Sex Reversal in Amphibians

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Key Words

Amphibians · Sex differentiation · Steroids · Temperature

Abstract

Amphibians have been widely used to study developmental biology due to the fact that embryo development takes place independently of the maternal organism and that observations and experimental approaches are easy. Some amphibians like *Xenopus* became model organisms in this field. In the first part of this article, the differentiation of the gonads in amphibians and the mechanisms governing this process are reviewed. In the second part, the state of the art about sex reversal, which can be induced by steroid hormones in general and by temperature in some species, is presented. Also information about pollutants found in the environment that could interfere with the development of the amphibian reproductive apparatus or with their reproductive physiology is given. Such compounds could play a part in the amphibian decline, since in the wild, many amphibians are endangered species.

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Among tetrapod vertebrates, the class Amphibia includes 3 orders: frogs (Anura), salamanders (Caudata), and caecilians (Gymnophiona). Caecilians (~200 species), which resemble giant earthworms, are the smallest and the least studied group. The 2 other orders are more

numerous (~6,620 species of frogs and 680 species of salamanders) and have been studied more extensively (www.amphibiaweb.org). A recent study found that 32% of the amphibian species are threatened worldwide (<http://www.iucnredlist.org/initiatives/amphibians>). As many as 159 amphibian species may already be extinct, and at least 42% of all known species are declining in population.

Some species like *Salamandra salamandra* are viviparous, and the female releases the young larvae into water. *Eleutherodactylus jasper* is an ovoviviparous frog which retains developing embryos within eggs in the oviduct, giving birth to live froglets. Other species like the American salamander *Desmognathus aeneus* have direct development that does not contain a free-living larval stage. However, the majority of amphibian species are oviparous. Their development includes an embryonic period and a larval period that ends at metamorphosis and then juveniles still evolve up to adulthood. Since embryo development often takes place independently of the maternal organism and since observations and experimental approaches are easy, amphibians are very interesting species to study developmental biology, and some of them like *Xenopus laevis* became model organisms in this field [Tadjudje and Heasman, 2010]. Among developmental processes, gonad differentiation and sex reversal have been well studied in amphibians, but we have less knowledge about the molecular aspects than in other groups of vertebrates.

Gonad Formation

Germ Cell Specification

Sex differentiation is an important event during which the gonads differentiate either into an ovary or a testis. Two cell lines are required for that: the germ cells and the somatic cells, both of them originating from the same cell, the zygote. Two kinds of mechanisms govern germ cell specification. In anuran amphibians, germ cell specification is the result of maternal determinants that are already present in the oocyte before fertilization. In *X. laevis*, this consists of mRNAs such as *nanos 1* (formerly *Xcat2*) and *Xdazl*, which are associated with a structure called the mitochondrial cloud (containing not only unique mRNAs, but also proteins, granules, the endoplasmic reticulum, and mitochondria) that lies close to the nucleus in stage I oocytes and then migrates to the vegetal cortex of the oocyte at stage II of oogenesis [Kloc and Etkin, 2005]. The germ plasm will be partitioned asymmetrically during cleavage and will be concentrated in the cells located at the vegetal pole that become primordial germ cells (PGCs) and have an endodermal origin. This mechanism called preformation is also known in *Drosophila* or *Caenorhabditis* for instance [Seervai and Wessel, 2013].

In contrast, in urodele amphibians, primordial germ cells are induced from pluripotent cells by complex extracellular signals [Dumond et al., 2008a]. Primordial germ cells develop within the mesoderm, and classic embryology studies have reported their induction from the primitive ectoderm (animal cap). The signaling factors involved in this process are not well defined, but in the axolotl, Fgf and Bmp4 have been identified as major players [Chatfield et al., 2014]. This mechanism called epigenesis is also known in mammals where it is well described [Seervai and Wessel, 2013].

The Undifferentiated Gonad

In all cases, germ cells appear in an extragonadal region located in the caudal part of the embryo and have to migrate to reach the embryonic gonad [Terayama et al., 2013]. This migration follows the dorsal mesentery that links the intestine to the dorsal wall of the abdominal cavity. Migration takes place until the germ cells reach the genital ridges that are the earliest stage of the developing gonads. Primordial germ cells are easily detected among somatic cells due to their large size, their polylobulated nucleus, and the presence of yolk platelets in their cytoplasm, whereas somatic cells have already used them [Dournon et al., 1990a]. The genital ridges are symmetric masses on each side of the dorsal mesentery. They are sur-

rounded by the somatopleural epithelium and consist at the beginning of one germ cell and a small number of somatic cells derived from the neighboring mesonephritic blastema. During the later steps of embryo development, germ cells and somatic cells proliferate, leading to an increase in the size of the embryonic gonads. During a period of time, the gonads have the same appearance in both males and females and are named undifferentiated gonads. At this stage and in all cases, the germ cells are always located in the cortical part of the gonads, and there is no histological sign of differentiation.

