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The Reversible Sex of Gonochoristic Fish: Insights and Consequences

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Environment · Epigenetics · Genes · Sex reversal · Wild populations

Abstract
Fish sex reversal is a means to understand sex determination and differentiation, but it is also used to control sex in aquaculture. This review discusses sex reversal in gonochoristic fish, with the coexistence of genetic and environmental influences. The different periods of fish sensitivity to sex reversal treatments are presented with the mechanisms implicated. The old players of sex differentiation are revisited with transcriptome data and loss of function studies following hormone- or temperature-induced sex reversal. We also discuss whether cortisol is the universal mediator of sex reversal in fish due to its implication in ovarian meiosis and 11KT increase. The large plasticity in fish for sex reversal is also evident in the brain, with a reversibility existing even in adulthood. Studies on epigenetics are presented, since it links the environment, gene expression, and sex reversal, notably the association of DNA methylation in sex reversal. Manipulations with exogenous factors reverse the primary sex in many fish species under controlled conditions, but several questions arise on whether this can occur under wild conditions and what is the ecological significance. Cases of sex reversal in wild fish populations are shown and their fitness and future perspectives are discussed.

In vertebrates, sex reversal has been defined as a mismatch between the phenotypic and the genetic sex. In fish, as well as in other poikilotherm vertebrates, sex reversal can be easily induced by hormonal and sometimes by environmental treatments. Because sex-reversed individuals can be used to produce genetic all-male or all-female populations, they have long been applied in aquaculture and for research on sex determination and differentiation. Following the identification of sex-linked markers, sex-reversed individuals have been identified more recently in wild populations of various fish species. Although their prevalence has still to be better characterized for most of these species, modelling studies have recently underlined the possible ecological and evolutionary consequences of sex reversal on wild fish populations [Stelkens and Wedekind, 2010; Wedekind, 2010; Wedekind and Stelkens, 2010; Senior et al., 2012; Senior et al., 2015]. Following a brief overview of sex determination and differentiation, the present review will therefore ana-

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lyze fish sex reversal in both domesticated strains and wild populations, the associated mechanisms, and its possible consequences on the dynamics of wild populations.

As reported by many authors, sex determination systems are conserved and stable within birds and mammals with a few exceptions [Schartl, 2004; Ellegren, 2010; Veyrunes et al., 2010], whereas they appear to be highly variable among poikilotherms, including fish species [Desjardins and Fernald, 2009; Mank and Avise, 2009]. Based upon their sexuality traits [Baroiller et al., 1999; Devlin and Nagahama, 2002; Kobayashi et al., 2013], fish species have been categorized into (1) hermaphroditic: individuals in a population produce both male and female gametes simultaneously or successively through a natural sex reversal process; (2) gonochoristic: each individual is either a male or a female, and this fixed sex will be kept permanently; and more rarely (3) unisexual: populations are composed exclusively of gynogenetic females.

Sex reversal in hermaphroditic fish species has been reviewed by Todd et al. [this issue]; therefore, our review will focus on gonochoristic fish that represent most of the teleost species.

Gonochoristic fish display an amazing diversity of sex determining systems, ranging from a strict genetic control (genetic sex determination, GSD) to an environmental control (environmental sex determination, ESD) or the joint action of both factors [Baroiller et al., 1999; Volff et al., 2007; Heule et al., 2014]. GSD was originally reported in medaka [Aida, 1921] but has been shown to be widespread within gonochoristic teleost species. In most fishes, sex is determined at fertilization by sex chromosomes with a monofactorial system often based on a XX/XY male heterogamety or less frequently a ZZ/ZW male homogamety [Devlin and Nagahama, 2002; Volff et al., 2007; Kobayashi et al., 2013; Heule et al., 2014]. Sex in some fishes as well as in a few other animal species relies upon a polyfactorial system where the sum of various independent loci determines the sexual phenotype [Kosswig, 1964; Moore and Roberts, 2013; Meisel et al., 2016]. Contrary to mammals [Graves, 2006] and birds [Takagi and Sasaki, 1974], where the sex chromosome pair can be identified by its heteromorphy with a decayed 'heterogametic sex chromosome', in most of the poikilotherm vertebrates including fish, sex chromosomes are homomorphic and poorly differentiated [Grossen et al., 2011; Heule et al., 2014, Chalopin et al., 2015]. Because they cannot be directly identified in fish through karyotype analysis, indirect approaches have been developed to search for the sex chromosomes of various species in order to analyze or control sex determination. More recently, molecular sex-linked markers have been identified in some model fish and aquaculture species (see section New Actors of Sex Determination and Differentiation). All-male or all-female populations (also known as monosex) have been produced for various aquaculture species to benefit from the best sex-linked dimorphisms (e.g., growth, age at puberty) [Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002]. The sexual phenotype in fish is a plastic trait that can be easily manipulated by hormonal treatments (induction of sex reversal) during critical stages. Complete and functional sex reversal was produced by oral administration of androgens to masculinize or estrogens to feminize medaka fry [Yamamoto, 1969]. The use of Y-linked color genes brought evidence that part of the phenotypic males from the masculinization groups were neomales (genetic females masculinized by the treatment = mXX). Similarly, in the feminization process, part of the phenotypic females were neofemales (genetic males feminized by the estrogens = fXY). By mating mXX with classic fXX females, all-female progenies have been obtained (mXX × fXX → fXX). Conversely, by mating fXY with mXY, viable and fertile YY males (mYY) were obtained (mXY × fXY → 1 fXX + 2 mXY + 1 mYY), and these YY males when mated with classic fXX breeders sired only male progenies (mYY × fXX → mXY) [Yamamoto, 1969]. Following this pioneer work, numerous studies have challenged the hypothesis that estrogens and androgens could be the natural 'gynoinducers' and 'androinducers', respectively. These studies mainly relied upon in vivo treatments using steroids, steroid enzyme inhibitors, or steroid receptor antagonists, in vitro steroid metabolism, steroid enzyme immunodetection, and gene expressions [Baroiller et al., 1999; Devlin and Nagahama, 2002], concluding that aromatase and estrogens are the ones which play a key role in fish sex differentiation [Guiguen et al., 1999, 2010; D'Cotta et al., 2001a]. All the hormonal sex reversal treatments that have since been applied to numerous fish species are based upon these pioneer studies. As reported by Göppert et al. [2016], this plasticity of sex differentiation can also lead to xenobiotic-induced sex reversal in fish due to pollution. Since these endocrine disrupting effects have been recently reviewed [Bhandari et al., 2015; Senthilkumaran, 2015], they will not be discussed further in the present review.

Identification of an ESD system is more recent in gonochoristic fish species [Conover and Kynard, 1981]. ESD implies that the environment experienced by the offspring during early ontogeny determines the sex of a progeny. Temperature was the first environmental factor

demonstrated to be able to determine the sex of an Atlantic silverside *Menidia menidia* progeny through a temperature-dependent sex determination (TSD) [Conover and Kynard, 1981]. Fifteen years later, a temperature-induced sex reversal (TISR) was first evidenced in the Nile tilapia *Oreochromis niloticus*, with high temperatures being able to masculinize sensitive progenies despite the fact that sex in this species is usually determined by major and/or minor genetic factors [Baroiller et al., 1995]. TSD was then found in the pejerrey *Odontesthes bonariensis* [Strüssman et al., 1996]. Together, these studies strongly stimulated research on TISR/TSD in various other fish species. EISR/ESR (environmentally induced sex reversal/environmental sex reversal) and especially TISR have been obtained in an increasing number of fish species under controlled conditions [Baroiller et al., 1999; 2009a]. This included species with (i) a male heterogametic GSD system, such as the Japanese flounder *Paralichthys olivaceus* [Yamamoto, 1999], the medaka *Oryzias latipes* [Hattori et al., 2007], and the Southern flounder *P. lethostigma* [Luckenbach et al., 2009]; (ii) a female heterogametic GSD system, such as the blue tilapia *O. aureus* [Desprez and Mélard, 1998; Baras et al., 2000], the turbot *Scophthalmus maximus* [Haffray et al., 2009], or the half-smooth tongue sole *Cynoglossus semilaevis* [Shao et al., 2014]; or (iii) a polygenic GSD system, such as the seabass *Dicentrarchus labrax* [Pavlidis et al., 2000; Saillant et al., 2002] or domestic strains of the zebrafish *Danio rerio* [Sato et al., 2005; Hattori et al., 2007].

Coexistence of Genetic and Environmental Influences

Reviews on the environmental effects on fish sex determination/differentiation [Baroiller et al., 1999; Strüssmann and Patiño, 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Conover, 2004] have generated debates about TSD in gonochoristic fish as well as in other vertebrates. Mainly based upon reptile sex determination, criteria have been proposed for identifying TSD in vertebrates [Valenzuela et al., 2003]. Amongst them, 2 main criteria have been used by Ospina-Alvarez and Piferrer [2008] to reclassify 19 out of the 27 fish species previously reported to be TSD species as GSD + environmental effects. This was based on (a) the existence of sex chromosomes which was considered a signature of an exclusive GSD; and (b) for some of the fish species the efficient temperature treatments which skewed the sex ratios were considered to be outside the natural range specifically encountered by the fry or juveniles of these species during their critical period.

Concerning these 2 criteria, it is important to enrich the debate with recent data concerning GSD in vertebrates and TSD in poikilotherm vertebrates which have led to the deconstruction of some common myths about the evolution of sex determination in the animal kingdom [Bachtrog et al., 2014]. Mainly based upon few mammal models and to a lesser extent upon bird models, sex in animals has long been considered to be stable and determined by sex chromosomes. This predominant misconception that sex chromosomes are necessarily associated only to GSD has for long led to underestimate that at least in poikilotherm vertebrates, genetic systems of sex determination without sex chromosomes or with the presence of both major and minor genetic factors can also exist, especially in fish [Mank and Avise, 2009; Bachtrog et al., 2014]. Indeed, in amphibians, reptiles, and teleosts 'sex-determining mechanisms are diverse and can evolve rapidly' [Bachtrog et al., 2014], with multiple evolutionary transitions between TSD and GSD having occurred in reptiles and amphibians and therefore, the line between a sex determination system relying on pure genetics or on pure environmental factor(s) is faint [Sarre et al., 2011; Bachtrog, et al., 2014; Holleley et al., 2015]. Following the theoretical model of Bull and Charnov [1977], ESD and GSD have for decades been considered as 2 mutually exclusive systems [Bull, 1980; Valenzuela et al., 2003]. However, based upon extensive studies in 2 lizard species, *Pogona vitticeps* and *Bassiana duperreyi* [Shine et al., 2002; Sarre et al., 2004; Radder et al., 2008; Holleley et al., 2015; Bull, 2015], unambiguous evidences have shown that GSD with sex chromosomes (respectively ZZ/ZW and XX/XY) and TSD can perfectly coexist. Furthermore, *B. duperreyi* has even a heteromorphic pair of sex chromosomes [Shine et al., 2002].

