for testis differentiation in fx the medaka-related luzon rice fish from the Philippines.	<i>Oryzias luzonensis</i> (luzon rice fish)	[78]
	<i>df6Y</i>	This growth and differentiation factor encoded by a gene on the Y chromosome seems to be the MSD in the shortlived killifish from Zimbabwe.	<i>Nothobranchius furzeri</i> (turquoise killifish)	[79]
	<i>Bmpr1bb</i>	This factor belonging to the group of Bone morphogenetic protein receptors is a strong MSD candidate in the hering. There are only a few genes in the sex-determining region of the Y chromosome, and BMP's are part of the testis differentiation cascade in many species.	<i>Clupea harengus</i> (atlantic herring)	[80]
	<i>AMH</i>	Anti-müllerian hormone (AMH) is secreted from the Sertoli cells and is known to induce regression of the Müllerian ducts in male mammals and birds. However, studies in fish have shown that AMH may be the MSD factor in several teleosts.	<i>Odontesthes hatcheri</i> (patagonian pejerrey)	[81]
	<i>AMHR2</i>	Also expression of the AMH receptor 2 is necessary for normal sex differentiation in vertebrates, and. seems to be the MSD in tiger pufferfish and a couple of closely related species.	<i>Takifugu rubripes</i> (fugu = tiger pufferfish)	[82]

### 5. Possible Effects of Hormones on Sex Determination or Differentiation in Swordtails

It is well known from many species that the temperature [83] and hormones [67] may determine or modify the sex. Oestrogens play a role in sexual differentiation of the gonads, as well as in secondary sex characteristics, in birds [67]. Oestrogens act on oestrogen receptors on the outer cortex of the left gonad, stimulating it to develop into an ovary, while the right gonad regresses in female birds (Both gonads develop into testicles in male birds.). However, several facts support the presence of a direct genetic effect in birds, in addition to the hormonal effect, on gonads, as well as body sex. Thus, ZZ birds feminized by an injection of oestrogens into their eggs underwent gonadal reversal from a testicular to an ovarian structure. Another argument comes from gynandromorphic sex chimera birds, in which the majority of cells on one side of the body show a ZZ sex chromosome constitution and the majority of cells on the other side of the body show a ZW constitution. On the ZZ side, the birds show a male phenotype with a comb, a wattle and spurs, while they show a

female phenotype on the ZW side. If sex hormones had played an absolutely decisive role, the phenotypic differences between the two sides would have been more indistinct.

In the guppy *Poecilia reticulata*, another ovoviparous fish, endocrine-induced sex reversal has been demonstrated [35]. Usually, male guppies have a XY and females a XX sex chromosome constitution. However, it is possible to produce homogametic males and heterogametic females by feeding the fish a hormone-supplemented diet. Kavumpurath and Pandian [35] produced a population of XX, as well as XY, females by the administration of oestrogens to their parents before parturition. This population was mated with XY males, and again, oestrogens were administered through the food before delivery of the next generation. In this way, it was possible to produce YY females, which, together with normal XY males, gave birth to only male offspring [35]. Such observations show that not only structural genes determine the sex in guppies, since hormones also play a central role. It is natural to question if such “plasticity” can also be observed in other *Xiphophorus* species such as fx. southern swordtails, where fx. endocrine disrupters such as nonylphenol and bisphenol A seem to cause the degeneration of spermatogenesis in *X. helleri* [1].

## 6. Sex Reversal or Sex Change in Swordtails?

Sex changes are well known in nature. Some species are sequential hermaphrodites, either protogynous (starting as females and ending as males) or protandrous (starting as males and ending as females) [84]. Sex changes may be induced by behaviour [85] or age [86]. In several species of coral reef, fish the dominating female changes its gonadal histology, external phenotype and behaviour and develops into a male within a few weeks, when the “old” male dies [87]. Recently, it has been shown that sex changes in clownfish (*Amphiprion percula*) are preceded or accompanied by molecular changes in the brain [88]. Thus, sex changes include the change of sex according to gonad histology and function, phenotype and brain structure and function, as well as behaviour [87]. Sequential hermaphroditism is found in at least 27 teleost families, mainly species found in coral reefs [84].