Gonad Differentiation

When differentiation takes place in case of testis development, spermatogonia migrate from the cortex (which will be reduced and become the testis envelope albuginea) to the central medulla where they form cysts included in lobules that are the homologs of the testis cords observed in other species like mammals (fig. 1). In *Silurana* (*Xenopus*) *tropicalis*, testis differentiation seems to occur along an antero-posterior gradient [El Jamil et al., 2008]. Meiosis entry is not observed until after metamorphosis [Wallacides et al., 2009]. At this time, in a cyst, all germ cells enter meiosis and progress along the different phases simultaneously [Flament et al., 2009]. However, several cysts belonging to the same lobule do not show simultaneous meiotic entry of germ cells.

In case of ovary differentiation, oogonia stay in the cortex where they proliferate and some of them enter meiosis. In *Pleurodeles waltl*, retinoic acid has been shown to be involved in regulation of meiotic entry, with retinoic acid being able to trigger meiosis entry of male germ cells prior to metamorphosis [Wallacides et al., 2009]. Oocytes are arrested at the diplotene stage of prophase of the first meiotic division. Meiosis is reinitiated in adult females upon progesterone stimulation, with a second blockade occurring at metaphase II stage where fertilization can be performed. *X. laevis* is a very good model to study oocyte maturation and meiotic arrests that prepare the female gamete for fertilization [Flament et al., 1996; Bodart et al., 1999; Delobel et al. 2002]. The germ cells that do not enter meiosis during ovary differentiation can be easily recognized because of their polylobular nucleus which is very different from the round nucleus with condensed chromatin of oocytes I. In the differentiating ovary, the medulla is completely modified: several cavities occur and then merge, leading to a central cavity (fig. 1). Thus, in both anuran and urodele amphibians, the ovary is an ovisac [Dumond et al., 2008a; Piprek et al., 2012]. In *S. tropicalis*, ovary differentiation seems to occur along an

Fig. 1. Histology of the gonads and genital tract of *Pleurodeles waltl* after metamorphosis (stage 56). **a** The testis is made of a medulla containing spermatogonia (Sg) surrounded by somatic cells and is limited by a thin layer, the albuginea (Al), derived from the cortex. **b** The ovary contains mainly follicles made of prophase I-arrested oocytes (Oo) surrounded by follicular cells (FC). These follicles are located in the cortex surrounding the central ovarian cavity (OC) resulting from medulla regression. **c** Male genital tract showing a large Wolffian duct (WD) and a small Müllerian duct (MD) that will persist without differentiation in juveniles and adults. **d** Female genital tract with a Wolffian duct (WD) that persists since it is used for urine elimination, and a Müllerian duct (MD) that is already more developed than in males at this stage and will develop as an oviduct. Scale bars = 50 μ m in **a** and **b** and 100 μ m in **c** and **d**.

antero-posterior gradient [El Jamil et al., 2008]. The different steps of ovarian differentiation in anurans have been described in detail in a comparison of 12 species [Ogielska and Kotusz, 2004]. The authors distinguished 3 types of ovary differentiation rate: basic (most species), retarded (genus *Bufo*), and accelerated (green frogs). Moreover, regarding sexual differentiation, it is important to know that in amphibians there are 3 types of species [Chardard et al., 1996]. Indeed, in differentiated species like *Lithobates sylvaticus* (formerly *Rana sylvatica*), *L. catesbeianus* (formerly *R. catesbeiana*), or *P. waltl* the gonads differentiate directly in testes or ovaries according to the sexual genotype, and at the time of metamorphosis the sex ratio = 1. In semi-differentiated species like *Hynobius retardatus*, both females and intersex gonads are ob-

served at metamorphosis, but the latter evolve into testes after metamorphosis. In undifferentiated species like *R. temporaria*, the gonads of genetic males differentiate first as ovaries so that at metamorphosis, the progeny looks like an all-female progeny. Then a period of intersexuality (longer than in semi-differentiated species) is observed in male individuals before testis differentiation.

In amphibians, the differentiation of a fat body is associated with the differentiation of the gonads. It is located in the anterior part of the gonad in anurans, whereas it is situated in the lateral part of the gonad in urodeles.

It seems that in amphibians germ cells do not play a major part in gonad differentiation. Indeed, in case of germ cell ablation obtained after UV irradiation of an embryo at the 2-cell stage, no significant deviation of the sex ratio was observed in *R. japonica* [Shirane, 1982].

In *P. waltl*, germ cell ablation was obtained by treatment of tail-bud embryos with busulfan [Al-Asaad et al., 2012]. Once differentiated, gonads in both sexes were similar to control gonads.