GSD systems can become sensitive to the environment and then present genotype-by-environment interactions or evolve towards TSD, allowing the coexistence of environmental and genetic influences [Sarre et al., 2004]. As hypothesized previously [Bull, 1981; Quinn et al., 2011; Schwanz et al., 2013], the major role of sex reversal to induce this type of transition has been recently confirmed in the Australian lizard *(P. vitticeps)* by an experimentally temperature-induced rapid shift between a predominantly GSD (with heteromorphic sex chromosomes ZZ/ZW) and a TSD system using sex-reversed ZZ females identified in the wild [Holleley et al., 2015]. Although the W chromosome was eliminated in one generation under laboratory conditions, a similar process should be much

more gradual in wild populations, and therefore the transition between GSD and TSD will probably need multiple generations [Holleley et al., 2015]. Altogether, these recent data confirmed that at least in these lineages TSD and GSD are not mutually exclusive systems but the 2 extreme endpoints of a continuum, underlying the coexistence of both genetic and environmental influences in the middle of this spectrum: 'many species have differentiated sex chromosomes, but also show a temperature override, where genes and environment interact to determine sex' [Holleley et al., 2015]. In fact just recently, a three-ended continuum that can be visualized as a triangle has been proposed [Perrin, 2016] in which genes, environment, and random factors (i.e., any stochastic fluctuation in the expression of key genes of the cascade) could interact to determine sex.

Regarding fish, such a continuum with possible coexistence of genetic and environmental factors has also been proposed by some authors [Baroiller et al., 2009a, b; Grossen et al., 2011; Yamamoto et al., 2014; Senior et al., 2016]. In the Atlantic silverside, whose TSD system reached a consensus, thermal sensitivity of the wild populations varies from high to low levels within a natural thermal gradient [Conover and Heins, 1987]. Because large females of *M. menidia* have a better reproductive fitness than large males, long growing seasons are more beneficial to females than to males. Therefore, at low latitudes when growing seasons are longer, early spawning (low temperatures) favors female differentiation. Temperature is a reliable cue of the future length of the growing season. Sex determination in Southern (high thermal sensitivity with an adaptive role of TSD) or in Northern (low sensitivity) populations, respectively, relies upon a polygenic control, with both temperature and genetic factors interacting, or upon major genetic factors. Between these 2 extreme conditions, a mixture of TSD and GSD determines the sex of individuals [Duffy et al., 2015]. Further studies analyzing 31 wild populations have demonstrated that the pattern of sex determination in the Atlantic silverside is actually non-linear, with none of the studied populations displaying a complete TSD or pure GSD, since in the first case some genetic component always exists, while in all the GSD populations some thermal sensitivity still persists. Hence, fish TSD relies upon genetic × environmental interactions with gradual shifts in sex ratio, unlike the abrupt pattern found in many reptiles [Conover, 2004; Duffy et al., 2015]. Furthermore, the identification of a major sex determinant (the *amhy* gene) in another species, the pejerrey *O. bonariensis* with a high thermal sensitivity [Yamamoto et al., 2014] again underlines that in sensitive fish species, both genetic and environmental factors can determine the sexual phenotype. Finally, it is also important to remember that differences exist in TSD traits between reptiles and fish. In reptile TSD species, especially in turtles and crocodiles, specific temperatures usually determine the sex of all individuals, producing therefore all-male or all-female progenies. Conversely in fish with TSD, such as *M. menidia,* extreme temperatures usually do not induce 100% male or female broods [Conover and Heins, 1987; Duffy et al., 2015]. For some individuals in a progeny, sex is therefore not determined (or not only) by temperature. Similarly, apart from the pejerrey [Strüssmann et al., 1996], temperature-induced monosex populations are very rarely reported in the Nile tilapia [Baroiller et al., 2009b], the European sea bass [Piferrer et al., 2005], as well as in other thermosensitive species [Baroiller et al., 2009a]. Besides, high between-family variance in sex ratios have been reported in tilapia, with contributions from both male and female breeders [Baroiller and D'Cotta, 2001; Baroiller et al., 2009b]. Because skewed sex ratios are strongly heritable, they can be selected [Wohlfarth and Wedekind, 1991]. Moreover, in the same species, these 3 characteristics can also be applied to its thermosensitivity [Baroiller et al., 2009b; Wessels and Hörstgen-Schwark, 2007]. Skewed sex ratios have also been shown in the rainbow trout, *Oncorhynchus mykiss*, following high temperature exposure (18°C treatments applied from 42 days post-fertilization [dpf] onwards) [Magerhans et al., 2010]. In this species there is a strong genetic XX/XY sex determination where the major sex determining gene *sdY* has been identified [Yano et al., 2012], but the presence of a minor loci (a recessive mutation), which was called *mal,* associated or not to other loci and/or to environmental variations can lead to spontaneous masculinization [Quillet et al., 2002]. The study of this transmission was performed through several generations showing that it was a heritable trait [Quillet et al., 2002]. High temperatures of 18°C were able to masculinize XX females that carried *mal* in gynogenetic doubled haploid individuals [Valdivia et al., 2014].

In tilapia (but probably also in other thermosensitive species) thermosensitivity has been demonstrated to be highly heritable (h = 0.69), and therefore, it can be selected [Wessels and Hörstgen-Schwark, 2007, 2011; Baroiller et al., 2009b]. In the turbot, *S. maximus*, sex determination mainly relies on a female heterogametic system (ZZ/ZW), but again, thermosensitivity and family temperature interactions have been reported at least in some progenies [Haffray et al., 2009]. These types of sex systems resemble polyfactorial systems. Until now, very few

cases of 'true' polyfactorial sex determination have been reported in fish. However, according to Bull [1983], a polygenic sex determination is characterized by a large between-family variance in sex ratio, paternal and maternal effects on sex ratios, and a sex ratio response to selection. Interestingly, species such as rainbow trout and tilapia that are usually classified as GSD can present some of these characteristics. It is also interesting to draw a parallel between the hypothesis of an ancestral polygenic sex determination in fish [Kirpichnikov, 1981] and the recent demonstration that domesticated strains of zebrafish (classified as polygenic sex determination by Liew et al. [2012] and Liew and Orban [2014]) have lost the natural sex determinant present in wild zebrafish populations that rely upon a classic monofactorial GSD of ZZ/ZW type [Wilson et al., 2014]. Interestingly, besides the ZZ/ZW system, other factors might be at play, because sex-reversed females have been reported. As suggested by the authors, domestication or/and selection or/and inbreeding of zebrafish laboratory lines have resulted in the evolution of the sex determination system possibly by using cryptic minor genetic factors. Such minor factors could coexist with the sex determinant in the wild population and partially explain why sex-reversed zebrafish can be found in both wild populations and domestic strains. These sex reversals could also be associated with zebrafish sensitivity to various environmental conditions (oxygen, density, temperature, thermocycle, poor nutrition), and/or to the unveiling of minor factors [Hattori et al., 2007; Sato et al., 2005; Shang et al., 2006; Wilson et al., 2014]. In haplochromine cichlids from Lake Malawi in East Africa, where multiple interacting loci control sex determination, sex-determining loci or sex determination systems can hide other loci or systems through epistatic dominance mechanisms or sex determination hierarchies [Ser et al., 2010]. Altogether, this could explain why in tilapia but also in species considered to have a strong GSD (e.g., medaka, rainbow trout, common carp), minor factors have been detected at least under extreme genetic (i.e., clonal lines in the rainbow trout, Quillet et al. [2002]) or environmental (including those that are 'out of the normal incubation range', Ospina-Alvarez and Piferrer [2008]) conditions. Vertebrate sex-determining systems are therefore probably more complex than previously thought [Radder el., 2008; Bachtrog et al., 2014], especially in poikilotherms, and the hypothesis of simultaneous involvement of multiple interactive factors should not be ignored even in species where major factors have been identified. Often considered as a transient state, polygenic sex determination is nevertheless stable over time at least in the house fly, *Musca domestica* [Meisel et al., 2016].

Regarding the second criteria that sex ratio deviation should occur within a range of temperatures naturally occurring during decisive developmental steps, recent data also bring some new perspectives. In the Californian grunion fish, *Leuresthes tenuis* [Brown et al., 2014], both temperature and photoperiod can influence the sex ratio. However, because the seasonal thermal variation is very low (4°C) along the North American Pacific coast, photoperiod has become a more informative cue of season length than temperature. Longer day lengths and lower temperatures induced higher proportions of females. This is the first case of a photoperiod-dependent sex determination system in fish [Brown et al., 2014]. Temperature and photoperiod seem to act in synergy in this fish to determine the sexual phenotype. This suggests that (i) temperature in fish is not the only environmental factor involved in ESD systems; (ii) in some environments, photoperiod might be a more informative factor of the future environment of the fry or juveniles than temperature; and (iii) in species for which at least 2 environmental factors might have synergetic effects on sex ratios, application of a treatment of only one environmental factor probably requires higher levels of this factor to obtain a similar effect than when the 2 factors are applied. Therefore, in sensitive species the effects of additional environmental factors on sex ratios have to be searched for. If other factors (at least a second one) are also efficient to influence the sex ratio, then synergic effects will have to be searched by applying decreasing values of temperatures.

Because minor and/or environmental factors can both induce sex reversal in gonochoristic fish, we consider that their sex reversal can be defined as a mismatch between the phenotypic sex and the sex theoretically governed by the major genetic factor(s).

The Use of Sex Reversal to Understand and Control Fish Sex Determination or Differentiation

Sex control is often a good answer to various aquaculture problems mainly related to sex-linked dimorphisms or for reproduction management [Piferrer et al., 2012; Senior et al., 2016]. Depending upon the species, one sex grows faster than the other, which can be the case for males (tilapia) or for females (e.g., flatfish, sea bass, turbot). The sex-linked dimorphism can also concern the color or the body shape of ornamental fish species, such as the guppies, the Siamese fighting fish, or the platyfish.

Table 1. Sex reversal as a way to produce monosex populations for aquaculture purposes

	XX/XY species			ZZ/ZW species	
	female monosex population	male monosex population	male monosex population	female monosex population	male monosex population
Specific breeders	XX males	YY males	YY females	WW females	ZZ females
Obtention of these breeders	androgen treatment + mating + progeny testing; gynogenesis	estrogen treatment + mating + progeny testing; androgenesis	estrogen treatments on progenies obtained from a YY male father and a XY female mother	androgen treatment + progeny testing; gynogenesis	estrogen treatment + progeny testing; androgenesis
Final matings	fXX × mXX → fXX	mYY × fXX → mXY	mYY × fYY→ YY	fWW × mZZ → ZW	mZZ × fZZ → ZZ
Species	salmonids, carps	Nile tilapia	Nile tilapia	turbot	blue tilapia
References	Piferrer [2001]; Cnaani and Levavi-Sivan [2009]	Baroiller and D'Cotta [2001]; Baroiller et al. [2009b]; Cnaani and Levavi-Sivan [2009]		Haffray et al. [2009]	Desprez et al. [2003a, b]

Besides, skewed sex ratios can be a means to optimize reproduction like in tilapia where a 1:3 (M:F) ratio is usually recommended for fry production. Furthermore, in salmonids flesh quality is affected by the endocrine changes associated with sexual maturity, and consequently, it is more interesting to rely upon female production because they mature later than males. Hence, studies on sex determination and sex differentiation have been strongly stimulated in various aquaculture species due to the benefits generated by controlling sex (i.e., better management and profitability). In some laboratory models and aquaculture species, sex reversal has been used to investigate the process and mechanisms of sex differentiation and/or to develop sex control approaches.