When it is beneficial to be big to produce enough oocytes, it may be an advantage to be protandrous, and if it is beneficial to be a big male to be able to conquer the females, protogynous species may have an advantage [89]. Southern swordtail females usually have a larger body size than the males, and therefore, it might be an advantage to reach a big size before differentiating into males (as described later) or maybe even undergo a sex change to males after having been living as females, which has been suggested in older studies, as described below.

Sex change/reversal in *X. helleri* has been described based on old observations by Essenberg [90]. The original catching places were not mentioned in the study by Essenberg [89], and since the classification of swordtails has changed since the publication of the “sex reversal observations”, it cannot be guaranteed that the data were based on studies of *X. helleri*. Furthermore, the fish observed seemed to have been formed by mixing stocks of different origins [90].

However, cases of swordtails that had given birth to at least one litter were found to change into fertile males able to father offspring from virgin females [90]. Sex reversal in swordtails was described to happen in three phases: in the first, the black spot (pigment around the ovary) disappeared, and the anal fin enlarged; in the second phase, the gonopodium was formed and, in the final phase, the sword was developed. Gonadal changes into males were documented by histological examinations [91]. *X. helleri* individuals undergoing sex reversal were described to be sensitive, inactive and without an appetite for food during the process [91].

Essenberg [89] also observed a dramatic increase in the sex ratio ( $\sigma/\varphi$ ) from 1/3 to 3 during the development from young immature swordtails to adult fish. Similar findings have since been observed in other studies. However, as described later, some males develop phenotypical sex characteristics late and, therefore, may be erroneously mistaken

for females, although they have no ovaries (detection of a black spot by illumination of the peritoneal cavity in front of the anal fin) [11].

Gomel'skiy and Fetisov [92] found nearly identical sex ratios in the comparisons of the gonadal histology of 2- or 3-month-old fish and sexually mature fish with the same parents. They compared three strains of *X. helleri* and suggested different sex ratios within different strains. However, each of the three strains was only represented by one pair of fish, which was not necessarily fully representative of the strain. Thus, if males, as well as females, have several different sex chromosome systems, it might explain the large variations in sex ratios in the offspring of different individuals within each *X. helleri* strain.

## 7. More Than One Male Phenotype

The presence of more than one male phenotype, so-called “dimorphism”, can be observed in many animal species, from fish to spiders [93]. In swordtail species, different categories of males are present. They may differ according to size and colour (*X. nigrensis*) [31] or sword colour (*X. alvarezi*) [29]. In the northern swordtail *X. nigrensis* living in the so-called “pygmaeus complex”, at least three different male phenotypes can be distinguished: large (body size > 30 mm), intermediate (24 mm < body size < 32 mm) and small body sizes (body size < 26 mm) [94].

The two larger types, in addition, have the biggest swords and are preferred by the females. Thus, the large males attract females through courtship, while the smaller ones hide and sneak around to copulate with the females when they get the chance. The sexual behaviour for the intermediate males seems to be a combination of the two behavioural patterns [94].