Among amphibians, it is also interesting to describe the situation of the bufonids that are characterized by the presence of the Bidder's organ. This structure differentiates as a rudimentary ovary in the anterior part of the genital ridge. It appears in both sexes early in larval life, prior to gonad differentiation [Sassone et al., 2015]. The Bidder's organs are maintained in adults of both sexes in species like *Bufo bufo* and *Rhinella icterica*, whereas they disappear in adult females of species like *R. marina* and *R. arenarum*. In all bufonid males, the Bidder's organ is located in the anterior part of the testes and contains ovarian-like previtellogenic oocytes at different stages of development. However, in case of testes removal, the Bidder's organ develops into a functional ovary whose oocytes reach vitellogenic stages and complete maturation [Pancak-Roessler and Norris, 1991]. This indicates that during the differentiation process, the anterior part of the genital ridge is not influenced similarly to the posterior one.

Differentiation of the Genital Tract

Once differentiated, the gonads will secrete hormones that influence the differentiation of the genital tract. Like in amniotes, 2 kinds of genital ducts are formed during amphibian development: the Wolffian ducts and the Müllerian ducts [Gallien, 1973]. However, their evolution after the step of gonad differentiation is different from those observed in amniotes. The Wolffian ducts differentiate as uro-spermiducts in males (due to the absence of a mesonephros and secondary ureter in anamniotes) and

they do not disappear in females where they are uroducts (fig. 1). The Müllerian ducts differentiate as oviducts in females and disappear in males of anurans probably due to the action of the anti-Müllerian hormone (Amh). Indeed *amh* is expressed in several anuran species including *Glandirana* (formerly *Rana*) *rugosa* and *Silurana* (*Xenopus*) *tropicalis* [Kodama et al., 2015; Jansson et al., 2016]. The Amh protein has also been immunodetected in gonads of *X. laevis*, *Bombina bombina*, *B. viridis*, *Hyla arborea*, *R. arvalis*, and *R. temporaria* using a polyclonal antibody raised against the human protein [Piprek et al., 2013]. *amhr2* is also expressed in *S. tropicalis* [Jansson et al., 2016]. However, the induction of Müllerian duct regression by this hormone has not been demonstrated in amphibians yet. Müllerian ducts are maintained without differentiation in males of urodeles (fig. 1) [Dumond et al., 2008a]. The latter observation could suggest that Amh was not present in urodele amphibians. Nevertheless, evidence of Amh expression and synthesis has been shown in *P. waltl* [Al-Asaad et al., 2013]. This suggests that in urodeles, Amh could only play a role in gonad differentiation (probably as a negative regulator of germ cell proliferation like in teleost fishes) rather than in Müllerian duct regression [Pfennig et al., 2015]. The persistence of Müllerian ducts in males of *P. waltl* can be demonstrated by E2 treatment which triggers oviduct differentiation [Dumond et al., 2008a].

Genetic Sex Determination

Sex Chromosomes

What are the mechanisms governing sex determination and sex differentiation in amphibians? All species of amphibians studied to date display a genetic sex determination. Males can either be ZZ and produce only 1 type of gamete, being homogametic (females are ZW), or males can be heterogametic (XY). Male heterogamety seems more common, but phylogenetic analyses suggested that female heterogamety was the ancestral state with several independent evolutionary switches to male heterogamety [Hillis and Green, 1990; Schmid et al., 2012]. XX/XY and ZZ/ZW systems of sex chromosomes can be observed not only among closely related species but also within the same species. This is very well illustrated in one frog species, the Japanese wrinkled frog, *G. rugosa* [Uno et al., 2008]. The ancestral populations of this anuran are located in East Japan and West Japan and display a XX/XY system, whereas derived populations are found in Central Japan and display a ZZ/ZW system [Ogata et al.,

2008; Miura et al., 2016]. Very recently, Roco et al. [2015] provided evidence that in *S. tropicalis* at least 3 sex chromosomes exist, leading to YZ, YW, and ZZ males and ZW and WW females. The fact that 3 sex chromosomes coexist in the same population suggests a recent emergence of the third sex chromosome and an ongoing process of homologous transition [Schartl, 2015].