YY Male Production

The pioneer studies of Yamamoto [1969] demonstrated that viable and fertile YY males can be produced in the medaka *O. latipes* through estrogen feminization and breeding fXY × mXY. Since then, YY 'super-males' (table 1) have been developed in various species either through this hormonal approach coupled with progeny testing or through alternative procedures such as androgenesis of XY males or gynogenesis of fXY (sex-reversed females by estrogen treatment) in numerous fish species [Cnaani and Levavi-Sivan, 2009]. In some cases a large scale production of YY individuals exists. Good survival rates of YY males have been reported in rainbow trout, tilapia, and yellow catfish, whereas low survival rates or non-viability of the YY individuals have been reported in the medaka and the guppy versus *Betta splendens* [George et al., 1994] and *Amatitlania nigrofasciata* [George and Pandian, 1996]. In 2 important aquaculture species, the Nile tilapia and the rainbow trout, XX and YY males have been generated in order to produce female (XX) and male (XY) monosex progenies, respectively, through a genetic approach.

WW Female Production

Because of important sex-linked growth dimorphism in turbot, the use of all-female populations could enhance farming profitability. As reported in our previous results on turbot [Haffray et al., 2009], the breeding of androgen-induced neomales (mZW) with classic females (fZW) provided some 1:3 (M:F) progenies, suggesting the viability of WW females (mZW × fZW → 1 mZZ + 2 f ZW + 1 fWW). However, due to the late sexual maturity of turbot, this 3-generation process requires at least 6 years to produce fWW (and more to also obtain mWW for WW mass production) in the absence of reliable sex chromosome markers. Moreover, turbot sex also relies upon minor genetic and thermal factors. Therefore, although this approach is technically feasible, it has not been yet developed at a commercial level neither in turbot nor in any other ZZ/ZW commercial species displaying a better female growth rate.

Fish Sensitivity to Sex Reversal Treatments

Until recently, based upon the results of Yamamoto [1969], it was generally assumed that effective sex reversal treatments had to be applied during a critical neutral time window which was species specific and generally different for feminization and masculinization, since ovarian differentiation happens prior to that of testis. This window precedes the onset and covers the first steps of gonadal sex differentiation. Once the natural sex differen-

tiation process had started, a complete and functional sex reversal was considered as no longer possible [Yamamoto, 1969; Hunter and Donaldson, 1983]. Species-specific characteristics of efficient treatments have been extensively described previously [Piferrer, 2001; Cnaani and Levavi-Sivan, 2009]. Our review will therefore focus on more recent aspects of gonad sensitivity to sex reversal treatments.

In cichlid and cyprinid species, where histological sex differentiation usually begins after first feeding, treatments mainly relied upon dietary administration of steroids or inhibitors of steroidogenic enzymes leading to androgens or estrogens [Devlin and Nagahama, 2002; Cnaani and Levavi-Sivan, 2009]. Interestingly, based upon the expression analysis of several candidate gene profiles, the inhibition of endogenous estrogen synthesis could even result in the production of neomales that are physiologically more similar to genetic males than phenotypic males produced by androgen treatments [Vizziano et al., 2008].

In the Nile tilapia, the standard and very efficient treatment used by most farmers to produce male monosex populations is based on a 3–4 weeks dietary administration of 17α-methyltestosterone (17MT) from the first feeding (around 10 dpf). A precocious sensitive period for sex reversal might also exist in tilapia monosex embryos, since the proportion of females or males increased (20–25%) with 17MT or EE2 immersion treatments of <12 h on XX or XY eggs [Rougeot et al., 2008a, b; Gennotte et al., 2014]. Although similar androgen 11KT (11 ketotestosterone) treatment or treatment with an aromatase inhibitor resulted in only 10% masculinization [Gennotte et al., 2015]. Uptake and clearance rates showed that EE2 levels in the eggs dropped at 4 dpf, but the whole body levels were still elevated at 10 dpf [Gennotte et al., 2014]. Accumulation of hormones in the egg or vitellus could be delaying the effect on the embryo. However, this hypothesis does not explain the increase in male percentage when precocious high temperatures were applied [Gennotte et al., 2014]. Moreover, similar EE2 bio-concentrations were obtained with a single immersion treatment at 10 dpf resulting in lower female proportions. Surprisingly, no YY embryos were affected even at superior doses of 6,500 μg/l, although both XY and YY progenies were sex reversed (83.7–100%) at later stages of 10 dpf [Gennotte et al., 2014]. Perhaps a Y-linked repressor or an X-linked activator is acting at this embryonic stage, which may still be partially active later since female proportions increase but are very rarely 100% [Gennotte et al., 2014]. Together, these studies suggest a precocious sensitivity to external factors in tilapia embryos just before the beginning of the morphological differentiation of the brain (31 hours post-fertilization, hpf) and also before the possible identification of the primordial germ cells (46 hpf) in the Nile tilapia [Morrison et al., 2001]. An important challenge is to compare the mechanisms of the precocious versus classic treatments, considering that the precocious doses were very elevated. We assume that both treatments probably act at different levels of the cascade leading to sex differentiation. The use of species sensitive to environmental treatments will allow bypassing the question of steroid accumulation associated with precocious hormonal treatments.

Until now, identification of the critical inversion window mainly relied upon the empiric analysis of the sensitivity of sex differentiation to external factors (hormones, xenobiotics, and environmental factors) rather than upon specific and precise developmental, histological, or molecular traits. We believe that this imprecise period has to be revisited based on the present knowledge on sex determination/differentiation and using up-to-date genomic tools available for some model species. For example, histological sex differentiation in rainbow trout has been reported to occur around 5 weeks after hatching at 10°C (around 67 dpf) in genetic females, but the physiological or molecular sex differentiation of the gonads characterized by sexually dimorphic expression patterns [Vizziano et al., 2007] begins much earlier around hatching time (around 32 dpf).

Fish, like other vertebrates were supposed to have a neutral stage during which the gonads are bipotential before they differentiate towards ovaries or testis. As reported before, sex differentiation towards a specific sexual phenotype can be easily induced in teleost fish with exogenous treatments applied at the 'critical' sensitive window covering at least the beginning of the sex-differentiating process. Gonadal bipotentiality was supposed to be lost after sexual differentiation. However, since the evidence that oocytes can be generated from frozen type A spermatogonia of rainbow trout transplanted into female recipients [Lee et al., 2013], it is clear that bipotentiality still persists in adult teleost gonads. Similarly in adult mice gonads, Sertoli cells and granulosa cells can also be reprogrammed in the absence of their respective transcription factors, DMRT1 or FOXL2 [Uhlenhaut et al., 2009; Matson et al., 2011]. Based upon the hypothesis of the possible persistence of gonadal bipotentiality, functional masculinizations have been obtained in adults of Nile tilapia and medaka through long E2 depletion treatments using an aromatase inhibitor resulting in sex-reversed males that had functional testis and sperm as well as male specific

sexual behaviors [Paul-Prasanth et al., 2013; Sun et al., 2014]. Female-to-male sex reversal was also possible in adult zebrafish with a 5-month aromatase inhibitor treatment causing retraction of the ovaries after which testes-like organs appeared, and cyst structures filled with spermatozoa-like cells were observed in sections of these tissues [Takatsu et al., 2013]. Therefore, sexual plasticity still exists in adult gonads of teleosts, but functional sex reversal in adults needs longer treatments (3–6 months in tilapia and zebrafish; 2 months in medaka) than in fry (2 weeks 1 month in tilapia).

Based on these results, we assume that several steps of the gonadal development are therefore probably sensitive to external influences but also that sex reversal can be obtained either through a control of sex differentiation or through the inhibition of the maintenance of the phenotypic sex (especially femaleness).

Mechanisms of Sex Reversal

Sex reversal can be considered as a change in the sex threshold trait [Heule et al., 2014]. In fish this threshold appears to be extremely susceptible to environmental modifiers (e.g., temperature, pH, density) shown to act at cellular and/or gene levels. Modifiers that affect the onset of ovarian meiosis [Yamaguchi and Kitano, 2012], germ cell proliferation [Saito et al., 2007], or the number of germ cells [Nishimura and Tanaka, 2014] lead to sex reversal. As previously questioned, the number of germ cells could be an important factor or even the threshold modifying the gonad sex fate at least in some species [Kurokawa et al., 2007; Siegfried and Nüsslein-Volhard, 2008; Baroiller et al., 2009a]. The presence of germ cells is indeed necessary for ovarian formation and differentiation in some fish species [Nishimura and Tanaka, 2014]. Loss of germ cells induced by high temperatures or by other means in zebrafish [Uchida et al., 2004; Slanchev et al., 2005], medaka [Kurokawa et al., 2007], and fugu [Lee et al., 2009] induced sex reversal of females into males, with somatic cells seemingly predisposed to develop into male cells [Nishimura and Tanaka, 2014]. However, this was not the case in the goldfish [Goto et al., 2012]. In the TSD pejerrey, germ cell apoptosis by high temperatures alone [Strüsmann et al., 1998] or coupled to busulfan did not affect the sex ratio of the sterile gonad [Ito et al., 2008; Yamamoto et al., 2013]. Likewise, our group [Almin et al., unpubl. data] and Pandit et al. [2015] have shown that germ cell depletion by high temperatures does not apparently cause sex reversal in the Nile tilapia.

New Actors of Sex Determination and Differentiation

The first step at which environmental factors like temperature may be overriding the genetic sex is by somehow affecting the primary sex-determining gene. We are in a fascinating era with the discovery of several different master genes in teleosts that will all be valuable and reliable sources to establish the genotypic sex of wild populations of these species in order to find sex reversals (see Sex Reversal in the Wild). Most of these genes have emerged from duplicated copies of downstream sex-differentiating genes that have been recruited to become master sex determinants. *Dmy/dmrt1bY,* which arose from the testis *dmrt1* gene, was first identified in the medaka *O. latipes* [Matsuda et al., 2002; Nanda et al., 2002]. Spontaneous XY female medaka are attributed to mutations of the male-specific region, the *dmy* coding region, or a single nucleotide mutation in exon 3 resulting in a truncated *dmy* [Matsuda et al., 2002; Otake et al., 2006]. In another medaka, *O. luzonensis*, sex is directed by the male-specific expression of *gsdfY,* a Y allelic form of the gonadal soma-derived growth factor, a member of the TGF-β superfamily [Myosho et al., 2012]. *Gsdf* may also be the sex determinant in the sablefish *Anoplopoma fimbia* [Rondeau et al., 2013]. In the Patagonian pejerrey *O. hatcheri*, maleness is directed by a Y copy of the anti-Müllerian hormone *amh* gene also belonging to the TGF-β superfamily [Hattori et al., 2012]. Despite having a strong TSD, another pejerrey, *O. bonariensis,* also expressed *amhy* at early stages of male promoting temperatures followed by the expression of the autosomal *amha* gene, showing the coexistence of both temperature and genetic sex determination in this species [Yamamoto et al., 2014]. Two tandem Y copies of the *amh* gene have been identified in the Nile tilapia with a 233-bp deleted region in exon VII and a 5-bp insertion in exon VI that leads to a truncated *amh* gene lacking the TGF-β domain [Eshel et al., 2014; Li et al., 2015], defined as *amhΔY,* and another *amhy* having a missense SNP which may be determining sex in some strains, notably the Japanese strain [Li et al., 2015]. In several pufferfish *(Takifugu)* species an allelic variation in the *amhr2* gene, the *amh* receptor, has been shown to be responsible for maleness [Kamiya et al., 2012]. Surprisingly, in the rainbow trout it is a copy of an immune gene, the interferon regulatory factor 9, which has become the master *sdY* gene [Yano et al., 2012].

