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Amphibian sex determination and sex reversal

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Abstract. Amphibians employ a genetic mechanism of sex determination, according to all available information on sex chromosomes or breeding tests. Sex reversal allows breeding tests to establish which sex is heterogametic and provides an indication of the mechanism of sex determination. Cases of spontaneous and experimental sex reversal (by temperature, hormones or surgery) are reviewed and illustrated by previously unpublished studies on crested newts. These newts respond conventionally to temperature and hormone treatment but provide anomalous results from breeding tests. It is suggested that both the evolution from temperature dependency to a genetic switch and from ZZ/ZW to XX/XY are superimposed on a generally uniform mechanism of sex determination in all vertebrates.

Key words. Sex determination; sex reversal; temperature dependence; sex hormone; amphibia; *Triturus cristatus*.

Genetic sex determination

All amphibians that have been tested possess a genetic mechanism of sex determination. Examples of male heterogamety (XX/XY) or female heterogamety (ZZ/ZW) have been found repeatedly in both anurans and urodeles. Sex chromosomes have been identified in almost 50 species [1], and breeding tests have established the heterogametic sex in a dozen or so other species where chromosomal differences are inconspicuous or absent. There are three main categories of breeding test employed for this purpose. Most simply, the inheritance of sex-linked markers can be recorded from laboratory or even natural spawnings. Isozyme markers have been used in this way to deduce the XX/XY system in *Rana clamitans* [2], or to confirm the ZZ/ZW status of *Pleurodeles waltl* and that *Triturus alpestris* and *T. vulgaris* have XX/XY sex chromosomes (reviewed in [3]). Second, breeding from sex-reversed specimens, i.e. genetically female (XX or ZW) neomales or genetically male (XY or ZZ) neofemales, has been employed to deduce which sex is heterogametic from the distorted sex ratio of their progeny (fig. 1). All known cases of this procedure are considered here because they both demonstrate the type of genetic system and offer some insight into its mechanism, by showing how it can be overridden by environmental or hormonal influences. Third, using irradiated sperm to activate eggs which are then heat-shocked to arrest the second meiotic division and thus restore diploidy, which is equivalent to self-fertilization of a female or breeding from a neomale (fig. 1c or d), has been practised on several anurans. Such gynogenetic breeding resulted in virtually all-female progeny and thus an XX/XY system in five species of *Rana*, *Hyla japonica* and *Bombina orientalis* [4], but on average only 80% females in *Rana pipiens* [5].

Spontaneous sex reversal

The earliest evidence for an XX/XY mechanism of sex determination in *Rana* came from natural examples of intersexes or hermaphrodites. One such specimen of *R. temporaria*, obtained while acting as a male in amplexus, sired 774 offspring which were all female [6] and thus must have been an XX specimen (fig. 1c). Witschi [7] reported several other examples, complicated by temperature effects and the various races of *R. temporaria*. Artificial fertilizations using hermaphrodites as either parent (and occasionally for self-fertilization) generally fitted the conclusion that all adult females and hermaphrodites are XX, while most adult males are

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XY. That explanation probably also applies to the hybrid races known as *R. esculenta*, where most males are sterile but some can sire either all-female or all-male offspring [8], and hence to the parent species *R. ridibunda* and *R. lessonae*. Spontaneous XY neofemales have been reported in *Hyla japonica* [4].

Temperature sex reversal

Few enviromental influences on amphibian sex determination have been demonstrated, or indeed searched for in a systematic way. Most notably, Dournon and Houillon [9, 10] produced ZW neomales of *Pleurodeles waltl* by rearing larvae at high temperature, 30–31 °C. Breeding these specimens with normal ZW females produced offspring at the expected 3:1 ratio of females to males, including the novel WW genotype females (fig. 1d). The closely related species *P. poireti* is feminized by this treatment, albeit less efficiently [11]. The sex chromosomes of both species have only been distinguished by a group of lampbrush loops which are heteromorphic in normal ZW oocytes but not in the oocytes of ZZ neofemales [11, 12].

Several other amphibians are reported to be masculinized by high temperatures or feminized by low temperatures during larval life, but only according to the distorted sex ratios obtained at or soon after metamorphosis [4, 13]. Diagnosis of sex then is based either on sections of the gonad or on its gross anatomy under a dissecting microscope. The testis has expanded posteriorly into a pear-shaped opaque organ, whereas the ovary has formed a translucent tube in which oocytes are usually visible. The absence of breeding tests leaves room for doubt about such cases, particularly those of 'undifferentiated races' where all individuals possess ovaries at metamorphosis, but half of them normally convert the ovary into a testis later. Conversion to the definitive sex is apparently irreversible, so that young hermaphrodites can be considered as immature males, and conversion seems to be delayed even more than metamorphosis at low temperatures. On that assumption, high temperatures masculinize *Rana sylvatica* [14], *R. temporaria* and *Bufo vulgaris* [15], and the urodele *Hynobius retardus* [16]. Very low temperatures probably feminize *B. vulgaris* and some races of *R. temporaria*. Since temperature-dependent sex determination seems to be a widespread phenomenon in cold-blooded vertebrates [13], it is probably the method of choice to obtain fertile sex-reversed specimens.