However, in striking contrast with birds and mammals, sex chromosomes are rarely differentiated in ectothermic vertebrates. Indeed, less than 4% of amphibian species (among 1,500 species studied) show morphologically distinct sex chromosomes [Schmid and Steinlein, 1991; Eggert, 2004; Schmid et al., 2012]. In *G. rugosa*, the XX/XY ancestral populations have homomorphic sex chromosomes, whereas the derived populations (ZW, neo-ZW, and XY) have morphologically heteromorphic sex chromosomes ($n = 7$) [Miura et al., 2016]. In the absence of morphological differences, identification of the heterogametic sex cannot be deduced from karyotype examination and often requires other approaches. In the urodeles *P. waltl* and *P. poireti*, the microscopic observation of lampbrush chromosomes from prophase I-arrested oocytes (diplotene stage) was used to identify ZZ/ZW sex chromosomes (bivalent IV) [Lacroix, 1968a, b, 1970; Penrad-Mobayed et al., 1998]. In *P. waltl*, the biochemical analysis of peptidase 1, a sex chromosome marker, also allowed the identification of the sexual genotype [Dournon et al., 1988]. The male heterogamety of the European tree frog *H. arborea* was revealed by the sex-specific pattern of a microsatellite-like marker at locus Ha5-22 [Berset-Brändli et al., 2006]. The gene was the homolog of *med15*, a key component of the Mediator Coactivator Complex [Niculita-Hirzel et al., 2008]. The cell membrane-associated HY antigen (or a cross-reactive antigen) that is present in the gonad of the heterogametic sex (XY or ZW) in all vertebrates studied so far was used to determine the heterogametic sex in several amphibian species including *X. laevis*, *B. bufo*, *Pyxicephalus adspersus*, *Pelophylax ridibundus* (formerly *Rana ridibunda*), *Pelodytes punctatus*, *P. waltl*, *Ambystoma mexicanum*, and *Triturus vulgaris* [Schmid and Steinlein, 2001; Eggert, 2004]. In *P. esculentus* (formerly *Rana esculenta*), the Y chromosome could be differentiated from the X by having a late replicating region in experiments using incorporation of the thymidine analogue bromodeoxyuridine [Schempp and Schmid, 1981]. The analysis of the progeny of sex-reversed individuals was also useful. For instance, in the American salamanders *A. mexicanum* and *A. tigrinum*, embryonic gonad transplantations induced female-to-male sex reversal that were used to demonstrate female

heterogamety (ZW) (25% of males in the progeny after crossing with a standard female) [Humphrey, 1942]. In the urodele *P. waltl* [Gallien, 1951] or the anuran *X. laevis* [Chang and Witschi, 1955], male-to-female sex reversal was obtained by treating larvae with estradiol. Half of the neofemales when crossed with normal males produced all-male progenies, demonstrating female heterogamety (ZW). Studies on the progeny of sex-reversed females have also shown male heterogamety (XY) in some anuran species [Schmid and Steinlein, 2001].

A Master Sex-Determining Gene

No sex-determining gene had been identified for a long time in amphibians. However, a first evidence of a sex-determining gene was reported in *X. laevis* [Yoshimoto et al., 2008]. In this species with a ZZ/ZW system of sex chromosomes, the authors have isolated a W-linked gene named *dm-W*. It was identified during a Southern blot analysis of *dmrt1*, a gene that is involved in testis differentiation in several vertebrate species and that was cloned previously in *X. laevis* [Osawa et al., 2005]. The *dmrt1* probe identified a female genome-specific band which was distinct from the bands corresponding to *dmrt1* [Yoshimoto et al., 2008]. FISH analysis for *dm-W* revealed that the W chromosome corresponds to chromosome 3, whereas 2 signals on the autosomes (chromosomes 1 and 2) exist for *dmrt1*. These signals correspond to *dmrt1α* and *dmrt1β*. Indeed, *X. laevis* is tetraploid, because the genus *Xenopus* underwent a whole genome duplication during evolution, and *dm-W* is a paralogue of *dmrt1*.

dm-W and *dmrt1* mRNAs are expressed exclusively in the primordial gonads of *X. laevis* during the sex determination period: *dm-W* only in ZW tadpoles but *dmrt1* in ZZ and ZW individuals. Besides, the expression of *dm-W* is transient, whereas expression of *dmrt1* is also observed after the sex determination period. An immunohistochemical analysis revealed that Dm-W and Dmrt1 colocalize to the somatic cells surrounding the primordial germ cells of the ZW gonad [Yoshimoto et al., 2010]. Some transgenic ZZ tadpoles carrying an expression plasmid for *dm-W* displayed ovarian cavities and primary oocytes at stage 56 [Yoshimoto et al., 2008]. In loss-of-function experiments using short hairpin knockdown vectors for *dm-W* it was observed that a few transgenic ZW individuals had gonads with testicular structures at stage 56 (27%) and 6 months after metamorphosis (5%) [Yoshimoto et al., 2010].

The N-terminal region of Dm-W (amino acids 1–123 encoded by exons 2 and 3) which contains the DNA-binding DM domain shows high sequence identity with

the corresponding regions of Dmrt1α and β. In contrast, their C-terminal regions have no significant similarities. In Dmrt1 this region contains a transactivation domain that is not found in Dm-W [Yoshimoto et al., 2006]. Thus, Dm-W could act as a competitor of Dmrt1 for its target genes in the primordial ZW gonads. Interestingly, during the early stages of sex determination, the *dm-W* mRNA level is higher than that of *dmrt1* in the ZW gonad. This was reinforced by electrophoretic mobility shift assays using the mammalian *dmrt1*-binding DNA cis element which showed that in vitro translated Dm-W and Dmrt1 proteins could interact with the same DNA sequence [Yoshimoto et al., 2010]. It was also shown that Dm-W could dose-dependently antagonize the transcriptional activity of Dmrt1 in human embryonic kidney cells [Yoshimoto et al., 2010]. This competition could arise from Dm-W/Dm-W homodimers or Dm-W/Dmrt1 heterodimers acting on cis elements of target genes. Thus, *dm-W* seems to act as an anti-testis gene [Yoshimoto and Ito, 2011] and allowed to identify the sexual genotype of *X. laevis* tadpoles and to examine the level of expression of several genes during and after sex determination [Okada et al., 2009].