Sex-determining genes are being searched for in several other fish species with many advances achieved in identifying linkage groups (LG) with quantitative trait loci and SNPs associated to sex using genome-wide sequencing or restriction associated DNA sequencing

(RADseq). These techniques have, for instance in the Nile tilapia, narrowed down an inverted region on LG1 to 8,8 Mb that harbors a sex-determinant but not the *amh*/*amhy* and contains 8 genes amongst which is the Wilm's tumor protein homolog *wt1b* in the Manzala and Ghana strains [Palaiokostas et al., 2013; Gammerdinger et al., 2014]. In tilapia, sex-linked loci seem to vary in function of the strain and population [Cnaani et al., 2008; Ndiwa et al., unpubl. data]. Environmental and parental factors are additional factors that influence the phenotypic sex of tilapia [Baroiller et al., 2009b]. Genotyping of functional SNPs evidenced that 2 were heterozygous for males, while females were homozygous, with misassignments corresponding to XX males [Palaiokostas et al., 2013]. Associations were confirmed in a temperature-masculinized family which had a 66% male sex ratio [Palaiokostas et al., 2015]. Study of LGs linked to temperature-induced phenotypic male tilapias was performed by comparing a thermosensitive line versus a low or non-thermosensitive line showing a strong association of LG1, LG3, and LG23 [Lühmann et al., 2012]. A strong SNP association was also shown in LG20 in another family sensitive to temperature-masculinization using RADseq [Palaiokostas et al., 2015]. In zebrafish, sex-determining studies have followed a long windy road: RADseq revealed a polyfactorial system in domestic strains [Anderson et al., 2012], but it found a ZZ/ZW system in the wild populations with the sex determinant on chromosome 4R [Wilson et al., 2014]. Some female-to-male sex reversals (female genotype *f*/*m*) were found but not the reverse, suggesting that either there is a dominant *f* allele which is required but not sufficient for a female phenotype, or alternatively there is a 2-dose male 1-dose female hypothesis [Wilson et al., 2014]. The authors suggest that environmental factors might be behind these female-to-male reversals affecting the meiotic oocytes by inhibiting primordial germ cell proliferation, meiosis entry, or stimulating oocyte apoptosis.

Gsdf is a novel gene, specific to fish and possibly an endogenous inducer of testis development [Myosho et al., 2012; Imai et al., 2015]. In medaka, *gsdf* seems to be the target of *dmy* since they co-localize in somatic cells (pre-Sertoli cells). It is detected just after *dmy*, with a down-regulation seen in XY females treated with estradiol [Shibata et al., 2010; Chakraborty et al., 2016]. *Dmy* knockdown with RNAi resulted in male-to-female sex reversal induced by suppressing the male pathway genes *(gsdf* and *sox9a)* and upregulating the female pathway (R-spondin1, *rspo1*) [Chakraborty et al., 2016]. Complete female-to-male sex reversal of the medaka *O. sakaizumii* but not of *O. latipes* stimulated *gsdf* and subsequently *dmrt1* [Horie et al., 2016]. In tilapia, upregulation of *gsdf* transcripts were initially suggested to be at 8 dpf prior to *dmrt1* and localized also in the somatic cells surrounding germ cells [Kaneko et al., 2015]. However, CRISPR/Cas9 knockouts revealed that *gsdf* is in fact expressed after *dmrt1* and an aromatase inhibitor treatment rescued the testicular phenotype of XY gsdf$^{-/-}$ gonads [Jiang et al., 2016]. In vitro analysis showed that *dmrt1* co-transfected with *sf1* was able to activate *gsdf* in a dose-dependent manner [Jiang et al., 2016]. G*sdf* action in XY tilapia is thought to be related to the suppression of estrogen production possibly via the inhibition of ovarian-differentiating genes [Jiang et al., 2016].

Ontogeny expressions show that both the sex determining gene *amhy* and the autosomal *amha* gene are implicated in testis differentiation in the TSD pejerrey even in males lacking *amhy,* while in the GSD pejerrey *amha* is not implicated [Yamamoto et al., 2014]. In the Nile tilapia, *amhy* and *amhΔY* are expressed specifically in XY larvae from 9 dpf, but only the *amhy* knockdown resulted in male-to-female sex reversal [Li et al., 2015]. Overexpression of *amhy* in XX fish but not of *amhΔY* resulted in female-to-male sex reversal, suggesting that *amhy* is the sex determinant at least in the Japanese strain [Li et al., 2015]. A non-synonymous SNP in exon VI was found in one of the tilapia *amh* genes (but which of the 3 genes it corresponds to is unclear) associated to temperature-induced males, that could be useful to increase the proportion of males in the selective breeding of temperature-induced lines [Wessels et al., 2014]. *Amh* and *amhr2* together with *gsdf* appear to play important roles in primordial germ cell proliferation which might explain why they have been recruited to become primary sex determinants [Heule et al., 2014]. In the medaka XY *hotei,* a mutation in the *amhr2* gene causes hyperproliferation of the germ cells and male-to-female sex reversal [Morinaga et al., 2007] due to lack of Amh signaling on the mitotic self-renewing germ cells [Nakamura et al., 2012]. In tilapia *amhr2* knockout caused 100% male-to-female sex reversal but in contrast, only 60% were obtained with *amhy* due to F_o generation being mosaic with the mutation varying between individuals [Li et al., 2015]. Therefore, both *amhy* and *amhr2* are critical to determine sex in tilapia, but it remains to be known how the signaling occurs. Knockdown of both *amhy* and *amhr2* in XY tilapia causes upregulation of the aromatase *cyp19a1a* gene as well as higher E2 levels, but no increase in *cyp19a1a* expression was seen in *amhy*/*amhr2* overexpressed XX fish [Li et al., 2015].

Revisiting Old Players of Sex Differentiation

The classical view of hierarchy and a sex-differentiating pathway is being replaced by a scenario of a gene network with a complex interplay of signals acting in a modular way [Crews and Bull, 2009; Schwanz et al., 2013; Heule et al., 2014]. The underlying molecular mechanisms modifying testis or ovarian thresholds leading to sex reversal in fish are slowly being better deciphered with transcriptome microarrays studies [Baron et al., 2007; Eshel et al., 2014] or using next-generation sequencing techniques (NGS) [Yano et al., 2012; Shao et al., 2014; Tao et al., 2016] coupled to overexpression and knockdown studies [Imai et al., 2015; Li et al., 2015; Chakraborty et al., 2016]. The hormonal estrogen status has been known for a long time to be an important factor affecting the sex threshold towards ovarian differentiation via changes of aromatase, the estrogen-producing enzyme or its gene *cyp19a1a* [Guiguen et al., 1999, 2010; Kitano et al., 1999; D'Cotta et al., 2001a, 2001b]. The critical role of estrogens with the earlier upregulation of the female pathway in fish sex differentiation but not androgens has been reconfirmed [Tao et al., 2013].

Depletion of estradiol levels was the prerequisite for adult sex reversal in both Nile tilapia and medaka [Paul-Prasanth et al., 2013; Sun et al., 2014]. In both species the aromatase inhibitors first caused female germ cell degeneration, depletion of E2 levels followed by the development of testicular portions where interstitial cells became steroidogenic-active Leydig cells and only later the rise in 11KT levels, effects that were rescued with E2 supplement. In this transdifferentiation, granulosa became Sertoli-*dmrt1* positive cells with suppression of female-specific genes followed by upregulation of male-specific genes [Paul-Prasanth et al., 2013; Sun et al., 2014]. In medaka some cyst germ cells were germline stem cells with bipotential activity that differentiate into oogonia but in the absence of E2 are capable to differentiate into spermatogonia [Paul-Prasanth et al., 2013]. The mode of action of feminizing EE2 (ethinylestradiol) treatment was studied in rainbow trout fry/juveniles with a meta-analysis of microarray data showing a rapid and strong decrease of key testis genes, notably those involved in androgen synthesis [Depiereux et al., 2015]. It highlighted new pathways such as the progesterone-oocyte maturation pathway with several genes of the germline, cell division and oocyte meiosis represented and the peroxisome proliferator-activated receptor (PPAR) signaling pathway containing genes from the nuclear hormone (steroid/thyroid/retinoid) receptor family that perhaps regulate aromatase levels.

Some studies suggest that induced masculinizations might not follow the same pathways as natural 'genetic' testis differentiation [D'Cotta et al., 2001b; Poonlaphdecha et al., 2013; Golan and Levavi, 2014]. Different fish masculinizing compounds, consisting of 17MT (an aromatizable androgen), dihydrotestosterone (DHT, a non-aromatizable androgen), and fadrozole (the aromatase inhibitor) were evaluated for their androgen activities, inhibition of aromatase activity, and efficiencies in female-to-male sex reversals in tilapia and using a species for which the male blue coloring is associated with high androgen levels [Golan and Levavi, 2014]. The masculinizing actions of 17MT and DHT are most likely due to binding to androgen receptors seen by the inhibitory effects and sex reversal capabilities when combined with the androgen receptor agonist flutamide. The fadrozole action (the most potent in inhibiting aromatase activity) might be an excess of un-aromatized androgens accumulated in the differentiating gonad [Golan and Levavi, 2014]. Masculinization treatments with androgens (11βOHΔ4) of XX rainbow trout [Baron et al., 2007] or long aromatase-inhibitor treatments of XX tilapia [Paul-Prasanth et al., 2013; Sun et al., 2014] caused a 'transdifferentiation' with a rapid inhibition of the female-specific genes followed by upregulation of male genes but with disrupted expression profiles.