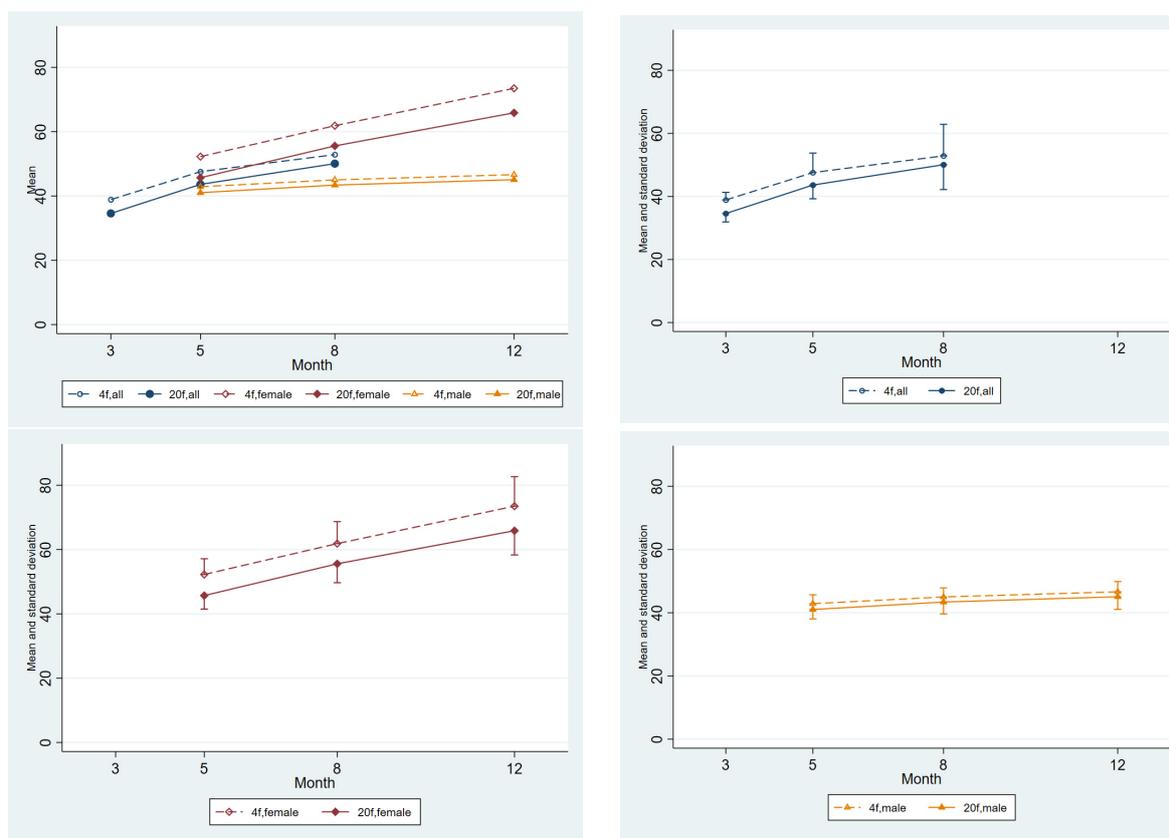
The variation in the sizes of the males seems to be explained by genetics. Bigger males seem to have more repeats of a sudden allele called “B” on the Y chromosome compared to small males, which only have one copy of “B” in the melanocortin 4 receptor (Mc4r) gene on the Y chromosome, or to an XX sex chromosome system like females [32,95].

In southern swordtails, the sword may have different colours, depending on the species and population, but the coloured area is always surrounded by a black edge. The colours may be green (due to a sepiapterin pigment), yellow, orange (due to carotenoid pigment) or red [29]. Since the sword colour may be sex-linked, observations of the proportions of sword colours (inherited dominant or recessive) have been used to confirm that female heterogamety is present in, for example, *X. alvarezi* [29]. In such studies, it is possible to reveal the genetic characteristics in females by the induction of sword-like structures using androgens.

Since different variants of males within one species may have different strengths and limitations, there is usually room for all. However, in an aquaristic environment where the room is very restricted and predators may not be present, unnatural selection may lead to one male variant being the dominant or even the only male category present.

From an old study of Peters [28], it was suggested that early sex-differentiated (the so-called “Frühhännchen”) and late sex-differentiated males (the so-called “Spätmännchen”) make up two clearly distinguished categories in some subpopulations of *X. helleri*, while the phenotypes of males form a continuum in other subpopulations [28]. Since gonadal sex was found to be determined during the first living weeks, the differences in male differentiation seem to be according to the growth and development of secondary sex characteristics (body sex) [28].