dm-W orthologues have been isolated in various species of *Xenopus* [Bewick et al., 2011]. It seems that *dm-W* evolved in this genus after its divergence from the genus *Silurana* (which is diploid) but before the divergence of *X. laevis* and *X. clivii* (tetraploid). *dm-W* was also found in *X. itombwensis* and *X. vestitus* (octoploids).

Importance of Steroids

Steroid hormones are important players for sex differentiation in amphibians. This has been suspected from numerous experiments in which sex reversal occurred following addition of exogenous steroids to the rearing water or injection to tadpoles [for review, see Nakamura, 2010; Flament et al., 2011]. The sensitivity of several anuran species to testosterone and 17β-estradiol was studied recently [Piprek et al., 2012]. It was also shown that testosterone propionate and estradiol could alter sex ratios in the Indian skipper frog *Euphlyctis cyanophlyctis* [Phuge and Gramapurohit, 2015]. Urodele amphibians are also sensitive to exogenous steroids. For instance, *P. waltl* can be sex reversed by the use of estradiol or androgens [Gallien, 1951; Chardard et al., 2003]. In a recent study, it was reported that the ancestral forms of *G. rugosa* were highly sensitive to steroid-induced sex reversal, whereas the derived forms were resistant [Miura et al., 2016]. Nevertheless, the concentrations used in these various sex reversal experiments were probably not physio-

logical, since our knowledge about the timing and the level of steroid production in amphibian gonads during development is very poor. It is surprising that a single steroid can have opposite effects depending on the species (either masculinizing or feminizing) and that in the same species a steroid can have opposite effects according to the concentrations used. An exogenous androgen like testosterone, once entered into the male organism, can be transformed in estradiol due to its enzymatic aromatization that could explain the feminizing effect of this androgen [Kuntz et al., 2003a]. In *P. waltl*, parabiosis experiments were performed in order to study the effects of physiological concentrations of the sexual hormones [Dumond et al., 2008b]. However, in this experimental model, hormones other than steroids can also influence gonad development, and it is impossible to conclude about the real effects of steroids. Indeed, Amh that is produced in this species by the ZZ gonad probably inhibits female gonad development as in the case of freemartins in mammals [Capel and Coveney, 2004; Al-Asaad et al., 2013].

The study of key enzymes of steroidogenesis was more informative from a physiological point of view. This was performed either by the use of inhibitors or by the analysis of their expression and activity during gonad development.

Aromatase or Cyp19, the estrogen synthesizing enzyme, was studied in several species. In *L. catesbeianus*, female-to-male sex reversal was observed when female tadpoles were implanted intraperitoneally for 3 months with capsules of the aromatase inhibitor 4-hydroxyandrostenedione [Yu et al., 1993]. The same inhibitor induced sex reversal in *G. rugosa* [Ohtani et al., 2003]. Another inhibitor, fadrozole, induced masculinization of gonad tissues in *X. laevis* [Miyata and Kubo, 2000]. Fadrozole was also used in *S. tropicalis* [Duarte-Guterman et al., 2009; Olmstead et al., 2009]. In this species, when applied at 2 μ M from stage 12 to stage 60, 55% of the individuals differentiated as males, 15% were intersex having testicular oocytes, and 30% were females. In caudate amphibians, aromatase inhibitors were studied mainly in *P. waltl*. In this species, the treatment of ZW larvae with fadrozole led to their differentiation into male individuals at metamorphosis [Chardard and Dournon, 1999]. When some ZW individuals escaped the fadrozole effect, they showed a delayed increase in aromatase activity and differentiated as phenotypic females [Chardard and Dournon, 1999; Kuntz et al., 2003a].