Temperature-masculinizing treatments in tilapia caused a very quick and strong overexpression of the main male-differentiating genes *amh* and *dmrt1* to levels above those found in differentiating genetic XY males, followed only after by the repression of the principal female-differentiating genes *cyp19a1a* and *foxl2* [Poonlaphdecha et al., 2013]. High temperature accelerates the molecular mechanisms leading to testis development, since other male genes also showed earlier upregulations such as *sox9a*, *sox9b*, *dax1,* and particularly *cyp11dh* [Poonlaphdecha et al., unpubl. data] which encodes 11-β hydroxylase that participates in 11KT production, suggesting that an increase of androgens might be required for temperature-induced masculinization [D'Cotta et al., 2001b]. This suggests that temperature-induced masculinization requires the male-differentiating pathway to be turned on and only after that, the ovarian-differentiating pathway is suppressed. In the TSD pejerrey, a heterologous microarray study revealed that in the masculinization at male-promoting temperature (MPT) there is an interplay of pro-apoptotic and anti-apoptotic genes [Fernandino et al., 2011]. In the sea bass, high temperatures (HT) also promote males, and microarray analysis showed elevated *dmrt1* expressions in sex-differentiating HT fish

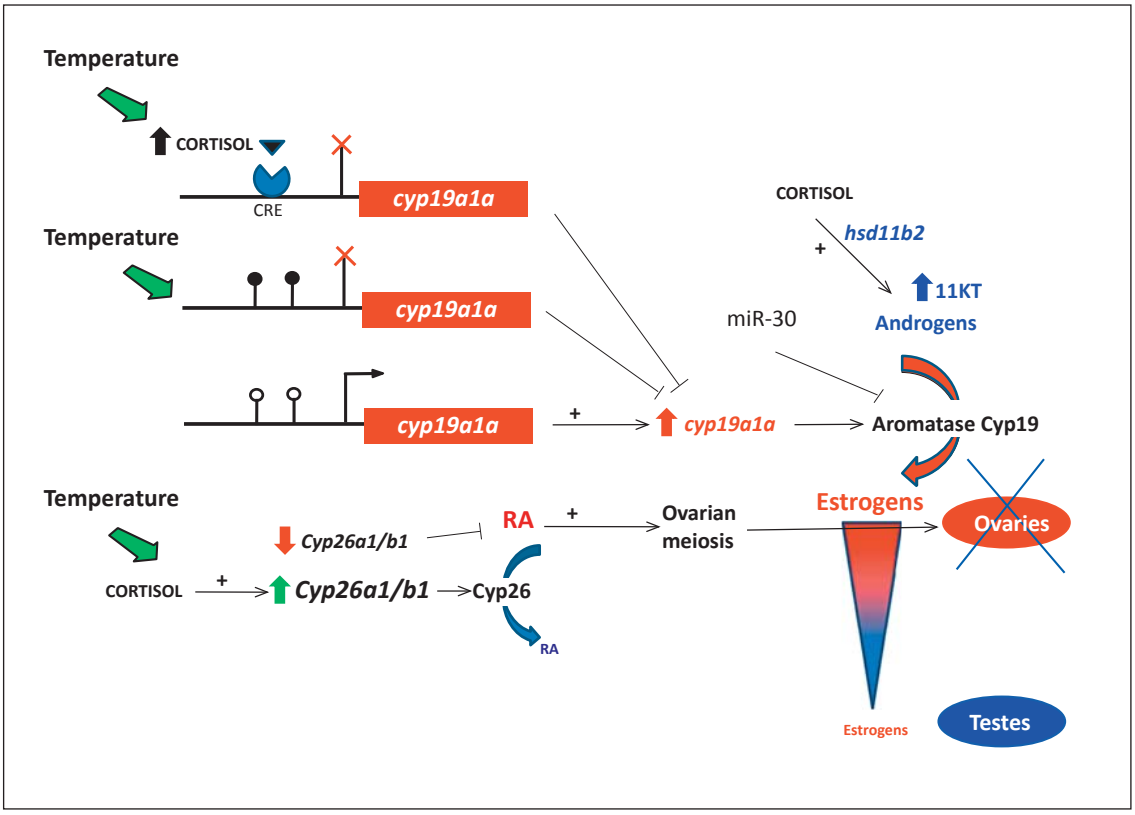

Fig. 1. Schematic representation of different regulating mechanisms that have been described amongst fish species in temperature-induced males, of cortisol action, cortisol binding, and DNA methylation on the aromatase *cyp19a1a* gene promoter suppressing the gene expression, which affect aromatase activity and estrogen levels leading to testis differentiation. Cortisol also leads to lower retinoic acid which delays ovarian meiosis inducing testes differentiation.

together with increases in cholesterol transporting genes and stress response genes as well as upregulation of epigenetic regulatory genes, while low temperature fish showed high *cyp19a1a* levels [Diaz and Piferrer, 2015].

C*yp19a1a* expressions were compared during sex differentiation between a GSD Atlantic silverside population from Nova Scotia and another TSD population from South Carolina which were both treated at feminizing (15°C) and masculinizing (28°C) temperatures [Duffy et al., 2010]. In the GSD population, *cyp19a1a* expression was earlier, stronger, and clearly dimorphic and was not affected by temperature. In contrast, in the TSD population only the feminizing temperature treated fish had elevated *cyp19a1a* levels but they were considerably lower than the presumptive females of the GSD population. This suggests that *cyp19a1a* is perhaps predisposed to be suppressed easily in this population (lower threshold?) and perhaps under different regulating mechanisms. It is possible that a difference in DNA methylation exists in the *cyp19a1a* promoter region (fig. 1; see Epigenetics: A Link between the Environment, Gene Expression, and Sex Reversal) as shown in the sea bass HT males [Navarro-Martin et al., 2011].

Is Cortisol the Universal Mediator of Sex Reversal in Fish?

Fish sex reversal induced by elevated temperatures, hypoxia, pH, or density might be due to harsh/stressful conditions. Cortisol, the glucocorticoid stress hormone, may be an ovarian inhibitor and may be mediating sex reversal at several levels as schematically shown in figure 1 and also described by Todd et al. [this issue]. In the pejerrey, elevated cortisol levels were found in larvae at MPT and in larvae treated with cortisol reared at female-promoting temperatures (FPT) [Hattori et al., 2009]. Both induced masculinizing gene profiles with decrease

in the ovarian *cyp19a1a* gene and increase in the testes *amh* gene as well as apoptosis, a feature of pejerrey MPT [Hattori et al., 2009]. Cortisol mediated by the hydroxysteroid dehydrogenase *hsd11b2* gene was shown to promote 11KT synthesis [Fernandino et al., 2012]. A similar mechanism might be acting in the European eel *Anguilla anguilla* for which high density promotes male differentiation, since larvae have been shown to have both elevated cortisol and 11KT levels [Geffroy and Bardonnet, 2016].

Changes in the onset of ovarian meiosis lead to sex-reversed males, and estrogen contributes to the timing via the regulation of genes from the retinoic acid (RA) pathway [Yamaguchi and Kitano, 2012; Feng et al., 2015]. The *cyp26a1* or its paralog *cyp26b1* genes are considered as meiosis-inhibiting factors of the Cyp26 RA-degrading enzyme and have been shown to have an important role in the onset of germ cell meiosis in fish ovaries [Yamaguchi and Kitano, 2012; Rodríguez-Marí et al., 2013; Feng et al., 2015]. In temperature-induced males of the Japanese flounder, *cyp26b1* was upregulated by cortisol, leading to a delay in germ cell meiosis [Yamaguchi and Kitano, 2012]. This species had elevated cortisol levels during and after sex differentiation in XX fish masculinized at 27°C and showing suppressed *cyp19a1a* levels, and cortisol was able to rescue the cortisol inhibitory effect of metyrapone [Yamaguchi et al., 2010]. Exposure of medaka to high temperatures or cortisol causes female-to-male sex reversal with inhibition of germ cell proliferation and of *cyp19a1a* expression, but estrogen rescued the effects [Hayashi et al., 2010; Kitano et al. 2012]. Temperature masculinization might require an increase in androgens together with aromatase inhibition and apoptosis [Fernandino et al., 2013]. Cortisol implication has not been studied in the temperature-induced masculinization of Nile tilapia, but among various stress treatments on larvae, only temperature affects the sex ratios [Baroiller, unpubl. data].

Brain Implication in the Sexual Phenotype

Fish possess a large plasticity for sex reversal which is also evident in the brain with a reversibility existing even in adulthood [Godwin, 2010; Paul-Prasanth et al., 2013]. In contrast, brain sex differentiation in birds and mammals occurs only during embryo development and is shown to be mediated not only by gonadal steroids but also by genes on the sex chromosomes as well as epigenetic regulations [McCarthy and Arnold, 2011]. Several neurosteroids are produced in the teleost brain, notably estrogen with an extremely elevated aromatase activity that increases with age, probably for the continuous neurogenesis [Le Page et al., 2010]. Most teleosts possess 2 aromatase genes *(cyp19a1a* and *cyp19a1b)* generated during teleost genome duplication, with *cyp19a1b* being the predominant brain gene [Le Page et al., 2010]. The *cyp19a1b* gene is thought to be implicated in a feedback loop in the brain regulating teleost steroid production through the kisspeptin and the hypothalamo-pituitary-gonadal axis [Hofmann, 2006]. In mammals, testosterone exerts many physiological actions in the brain, affecting male sexual behaviors that are mediated by the aromatase neural conversion of androgens into estrogens and therefore, the brain aromatase is a critical factor in the masculinization of the brain [Balthazart et al., 2011]. Differentiating rainbow trout males had overall higher *cyp19a1b* expression levels in developing brains [Vizziano-Cantonnet et al., 2011]. During the onset of gonadal sex differentiation, female tilapia brains had higher aromatase activity than males [D'Cotta et al., 2001a], but no sex differences were found for *cyp19a1b* expressions [Kwon et al., 2001]. The precocious sex reversal of tilapia XY and YY into females caused an increase in brain *cyp19a1b* expression that was dependent on the EE2 dose and was also associated with an increase in both testosterone and estrogen concentrations [Gennotte et al., 2014]. Upregulation of *cyp19a1b* expression was also seen when we masculinized XX females with high temperature treatments, together with higher *erα* and *erβ* expressions [Ouedraogo et al., unpubl. data]. We saw different *cyp19a1b* expression profiles for the different phenotypic males with levels remaining low till adulthood in the 17MT males compared to fadrozole-treated males and temperature-induced males, all lower than genetic males. This study suggests that the gene endocrine environment is probably different between these phenotypic males, and it would be interesting to investigate if they display differences in their sexual behavior. In the medaka brain, *esr1* has a male-biased expression suggesting that it has a role in the masculinization or defeminization in medaka while *esr2β* and *arβ* were implicated in the feminization or demasculinization [Hiraki et al., 2012]. In this study the ventral telencephalic and preoptic regions showed strong female-specific expressions of ERs and ARs which were activated by estrogens and inhibited by androgens. In the TSD pejerrey, *cyp19a1b* is expressed in the brain at MPT before the onset of gonadal sex differentiation [Strobl-Mazzulla et al., 2008].