Although small fast-developed males clearly differ from larger late-developed males, the males in our *X. helleri* populations made up a continuum of males of varying sizes rather than being two sharply distinguished categories of males. In an experiment where the offspring of a *X. helleri* population originating from Rio Seco, Oaxaca, Mexico, were grown in 20-litre aquaria with 4 or 20 offspring, the fish grown in the high-density aquaria developed more slowly than the fish in the low-density aquaria, particularly before 3 months of age (Figure 6).



**Figure 6.** Growth in the body lengths of 96 offspring, each ages  $8 \pm 1$  days, of one of our *X. helleri* populations, which were grown in parallel in eight 20-litre aquaria: 4 aquaria with 4 fishes and 4 aquaria with 20 fishes each. The offspring were obtained from 3 virgin females ( $211 \pm 30$  days) paired with an early mature male of the same age, giving rise to 120 offspring delivered 48–49 days later. One female gave birth to the 40 fish in two aquaria and 8 fish in other two aquaria, while the final two each gave birth to 20 in one aquaria and 4 in another one. Thus, the fish in the low-density aquaria were always compared to the fish in the high-density aquaria with an identical genetic origin. The blue curves show all fishes ( $p < 0.001$ ), orange curves show fishes with a gonopodium (males;  $p = 0.076$ ) and red curves show fishes without a gonopodium (females;  $p < 0.001$ ); the dotted lines represent fishes from low-density aquaria, while fully drawn lines represent fishes from high-density aquaria. Body lengths are presented as means with standard deviations. A linear mixed effects model autoregressive structure was used to estimate the differences in growth between different time points. The offspring were fed with freshly hatched artemia larvae. From the second month, the living artemia were replaced by frozen red mosquito larvae every second day. The fishes were inspected daily, and their body lengths, with and without the tail fin, were registered at 3, 5, 8 and 12 months of age. Basic water quality measurements were performed weekly, and no significant differences were observed between the low- and high-density aquaria. In the low-density aquaria, 2 out of 16 (12.5%) died during the experiment, and in the high-density groups, 16 out of 80 (20%) died during the experiment.

All fish were given an abundant food supply, and the water was changed regularly. However, it cannot be excluded that an inhibition in growth among the small fish ( $<3$  months) in the high-density aquaria might be due to physiological stress [96,97]. Although the pH and  $\text{NO}_2$  and  $\text{NO}_3$  concentrations were maintained at the same levels in the low-density and high-density aquaria, it cannot be excluded that the water quality in the high-density aquaria—an addition to the higher “concentration” of fish in itself—might have influenced the growth rates.

While we usually observed a sex ratio of 1:1 among the offspring in our *X. helleri* populations (Figure 6), Peters [28] found a sex ratio of  $\sim 2:3$  in the offspring of early sex-

differentiated males in contrast to <1:2 in the offspring of late sex-differentiated males. She suggested that this might be explained by a higher “male strength” in the developed males. Probably, it could be due to genetic factors, but if a simple polygenetic background were the explanation, a continuum of male phenotypes would be expected.

Social influence on the growth rate, age at maturity and adult size have also been described in other species of *Xiphophorus*, including the platies *X. maculatus* [98] and *X. variatus* [99,100] and northern swordtails [97,101], but also in other fishes belonging to the Poeciliidae family [102,103].

## 8. Conclusions and Future Directions

While sex is determined by genes in mammals and birds, environmental factors such as temperature strongly influence sex determination in reptiles and some fish species. The master sex determination factors initially were localized on homomorphic chromosomes. Gradually, sex chromosomes become increasingly heteromorphic, since the genetic material is lost from one of the chromosomes due to the loss in recombination of the sex chromosome only present in one sex. In fish such as swordtails, the sex determination mechanisms are still so young that possible evolutionary mechanisms can be studied in such species. That swordtails are live-bearing and easy to raise in aquaria are huge advantages in performing experimental studies.

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