In *P. waltl*, a low aromatase activity was detected in ZZ and ZW gonad-mesonephros-interrenal organ complexes from stage 47. The enzymatic activity increased in fe-

males from stage 52, and at metamorphosis it reached 500-fold the activity measured in males [Chardard et al., 1995]. In *G. rugosa* tadpoles at stage I (prior to gonad differentiation), the rate of conversion of testosterone to 17 β -estradiol was also higher (~6-fold) in the indifferent gonad of females compared with males [Isomura et al., 2011]. This 17 β -estradiol synthesis was inhibited by fadrozole.

cyp19 expression was also studied. In *P. waltl*, in situ hybridization (ISH) studies showed that aromatase mRNAs were not present in the mesonephros [Kuntz et al., 2003b]. At stage 54, a female-enriched expression of aromatase mRNAs was observed on histological sections. A whole-mount ISH study performed at this stage confirmed the higher aromatase expression in female gonads [Ko et al., 2008]. Real-time PCR analyses showed a 4-fold difference in mRNA level at stage 54 and a 50-fold difference 30 days later [Ko et al., 2008]. Thus, the increase in aromatase expression level seems to be involved in the changes in the activity of the enzyme. Similarly, a dimorphic expression of *cyp19* was observed in *H. retardatus* in which it occurred 15 days after hatching, i.e., prior to the morphological differentiation of the gonads [Sakata et al., 2005, 2006]. Such a dimorphic expression of aromatase has also been reported in several anuran species, including *G. rugosa*, *X. laevis*, and *P. nigromaculatus* [Kato et al., 2004; Sakata et al., 2006; Maruo et al., 2008; Mawaribuchi et al., 2014; Xu et al., 2015], and was used to predict the phenotypic sex in *L. sylvaticus* and *S. tropicalis* [Navarro-Martín et al., 2012]. In *X. laevis*, the dimorphic expression of *cyp19A1* occurred just after sex determination in several masses of cells, each surrounded by a basement membrane, that were aligned along the anteroposterior axis [Mawaribuchi et al., 2014]. During sex differentiation, ovarian cavities formed inside each mass of *cyp19A1*-positive cells in the ZW gonads, whereas the mass-in-line structure disappeared during testicular development in the ZZ gonads.

In male gonads, 17 α -hydroxylase (*cyp17*) also displays an early increased expression during sex differentiation. This has been reported in *G. rugosa* where it is expressed in the indifferent gonad of males [Iwade et al., 2008]. The Cyp17 protein is present in somatic cells of indifferent gonads, and the conversion of [3 H]progesterone into [3 H]androstenedione is higher in males than in females [Sakurai et al., 2008]. This has also been described in *X. laevis* where *cyp17A1* expression is 5–40-fold higher in ZZ than in ZW gonads during early sex differentiation (stage 50–62) [Mawaribuchi et al., 2014].

In *G. rugosa*, the gene encoding the androgen receptor (*Ar*) is located on both Z and W chromosomes [Yokoyama et al., 2009]. Its expression is upregulated in the male gonad and was proposed as a candidate gene for testis differentiation [Yokoyama et al., 2009]. A recent study has shown that *Ar* is involved in male sex determination in *G. rugosa* [Fujii et al., 2014]. Indeed, a subset of transgenic female (ZW) frogs carrying an exogenous Z-*Ar* driven by the promoter region of both Z-*Ar* and *Ef1 α* genes formed ovotestes. The expression of *cyp17* and *dmrt1* was upregulated in these ovotestes. Testosterone added to the rearing water completed the masculinization.

Temperature-Induced Sex Reversal

Since the genetic sex was unknown in most experiments in anura, sex reversal was deduced from biased sex ratios and from both macroscopical and microscopical examination of gonads around metamorphosis. Initial experiments performed on *R. temporaria* indicated that high temperatures ($\geq 25^{\circ}\text{C}$) could induce female-to-male sex reversal, whereas low temperatures ($\leq 12^{\circ}\text{C}$) could induce male-to-female sex reversal [Witschi, 1914; Piquet, 1930]. However, the sexual race of the populations used in these studies was unknown. The experiments performed in *L. sylvaticus*, which is a sexually differentiated species, were more convincing [Witschi, 1929a, b]. When tadpoles from this species were reared at 32°C for 15–33 days, 53.9% of the individuals were phenotypic males, while 46.1% were intersex individuals, displaying ovotestes at the end of the treatment. High temperature administered during larval life had also a masculinizing effect in other anuran species, including *R. japonica*, *L. catesbeianus*, and *B. bufo* [Chardard et al., 2004]. Low temperature ($\leq 12^{\circ}\text{C}$) was suggested to have feminizing effects in *B. bufo* [Piquet, 1930].

In caudate amphibians, the first study indicating a sex reversing effect of temperature was performed in *H. retardatus*, a sexually semi-differentiated species. High temperature was first reported to have masculinizing effects [Uchida, 1937a, b]. However, more recent studies demonstrated clearly that high temperature (28°C) induced a male-to-female sex reversal [Sakata et al., 2005, 2006]. Temperatures from 20 to 26°C did not modify the sex ratio, and larvae reared at temperatures higher than 28°C could not survive [Sakata et al., 2006]. Sexually differentiated species were also studied: *P. waltil* and *P. poireti* [Dournon and Houillon, 1984, 1985; Dournon et al., 1990b], and 2 subspecies of *T. cristatus* (*T. c. cristatus* and *T. c. carnifex*) [Wallace et al., 1999; Wallace and Wallace, 2000]. Sex reversal could be ascertained since the genetic

sex of all or some individuals could be identified (peptidase-1 analysis in *P. waltil* and C-banded chromosome analysis in *T. cristatus*).