We have shown in tilapia a very precocious sexual dimorphism of *amh* expression in male brains occurring

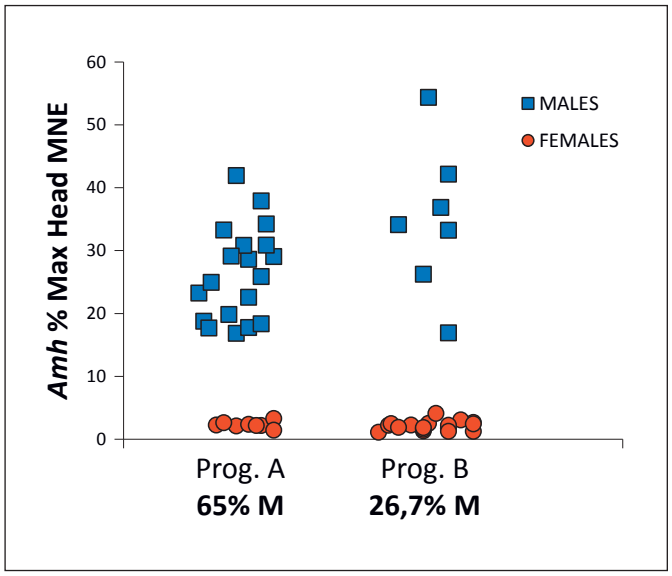

Fig. 2. *Amh* expression levels in the heads of Nile tilapia at 14 dpf found in 2 progenies treated at 27°C. In progeny A the *amh* expression identified 65% males and in progeny B 26.7% males (n = 30 samples/progeny analyzed), and both correlated with the male sex ratio obtained by gonadal squash at 4 months of age. Values are represented as %maximum mean normalization expression (%Max MNE). *Amh* expression in males is represented with blue squares and in females with red circles [Poonlaphdecha et al., unpubl. data].

between 10 and 15 dpf [Poonlaphdecha et al., 2011] which is the stage when the gonad aromatase *cyp19a1a* gene only starts to be expressed in differentiating females [Ijiri et al., 2008; Poonlaphdecha et al., 2013]. This suggests that masculinization of some brain regions might not be mediated by gonad steroids. It could be the result of *amh* present on the presumptive Y chromosome of tilapia, the *amhy* gene, although no brain expression was detected by Li et al. [2015]. We have developed a precocious sexing procedure for tilapia using brain *amh* expression that reflects the male phenotype of individuals at 14 dpf correlating accurately with the sex ratio of a progeny (fig. 2) [Poonlaphdecha et al., unpubl. data]. This procedure could be an efficient way to accelerate the selection of YY individuals or for generating a thermosensitive line.

The adult treatments of medaka and tilapia with aromatase inhibitors (AI) clearly showed the brain sexual plasticity since the XX treated fish displayed male-specific territorial and sex behaviors [Paul-Prasanth et al., 2013]. AI treatment of the African cichlid *Astatotilapia burtoni* adult females did not cause sex reversal of the ovaries retaining female gene profiles; however, partial masculinization was observed with changes in coloration, body shape and form, steroid levels, and notably modifications in the brain gene expressions which affected sexual behavior [Göppert et al., 2016]. *Cyp19a1a* present in the brain in this species was upregulated in AI females while both *arα* and *cyp19a1b* were downregulated, resembling the profiles of males which could be to compensate E2 levels or due to higher testosterone levels.

Epigenetics: A Link between the Environment, Gene Expression, and Sex Reversal

One of the underlying mechanisms leading to sex reversal in some fish species might be epigenetics. It is now evident that the environment can induce epigenetic modifications of histones affecting the compactness of the chromatin structure and DNA accessibility without causing actual nucleotide changes that play essential roles in gene regulation and ultimately modify the phenotype [Jaenisch and Bird, 2003]. One of the most studied epigenetic mechanisms is DNA methylation of cytosines (C5 position) at CpG dinucleotides because of its implication in a large number of cellular functions such as development and cell differentiation, tissue-specific gene expression, genome imprinting, X chromosome inactivation, and in disease susceptibility [Zhang and Ho, 2011]. Hypermethylation is associated with gene silencing, but methylation also acts at the level of transcript splicing [Shukla et al., 2011]. Higher DNA methylation levels have been found in sea bass gonads of differentiating males in the aromatase *cyp19a1a* promoter that were inversely correlated with the *cyp19a1a* gene expression [Navarro-Martin et al., 2011]. Exposure during early life to high temperatures (>17°C) induced a male bias and also increased the methylation levels in both males and females (fig. 1). Interestingly, sex-specific differences were seen for the 7 CGs sites, while only 2 showed temperature-induced differences. The in vitro study suggests that the hypermethylation blocked the stimulatory effect of SF1 and FOXL2 [Navarro-Martin et al., 2011].

Opposite methylation patterns were evidenced in the half-smooth tongue sole, with males having a low methylation rate at the *dmrt1* promoter (located on the Z chromosome) associated to an elevated *dmrt1* expression [Chen et al., 2014; Shao et al., 2014]. Tongue sole is a ZW/ZZ species but temperatures of 28°C lead to ~73% masculinized ZW females [Chen et al., 2014], and these phenotypic males show a demethylation of the *dmrt1* promoter [Shao et al., 2014]. Genome-wide DNA methyla-

tion and the transcriptome were studied in this species, permitting a much larger analysis of the methylation status of gene regulatory elements and gene expressions [Shao et al., 2014]. Differences in the methylation rates were found between ovaries and testes notably for the sex-differentiating pathway genes. Interestingly, higher levels of methylation were found in a 2-Mb region of the Z chromosome which differed from the W chromosome that clustered for testes, which might lead to some dosage compensation in ZW males. In addition, the tongue sole showed splicing alternatives but not silencing, for a female W gene *fig1a* (factor in the germ-line alpha) associated to differential expression between ZW females and ZW males [Shao et al., 2014]. Very recently, the global DNA methylation status was analyzed in the Nile tilapia between mature (>4 months) females, mature males, and from a matured mixed-sex group treated at high temperatures [Sun et al., 2016]. Overall higher CpG methylated domains were found in the gene bodies of females compared to males. High temperature treatments increased the methylation levels in both males and females. The orphan nuclear receptor *dax1 (nr0b1a)* gene had higher methylation levels in induced males when compared to control females, while the steroidogenic enzyme *hsd17b8* gene as well as the *erb2* and *gsdf* genes had lower levels.

Cytosine methylation is catalyzed by the DNA methyltransferase enzymes (DNMT). In mammals, DNMT1 is involved in the maintenance of the CpG methylation pattern on the new DNA strand after replication, while DNMT3A and DNMT3B are considered de novo methyltransferases implicated in the initial CpG methylation pattern [Jaenisch and Bird, 2003; Zhang and Ho, 2011]. DNMT3L is an additional member with no enzymatic activity that acts as a cofactor of DNMT3A and DNMT3B activity. In teleosts, due to the genome duplication, up to 6 *dnmt3* genes have been identified in zebrafish [Campos et al., 2012] which complicates the analysis of the Dnmt enzyme contribution. We have started to analyze the expression levels of Nile tilapia *dnmt* genes during sex differentiation. Sexual dimorphism and temperature differences were observed for some of the de novo DNA methyltransferases, *dnmt3* while *dnmt1* remained unchanged [Saboret et al., unpubl. data] which suggests that the *dnmt3a* and *dnmt3b* paralogues have specific roles during tilapia genetic sex differentiation and temperature-dependent masculinization. It might indicate differences in gene methylations occurring between genetic males and females but can also be induced by a temperature-induced masculinization. This stage coincides with repression of the c*yp19a1a* gene in temperature-induced males [D'Cotta et al., 2001a; Poonlaphdecha et al., 2013]. Whether its promoter is hypermethylated in tilapia males similarly to what has been seen in sea bass remains to be investigated.

DNA methylation associated with sex-determining and -differentiating genes has begun to be investigated in other fish species [Todd et al., this issue], but a whole avenue is now possible with genome-wide studies of the methylome associated with RNAseq which will enlighten how the methylation marks are activated or erased, implicated in transcript silencing or dosage compensation, between genetic sexes and sex-reversed fish. Epigenetic marks other than methylation are only starting to be analyzed in fish during sex determination and differentiation. For instance microRNA, small non-coding RNAs that inhibit at post-transcriptional level target-gene expressions, have begun to be profiled between differentiating ovaries and testes in the Nile tilapia [Eshel et al., 2014; Tao et al., 2016]. Integration of miRNA and mRNA was compared for early differentiating XX and XY Nile tilapia identifying 130 novel miRNA of which 49 were unique for XX and 45 solely found in XY gonads [Tao et al., 2016]. Sex-biased miRNA were negatively correlated with genes from the steroid hormone biosynthesis pathway *(Cyp11a1, Hsd3b, Cyp19a1a, Hsd11b)* and to the ovarian or testes differentiating network genes such as *Foxl2, Amh, Star1, Sf1, Dmrt1*, and *Gsdf*.

Epigenetic mechanisms can be reversible and heritable [Zhang and Ho, 2011]. The study of transgenerational epigenetic inheritance is easily accessible in fish, notably those with short life stages [Salinas and Munch, 2012], and a better understanding of these mechanisms will be possible by studying successive generations of sex-reversed individuals. It would be very intriguing to see how sex-reversed fish have adapted to natural high temperature regimes and how populations have been potentially affected in the wild, depending upon the presence or absence of such epigenetic marks.

Sex Reversal in the Wild

Many fish species have been manipulated with exogenous factors to reverse the primary sex under controlled conditions, but several questions are raised on whether this can occur under wild conditions, at the individual level, at what frequency, does it impact the population, and finally does it have an ecological significance [Stelkens and Wedekind, 2010]. Apart from *M. menidia* [Conover, 1984; Conover and Heins, 1987] where the existence of

Table 2. Relationship between the phenotypic sex and gene genotyping in some wild fish populations

Name	Species	Location	Genotype	Phenotype		Total (%)	Reference
				male (%)	female (%)		
Medaka	*Oryzias latipes*	Japan, Korea, China, Taiwan (n = 109)	*dmy*$^+$ *dmy*$^-$	1,438 15 (1)	26 (1.7) 1,525	1,464/3,004 (48.7) 1,479/3,004 (49.2)	Shinomiya et al. [2004]
Chinook salmon	*Oncorhynchus tshawytscha*	Clearwater, Kuskokwim, Colombia Rivers (USA)	*sdY*$^+$ *sdY*$^-$	45 0	13 (8.3) 144	58/202 144/202	Cavileer et al. [2015]
Pejerrey	*Odontesthes bonariensis*	Lake Kasumigaura (Japan)	*amhy*$^+$ *amhy*$^-$	37 3 (7.5)	1 (2) 49	38/90 (42.2) 52/90 (57.8)	Yamamoto et al. [2014]
Nile tilapia	*Oreochromis niloticus*	Lake Turkana and Lake Bogoria[a] (Kenya)	*amhΔY*$^+$ *amhΔY*$^-$	14 5 (26.3)	3 (21.4) 11	17/33 (51.5) 16/33 (48.5)	Ndiwa et al. [unpubl. data]
		Loboi Hot Springs[b] (Kenya)	*amhΔY*$^+$ *amhΔY*$^-$	27 5 (15.6)	2 (7.7) 24	29/58 (50) 29/58 (50)	

[a] Water temperature 27–28°C. [b] Water temperature 35–36°C.

TSD has been associated with a better reproductive fitness of one sex following in this way the model of Charnov and Bull [1977], possible advantages for the existence of environmental sex reversal in fish species are not known. It is first essential to study how frequent environmental sex reversals are in wild populations to then study their fitness and the effects of biased sex ratios on population dynamics. Because the majority of fish species with GSD have homomorphic sex chromosomes, karyotype analysis does not allow the study of mismatches between the genotypic and phenotypic sex. To study these discordances in wild fish populations requires sex-specific markers.