In *P. waltil*, high temperature (32°C) induced a female-to-male sex reversal [Dournon and Houillon, 1985; Chardard et al., 2004]. In *P. poireti*, high temperature has a feminizing effect: when larvae from a standard progeny were reared at 30°C from stage 42 to 54, some ZZ genotypic males were sex reversed: 65.7% females, 22.9% males, and 11.4% intersexes were observed at metamorphosis [Dournon and Houillon, 1984; Dournon et al., 1990b; Dorazi et al., 1995]. In *T. c. cristatus* and *T. c. carnifex*, which display XX/XY sex chromosomes, high temperatures have masculinizing effects whereas low temperatures induce feminization [Wallace and Wallace, 2000]. For instance, in *T. c. cristatus*, male biased sex ratios (61%) were observed when larvae were reared at 28°C and some individuals were diagnosed as XX neomales. Trials at and below 16°C resulted in a significant excess of females including XY neofemales.

The sex-reversing effect of temperature can be observed only if the modified temperature is applied during a limited window of larvae development called the thermosensitive period (TSP). The TSP is defined by the 2 stages of development between which a thermal (cold or heat) treatment can induce male or female sex reversal. If thermal treatment is performed before or after this period, it does not modify the sex ratio or only with a low efficiency. For instance, in *P. waltil*, the TSP extends between stages 43 and 54 and is approximately 2 months long [Dournon and Houillon, 1985]. Indeed, 100% of ZW larvae (genotypic females) were sex reversed when reared at 32°C between stages 43 and 54. When heat treatment (32°C) began after stage 42 or was stopped before stage 54, it did not yield 100% sex reversal. A temperature of 30°C applied during the whole TSP was less efficient than 32°C , since it did not induce sex reversal of all ZW larvae. In *T. c. carnifex*, low temperature (13°C) induced male-to-female sex reversal (78%) when applied from the uncleaved egg to metamorphosis (9–10 months duration), whereas no sex reversal could be detected when cold treatment was limited to feeding stages [Wallace et al., 1999]. However, male biased sex ratios were observed in case of thermal treatment starting at the ‘feeding larvae’ stage (67, 74, and 67%, respectively, at temperatures of 28, 30, and 31°C), and XX neomales were diagnosed in the 30°C trial. Here, the duration of heat treatment was only 3 months (at 28°C), suggesting that the TSP in *T. c. cristatus* was similar to that in *P. waltil* [Wallace and Wallace, 2000]. In *H. retardatus*, it

was shown that the TSP extends from 15 to 30 days after hatching [Sakata et al., 2006].

The molecular target of temperature leading to sex reversal is not yet identified. In *P. waltl*, estrogen treatment counteracts the masculinizing effect of temperature [Zaborski, 1986]. In this species, during temperature-induced female-to-male sex reversal, aromatase activity in gonads of ZW larvae remains at low levels similar to those observed in ZZ larvae [Chardard et al., 1995]. This cannot be due to a direct inhibition of the enzyme, since aromatase activity measurements are performed at 37°C, but it could be due to a repression of *cyp19* expression, since its mRNA levels do not increase in case of sex reversing heat treatment of ZW larvae [Kuntz et al., 2003b]. Similarly, in *H. retardatus*, when larvae were reared at female producing temperature, during the TSP the expression of aromatase increased [Sakata et al., 2005, 2006].

However, this effect of temperature on aromatase expression is probably indirect, and to better understand the sex reversal process, it is important to elucidate the regulation of aromatase expression in normal gonad differentiation and sex reversal. Among others, steroidogenic factor 1 (Sf1) has been studied. In *G. rugosa*, Sf1 binding sites were found in the promoter region of ovarian *cyp19*, but Sf1 did not activate *cyp19* expression in luciferase assays performed in HEK293 cells [Oshima et al., 2006]. In *P. waltl*, a female enriched expression of *sf1* was observed only at stage 56, i.e., after the elevation of aromatase expression and gonad differentiation [Kuntz et al., 2006]. Sox3 binding sites were also found in the promoter region of ovarian *cyp19* in *G. rugosa* [Oshima et al., 2009]. *sox3* is located on the Z and W chromosomes and directly up-regulates aromatase expression [Uno et al., 2008; Oshima et al., 2009]. Foxl2, a putative winged helix/forkhead transcription factor, is another potential regulator of aromatase expression as initially shown in mammals [Pannetier et al., 2006]. In *G. rugosa*, Foxl2 binding sites were also found in the promoter region of the *cyp19* gene [Oshima et al., 2009]. In this species, *foxl2* expression was upregulated in the gonads of female tadpoles before their differentiation, and Foxl2 could stimulate *cyp19* expression in luciferase assays performed in A6 cells [Oshima et al., 2008].