The role of *dmy*/*dmrt1bY* as the sex determinant in the medaka *O. latipes* was partially established by Matsuda et al. [2002] due to the finding of 2 wild-derived XY female mutants. The finding of spontaneous sex-reversed medaka led to the *Dmy*/*dmrt1bY* genotyping in 8 medaka strains to try to find discordance between the genotypic and phenotypic sexes [Nanda et al., 2003]. Both PCR analyses and FISH using a Y-specific BAC probe identified 40 males out of a total of 764 fish (5.2%) lacking the *dmy*/*dmrt1bY* marker which were further confirmed to be XX males when some were used in progeny testing giving female-biased offspring [Nanda et al., 2003]. Frequency of these XX males ranged between 3.7–15.4% of males in 6 strains analyzed, but no XY females were found amongst them. Existence of the XX males indicated that *dmy*/*dmrt1bY* and the Y chromosome were not indispensable for testis development, and this was explained by putative autosomal modifier genes overriding the sex chromosomes by either suppressing a female-determining locus on the X chromosome or by acting as male inducers [Nanda et al., 2003]. The absence of XX males or their presence in low frequency in some strains led the authors to suggest that the modifiers were polygenic and strain specific.

A larger *Dmy* genotyping was performed in a survey of 3,004 wild-caught and wild stock medaka from 109 sites in Japan, Korea, China and Taiwan [Shinomiya et al., 2004]. In the majority of fish, the phenotypic sex coincided with the genotypic sex, but in 8 localities 15 *dmy*$^-$ males, presumably XX males (1.03% of males), were found while 26 *dmy*$^+$ XY females were located in 13 sites (1.68% of all females) (table 2). A progeny testing by crossing these XY females with inbred XY males gave functional XY females in the F1 generation that all carried the maternal Y chromosome. This study showed not only that *dmy* was commonly found in wild populations of medaka but also the existence of wild XY sex-reversed females that either carried *dmy* or a mutated *dmy* gene which apparently was not lethal. The study to identify the genetic basis leading to the spontaneous XX female-to-male sex reversal showed that it was a recessive mutation,

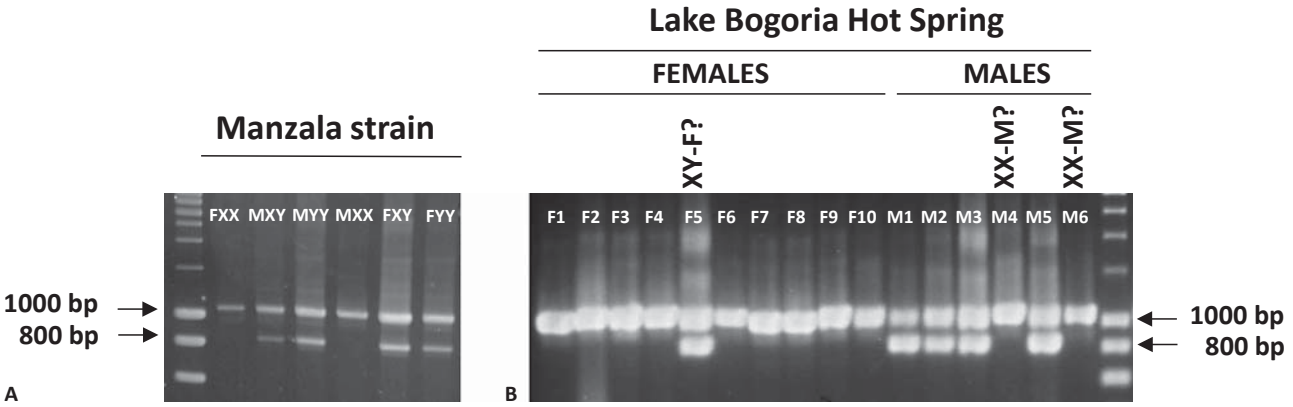

Fig. 3. PCR genotyping of Nile tilapia males (M) and females (F) using the *amhΔY* marker. **A** Presence of a 800-bp band corresponding to the putative 233 bp deleted *amhΔY* gene associated to the Y chromosome in XY and YY males and in XY and YY females of the Manzala strain. The 1,000-bp band was observed in all sexual genotypes and corresponds to 2 putative *amh* genes: the X chromosome *amh*, and the sex-determining Y chromosome gene *amhy* just downstream of the *amhΔY* gene [Eshel et al., 2014; Li et al., 2015]. **B** Mismatch between the phenotypic sex and the sexual genotype when using the *amhΔY* marker in some wild caught tilapia from Lake Bogoria Hot Spring (35–36°C) (Kenya) suggesting possible XX males and XY females [Ndiwa et al., unpubl. data].

controlled by the *sda-1* locus located on the autosomal LG8 [Shinomiya et al., 2010]. The absence of the Y chromosome in successive generations of medaka from the Shirone wild population suggests that LG8 has taken over the role of the sex chromosomes [Shinomiya et al., 2010]. The genetic basis of XY lacking *dmy* resulting in male-to-female sex reversal was found to be of 2 types, one due to mutations in the amino acid coding sequence of *dmy* and the other due to low expressions of *dmy* at 0 days after hatching at the critical sex-determining period while the coding sequence was normal [Otake et al., 2006]. This suggested that *dmy* expression below a threshold did not cause pre-Sertoli (*dmy*-positive cells) to develop into Sertoli cells, disrupting primordial germ cell proliferation and testis development leading to female phenotypes [Otake et al., 2006]. Regulatory mutations in the *dmy* flanking region might be the cause of the mutants having reduced *dmy* expression. In addition to the reduced *dmy* expression levels there might be autosomal loci associated with sex reversal [Otake et al., 2006].

The sex-determining gene *sdY* identified in rainbow trout was searched in several salmonids species with the finding of a single sdY^+ female in Chinook salmon *O. tshawytscha* and a single sdY^- male in lake trout *Salvelinus namaycush* [Yano et al., 2013]. *SdY* was used further to search for mismatches between the genetic and phenotypic sex in wild populations of Chinook salmon from Alaska, Idaho, and Washington [Cavileer et al., 2015]. While all males were sdY^+ (45/45 males), 13 (8.3%) out of 157 phenotypic females (table 2) were found to be also sdY^+. Seven of these putative XY females contained all 4 exons of *sdY* and were also positive for the Y chromosome male markers *OtY1* and *GHc*, suggesting possible unknown environmental factors causing sex reversal [Cavileer et al., 2015].

In the Nile tilapia the 233-bp deleted *amh* gene, which was initially called *amhy* [Eshel et al., 2014] and later *amhΔY* [Li et al., 2015], was identified in males and considered to be specific for the Y chromosome and was always associated to the male sex determinant *amhy* in this species. We analyzed the existence of the *amhΔY* in different sexual genotypes of the domesticated Manzala strain using primers that covered both the deleted and undeleted regions of the *amh* genes producing a fragment of 800 bp and 1,000 bp. respectively. XX females had only the 1,000-bp fragment, while XY and YY individuals had both (fig. 3A) [Ndiwa et al., unpubl. data]. Similarly, only the undeleted 1,000-bp fragment was observed in XX males whereas both were seen in XY females. The presence of the extra 1,000-bp fragment (the putative X-*amh*) in YY individuals was later explained when Li et al. [2015] showed that 2 tandem Y *amh* genes existed, one corre-

sponding to the 233-bp deleted *amhΔY* gene and the other, the sex determinant *amhy* gene with a missense SNP (C/T) and with a 5,608-bp deletion in the promoter. Using *amhΔY,* we genotyped wild-caught Nile tilapia from Lake Turkana and Lake Baringo (Kenya) living in 27–28°C waters and fish caught in the Loboi Hot Springs (Kenya) that are constantly at 35–36°C. The large majority of fish showed a good agreement between the phenotypic sex and the genotypic *amhΔY* sexing (fig. 3B; table 2). However, we found 10 out of 91 individuals (~11%) that were *amhΔY*$^-$ males (putative XX males) and 5 females (5.5%) that carried the *amhΔY*$^+$ gene (putative XY females), in both constant temperature regimes and hot springs (table 2) [Ndiwa et al., unpubl. data]. We do not know if individuals in the wild that carry the *amhΔY* also have the *amhy* gene, but both were linked to the presence of the Y chromosome in the Japanese strain. We have nevertheless found wild African populations that lack both the *amhy* SNP and the *amhΔY* [Ndiwa et al., unpubl. data]. The presence of *amhΔY* in these wild Kenyan populations indicates that it is an old truncated form that did not rise from Nile tilapia domestication. We are currently analyzing the discordance between the phenotypic and genotypic sexes by genotyping both *amhy* and *amhΔY* to confirm these surprisingly high proportions of putative sex-reversed tilapia. We are also performing progeny testings using wild individuals considered to be sex reversed.

The pejerrey *O. bonariensis* has been considered until very recently to be exclusively a TSD species, where 100% males are produced at 29°C (MPT) and 100% females produced at 17°C (FPT) [Strüssmann et al., 1996; Strüssmann and Patiño, 1999]. However, at intermediate temperatures of 25°C, mixed sex ratios ranging from 20 to 80% males have been found between progenies from different parents. The sex determining *amhy* gene identified in the GSD Patagonian pejerrey was searched and cloned in the pejerrey *O. bonariensis* and first tested on a laboratory broodstock to identify mismatches between the genotypic and phenotypic sex [Yamamoto et al., 2014]. Out of 24 broodstock fish, 14 carried the *amhy*$^+$ while 10 did not (*amhy*$^-$), with males being all heterozygous for *amhy* (represented here as *amhy*$^+$). Furthermore, an *amhy*$^+$ female was also identified and shown by progeny testing to be heterozygous (*amhy*$^+$). The *amhy* was then used to analyze possible TSD and GSD interactions of *O. bonariensis* progenies treated at intermediate temperatures [Yamamoto et al., 2014]. One of these progenies gave a male-biased sex ratio of 70% with 33 phenotypic males found to be *amhy*$^+$ while 34 were *amhy*$^-$, out of which 13 (38.2%) were female-to-male sex-reversed males indicating the coexistence of TSD and GSD in this species. This coexistence was also evidenced in wild populations, since *amhy* could be used to genotype pejerrey *O. bonariensis* from Lake Kasumigaura in Japan (table 2) with the finding of 3 *amhy*$^-$ males (7.5% of males) and 1 *amhy*$^+$ female (2% of females) [Yamamoto et al., 2014]. This study together with our work on tilapia raises the question on the impact of high temperatures due to climate change on wild populations and how they will adapt.