In *X. laevis*, real-time RT-PCR analysis also revealed that the gonadal expression levels of *foxl2* and *cyp19* were higher in ZW than in ZZ gonads during sex determination (stage 50) and later during sex differentiation (stage 53 and 57). In gonads of *dm-W* transgenic ZZ tadpoles, the pattern of expression of *foxl2* and *cyp19* displayed a ZW type [Okada et al., 2009]. In sex-reversed gonads of

ZW tadpoles carrying a knockdown vector for *dm-W*, *cyp19* and *sox3* displayed a ZZ pattern of expression, whereas *dmrt1* and *sox9* were upregulated [Yoshimoto et al., 2010].

Sex Reversal and Pollutants

Since many amphibian species are dependent upon an aquatic environment especially for reproduction and larval development, these animals are very sensitive to various pollutants. For instance, pollutants like heavy metals can influence amphibian reproduction and development at different steps. This is well illustrated by cadmium which inhibits metamorphosis of the urodele *P. waltl* and decreases fertilization rate and embryo cleavage in the anuran *X. laevis* [Flament et al., 2003; Slaby et al., 2016]. Cadmium and other metal chlorides can also inhibit progesterone-induced oocyte maturation in *X. laevis* [Marin et al., 2015]. Since steroids are important for gonad differentiation, amphibians will be also sensitive to pollutants acting as endocrine disruptors. For example, ethynylestradiol (EE₂) and bisphenol A (BPA) have been demonstrated to exert feminizing effects in different amphibian species [for review, see Bhandari et al., 2015]. EE₂, a component of contraceptive pills, is one of the most potent estrogenic compounds identified in the aquatic environment [Johnson et al., 2000]. In *R. temporaria*, 60 pM EE₂ induced a female-biased sex ratio [Pettersson et al., 2006]. In *S. tropicalis*, ZZ larvae exposed to EE₂ displayed partial or total male-to-female sex reversal [Hirakawa et al., 2013]. Indeed, ZZ ovaries were observed at 10 ng/l (2/15 individuals) and at 30 ng/l (7/11 individuals). This compound also induced a complete feminization in wood frogs (*L. sylvaticus*) with an EC₅₀ of 26 nM [Tompsett et al., 2013]. In addition to induce sex reversal, at high doses this compound can strongly interfere with the development of the genital tract. Indeed, a high percentage of female individuals of *S. tropicalis* lacked oviducts when they had been exposed to high doses during larval life [Pettersson et al., 2006; Gyllenhammar et al., 2009]. Even if such concentrations are not reached in the wild, the presence of different estrogeno-mimetic pollutants could have cumulative effects leading to such abnormalities. Other pollutants can have masculinizing effects. This is, for example, the case of trenbolone, an androgenic anabolic steroid used as a growth promoter in animal agriculture, particularly in cattle. This substance and its metabolites are widespread in surface water. Exposure to 17β-trenbolone (between 78 and 100 ng/l) during lar-

val life could induce male-biased sex ratios at metamorphosis in *X. laevis* [Olmstead et al., 2012]. This compound also induced female-to-male sex reversal at concentrations as low as 100 ng/l in *P. nigromaculatus* [Li et al., 2015]. Pollutants can also influence the genital tract. For instance, in case of exposure during larval life, EE₂ induced hypertrophy of the Müllerian ducts in *P. waltl* males, and 17 β -trenbolone induced Wolffian ducts hypertrophy in *X. laevis* [Flament et al., 2011; Olmstead et al., 2012]. Taken together, these different actions of various pollutants could account for the amphibian decline.

Conclusion

Sex determination in amphibians is rather complex with various systems of sex chromosomes that are not often differentiated. Our understanding of the molecular events considerably increased since the year 2000. A major finding was the discovery of *dm-W* which acts as an anti-testis sex-determining gene in a few anuran species. The search for *dm-W* paralogs in other species displaying female heterogamety has to be performed. Up to now, no sex-determining gene has been found in species displaying male heterogamety. In all cases, steroids still appear as major players of the sex determination/differentiation process. Nevertheless, we still have to determine if differences in the production of these hormones is a very early event or if it simply reflects a differentiation that started

earlier at the molecular level. It will be also important to study steroid production in larvae during the sex differentiation process. In some species, temperature can counteract the genetic determination process if temperature changes are applied during the TSP. Several studies indicate that steroid production is probably affected in case of temperature modification. This is clearly illustrated by changes in aromatase expression (which increases in case of a feminizing action of temperature and decreases in case of a masculinizing action). However, the exact target of temperature is still unknown. Besides, the existence of temperature-induced sex reversal in wild populations is difficult to appreciate. Finally, it is important to unravel sex determination/differentiation in amphibians in order to better understand the effects that pollutants could have on this process and in what extent this could account for the observed amphibian decline.

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