Consequences of Sex Reversal in Wild Populations

In some fish species, such as the Nile tilapia, the pejerrey *O. bonariensis,* and the Patagonian pejerrey *O. hatcheri* [Baroiller et al., 2009b; Hattori et al., 2012; Yamamoto et al., 2014], genetic factors determine the development of a phenotypic sex within a specific range of environmental conditions, but under extreme conditions, temperatures can override the genetic factors at least in some individuals. Due to TSD/ESR, some individuals will have opposite sexual phenotypes and genotypes. Both pejerrey and Nile tilapia have a XX/XY heterogametic system, and both sex-reversed XX males and XY females have been already described in some wild populations [Bezault et al., 2007; Baroiller et al., 2009b; Yamamoto et al., 2014]. Modelling studies have analyzed the possible effects of their reproduction on sex ratio, dynamics, and resilience of wild populations [Kanaiwa and Harada, 2002; Hurley et al., 2004; Stelkens and Wedekind, 2010]. The prerequisite for a sex-reversed breeder to impact a population is its relative reproductive capacity and fitness when compared to genetic males or females [Senior et al., 2012, 2013]. As already reported in this review, numerous sex-reversed individuals have been generated for sex control or research purposes (table 1): neomales (mXX or mZW), neofemales (fXY or fZZ), but also YY males, YY females, and WW females have been demonstrated to be viable and functional in most species analyzed [Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Baroiller et al., 2009a, b; Cnaani and Levavi-Sivan, 2009; Haffray et al., 2009]. Moreover, recent meta-analysis concluded that (i) induced sex reversals by exogenous hormones/endocrine disrupting chemicals (EDCs) can negatively affect both the size and the gonadosomatic index (GSI) of the fish during treatment. However, at least size was rapidly restored after the end of the treatment [Senior et al., 2012; Senior and Nakagawa, 2013]. As suggested by the authors, these negative effects are associated to EDCs rather

than to sex reversal; size and gonads of EDC-treated genetic males and EDC sex-reversed males should be equally affected; (ii) following the functional masculinization of genetic females by EDCs/androgens, sperm traits (ejaculate volume, sperm motility, duration or linearity) are similar between genetic males and sex-reversed males [Senior et al., 2016]. Altogether, these studies suggest that sex-reversed males could efficiently compete with EDC non-reversed individuals. Similar results could probably be expected from temperature-induced males.

Nile tilapia has been found to adapt to extreme thermal conditions in the wild, including warm temperatures in hydrothermal springs with temperatures up to 40°C [Bezault et al., 2007]. Most of the other wild populations are found in tropical/subtropical regimes where the temperature fluctuates between 28 and 34°C. In this mouthbrooding species, after a first guarding phase, the fry swim out of the mother's mouth during the day to search for food and stay mostly in shallow waters where temperatures are frequently higher than 32–34°C [Bezault et al., 2007]. As demonstrated earlier in the Nile tilapia, XX fry can be strongly masculinized by high temperature treatments (>32°C) applied during at least 7 days from 10 dpf onwards [Baroiller et al., 1995, 2009b]. Interestingly, fluctuating temperatures that mimic natural thermal regimes (35°C during the day and 27°C during the night) can also masculinize the sensitive progenies of the blue tilapia [Baras et al., 2000]. Working on different wild populations including one (Metahara) living under high temperature regime (between 32 and 39°C), Bezault et al. [2007] found male breeders that sired all-female or highly skewed female (98%) offspring under controlled conditions. This strongly suggested that these individuals were XX males. Moreover, wild fry shoals were also collected in shallow waters, their genetic structure was characterized, and the phenotypic sex determined [Bezault et al., unpubl. data]. Amongst them, some contained 98% sibling female fry. This indicates that the father had to be an XX male. More recently, analysis of sex-linked molecular markers also suggested the presence of sex-reversed XX males in other wild populations (see Sex Reversal in the Wild). Taken together, these results strongly suggest that XX sex-reversed breeders exist in the wild where they reproduce and sire all or almost all-female offspring. Similarly, under controlled conditions androgen-induced XX males and XY or YY males of Nile tilapia were found to have quite similar male behavior and sperm quality [Gennotte et al., 2012]. Besides, our previous unpublished data revealed that under controlled conditions, progeny testing of 19 single hormonally-induced sex-reversed XX males sired progenies strongly skewed toward females but in some of them, unexpected XX males (1–25.5%) were identified. Interestingly, these unexpected F1 males when crossed with genetic XX females also sired progenies with strong female bias but again containing some unexpected males (2.5–9.2%). Similar results have been reported in the medaka by Nanda et al. [2003]. This suggests that in the Nile tilapia and the medaka (i) the sex determining factor is not obligatory for producing a male phenotype; and (ii) the possible existence of minor/cryptic sex-related genes unveiled under extreme genetic or environmental conditions. Finally, in the Nile tilapia thermosensitivity has been demonstrated to be a heritable trait [Wessels et al., 2007; Baroiller et al., 2009b].

Based upon the modelling studies [Kanaiwa and Harada, 2002; Hurley et al., 2004; Stelkens and Wedekind, 2010; Senior et al., 2012, 2013] and taking into account the biologic specificities of the Nile tilapia, what could happen when a Nile tilapia population is confronted to a rapid increase of the water temperature due to climate change? The first effect will be a masculinization because of its thermosensitivity, with sensitive progenies becoming partially masculinized. Based upon our previous studies, important variance of thermosensitivity (proportion of males following a 36°C treatment during the critical period) exists within (61.4–94% males) and between (61.4–80.6% males) wild populations. This will increase the male proportion but also favor the production of XX sex-reversed males. Around 6 months later, sexual maturity will occur and sex-reversed XX males will be able to sire only XX offspring. Because reproduction in tilapia is a continuous activity (as long as temperature is higher than 23–24°C), a dominant male can reproduce daily for 8–9 months per year. For each reproduction, on average a female will produce between a few hundred to 3,000 eggs. A single male can fertilize several females per day (3–5). Therefore, a single XX male can potentially sire more than 1,000 individuals/day. Based on the brooding behavior, survival rates of these fry will be very high. Part of these XX offspring will then be masculinized, and part will stay females. As suggested by the models, this could progressively increase the proportion of the XX genotype and decrease the proportion of XY individuals. In an extreme scenario, the XY genotype could even disappear from the population [Kanaiwa and Harada, 2002; Hurley et al., 2004]. In tilapia, population thermosensitivity can be characterized by 2 parameters: the percentage of thermosensitive families (Tf) and the proportion of males obtained following temperature-induced masculinization (M%). Our previous results [Bezault et al., 2007] reveal

that thermosensitivity was also present in the Metahara wild population living in a hydrothermal spring (32–39°C) but, however, XY males were still identified. This can first result from a rapid adaptation of the thermosensitivity. Indeed, the Metahara population had an important Tf but the M% was lower than in the other wild populations living in more standard thermal regimes. Moreover, in other wild populations, we also identified through progeny testing a XY female probably resulting from the presence of a minor factor rather than from an environmental effect [Bezault et al., 2007]. The reproduction of such females with classic XY males leads to an excess of males (75% males) composed of 2/3 XY and 1/3 YY males. Probably because of these 2 mechanisms, the Nile tilapia might be able to adapt to important temperature changes. Current studies will further verify these hypotheses.

Very recently Holleley et al. [2015] experimentally demonstrated that climate change can strongly and quickly impact the sex determining system (GSD-TSD transition and loss of the W chromosome) of the Australian dragon lizard: under high temperature regimes, a TSD system can be fixed in small populations where the heterogametic sex chromosome (W) could disappear through the reproduction of the sex-reversed homogametic individuals with non-reversed homogametic breeders (ZZf × ZZm) leading to the decline and progressive extinction of the heterogametic genotype (ZW). This confirms that the resilience of some wild populations facing a rapid climate change is of importance. However, a rapid TSD adaptation could lead to stable balanced sex ratios. Therefore, it will be of major importance to understand and predict the evolution of thermosensitivity and subsequent sex ratios at the population level after the establishment of TSD. Finally, a strong plasticity of sex determination systems could favor an adaptation to a rapid climate change [Holleley et al., 2015].

Conclusion and Future Perspectives

In fish, induced sex reversals have been obtained in many species, and occurrence of spontaneous sex-reversed individuals has been reported in both domestic strains and wild populations. Sex reversal in wild populations can result either from genetic (e.g., minor factors, mutations) and/or environmental (e.g., abiotic factors, xenobiotics) factors. All these reported cases of spontaneous or induced sex reversal underline once again the amazing plasticity of the mechanisms of sex determination in fish. They suggest that underlying sex-determining loci/sex determination systems can be masked by already identified major sex determining factors through dominant epistasis or/and sex determination hierarchies [Ser et al., 2010]. Strong environmental cues (even when they are outside of the natural range) can reveal such hidden genetic variations [Georges et al., 2010; Holleley et al., 2015].

Altogether, the relative importance of sex reversal events in fish suggests that it can play an important role in the evolution of sex determination through 2 possible mechanisms: (i) facilitating rapid transitions within and between sex determination systems, eventually leading to sex chromosome elimination [Holleley et al., 2015] and potential impacts on wild population dynamics and resilience; and (ii) purging deleterious mutations on the heterogametic sex chromosome (Y or W) through XY or ZW recombinations in sex-reversed females as proposed by the 'fountain of youth' hypothesis [Perrin, 2009]. This could explain the high proportion of homomorphic sex chromosomes in fish as well as in amphibians.

In order to better understand sex reversal in fish, analysis should not only be considered at the species level but also at the population level (at least for some species), in order to identify possible different sexual genotypes (resulting either from the possible existence of multiple sex-determining loci or from environmentally-induced sex reversal) associated to a single phenotype. Relative fitness of these different genotypes associated to a single phenotype will have to be considered in order to better understand the effects and possible role of sex reversal at the population level. Comparisons have to be done between treated individuals that have been sex reversed or not.

Genomic tools have provided sex-linked markers that are particularly useful to identify sex-reversed individuals in wild populations, the mismatch between the sexual phenotype and the genetic sex. This will allow estimating the occurrence of sex reversal within and between wild populations. The next step will be to determine which factor has induced the sex reversal process. If the proximate cause is not a minor factor or a mutation, it is still difficult to discriminate between xenobiotic effects and environmental factors such as temperature, pH, photoperiod, etc. Analysis of otolith isotopic ratios associated with gene expression studies and sex-linked markers could permit this discrimination. We have shown in the Nile tilapia that permanent exposure of juveniles to 27°C and 36°C can be detected a posteriori using such an otolith isotopic approach [Revessat et al., unpubl. data]. Similarly, transient and persistent effects on growth and gene expression have been shown in tilapia following fry exposure to environ-

mental factors that induced sex reversal [Shved et al., 2007). They can be used to reveal precocious exposure to environmental cues.

Because a photoperiod-dependent sex determination has been recently reported in a species that is also TSD, we believe that an important challenge will be to revisit the thermal sensitive species in order to determine if other factors can also influence the sex ratio of their progenies with probable synergic effects, leading to a reclassification of ESD species.

Major advances on mammal sex determination arose from pathological sex reversal cases [McElreavey and Fellous, 1999; Vaiman and Pailhoux, 2000; Camerino et al. 2006]. Therefore, we assume that studies on spontaneous (under wild or controlled conditions) and/or induced (through hormonal or environmental treatments) sex reversals in fish may provide new clues to better understand the amazing diversity and evolution of fish as well as vertebrate sex determination.

NGS advances associated with transcriptomic, miRNome and gonadal methylome analysis will probably allow the discovery of additional factors as well as their interactions with already identified sex-determining loci. Comparative studies should be performed on complementary models supposed to rely upon different types of sex determination and/or having different environmental sensitivities such as the medaka, the rainbow trout, the tilapias (both *O. niloticus* and *O. aureus*), the zebrafish (both domestic and wild strains), the pejerrey, and many other non-model species.

Gonochoristic fish constitute promising models to better understand variations within (population level) and between sex determination of species. As reported by Duffy et al. [2015], until now TSD variation among wild populations has been reported only for 3 teleost species, *M. menidia* [Conover and Heins, 1987], *M. peninsulae* [Yamahira and Conover, 2003], and Nile tilapia [Bezault et al. 2007]. Without doubt, these exciting challenges will strongly stimulate studies on ESR.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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