We have subjected *Triturus cristatus* to a range of temperatures during larval life, mainly to see if this urodele with recognizable X and Y chromosomes [17] responds in the same manner as *Pleurodeles*. Larvae of *T. c. carnifex* reared in the range 16–26 °C routinely show a high survival rate and a 1:1 sex ratio at metamorphosis, when sex can be distinguished by the appearance of the gonads. An increased mortality outside this temperature range, associated with fungal infection at low temperature or decomposition of food at high temperature, often makes the sex ratio less reliable. Table 1 shows data derived from the highest survival trials, ignoring the characteristic 50% embryonic mortality which results from an autosomal balanced lethal system and which is not influenced by temperature [18]. Uncleaved eggs of *T. c. carnifex* kept at 20 °C from collection developed into feeding larvae in 3–4 weeks and mostly reached metamorphosis 3 months later. The duration of larval life was clearly dependent on temperature in the range 12–26 °C, which did not affect the sex ratio. The highest temperatures, however, reduced larval growth and delayed metamorphosis.

MATING	PARENTS	SEX RATIO	PARENTS	SEX RATIO
Normal	(a) XX x XY	1 : 1	(b) ZW x ZZ	1 : 1
Neomale	(c) XX x <u>XX</u>	all female	(d) ZW x <u>ZW</u>	3 : 1
Neofemale	(e) <u>XY</u> x XY	1 : 3	(f) <u>ZZ</u> x ZZ	all male

Figure 1. The Mendelian expectation for the sex ratio (female:male) of progeny from mating normal specimens or sex-reversed ones (underlined) depends on which sex is heterogametic. Mating (d) generates WW females and mating (e) generates YY males, so the ratios could be sensitive to the viability of these novel genotypes.

Table 1. Sex ratio of *T. c. carnifex* after temperature treatments from feeding larvae with three fingers or from uncleaved eggs to early metamorphosis.

Conditions	Female	Male	Unknown	% female
From feeding larva				
31 °C for 3–4 months	15	31	65	33*
30 °C for 3–4 months	10	29	7	26*
28 °C for 3 months	14	29	5	33*
26 °C for 2–3 months	44	36	33	55
24 °C for 2–3 months	80	72	10	53
20 °C for 3 months	110	106	26	51
16 °C for 6 months	86	88	15	49
14 °C for 7 months	32	38	18	46
12 °C for 8 months	11	9	32	55
From uncleaved egg				
14 °C for 6 weeks	22	20	4	52
14 °C for 6 months	17	17	14	50
13 °C for 3–6 months	39	52	26	43
13 °C for 9–10 months	32	9	30	78*

Numbers of specimens of each sex and of unknown sex (casualties with undifferentiated gonads) are shown, underlined when they include specimens diagnosed as neomales or neofemales. This diagnosis depends on identification of the sex chromosomes in tailtip biopsies from a sample of larvae, so the frequency of sex reversal is uncertain. The sex ratio (% female) ignores specimens of unknown sex, and significant departures from 50% are marked *.

Heat treatment

The sex ratio showed an excess of males in the high-temperature trials where enough specimens survived long enough for sex to be diagnosed (table 1). A sample of XX larvae from the 30 °C trial shown in table 1 was identified by larval tailtip biopsies and reared to maturity, when two of them showed typically male secondary sexual characteristics (a crest, tail-flash and swollen cloaca) for two breeding seasons. Each of these neomales courted a series of five females sufficiently for them to lay eggs which did not develop. Since all these females proved to be highly fertile when mated subsequently with standard males, this breeding failure suggested the neomales were sterile. Autopsy during the second breeding season confirmed that suspicion. Both neomales had Müllerian ducts stretching the full length of the body cavity, but no residual ovary. They had miniature testes consisting of 1–4 lobes completely embedded in a fat-body, and no obvious Wolffian duct. Sections of the testes revealed the presence of premeiotic spermatogonia, in contrast to the mature sperm which fill normal testes at that season, and spaces presumably left by degenerated spermatocytes. Females obtained after the same temperature regime were fully fertile. The simplest interpretation of these results is that a fertility factor on the Y chromosome of this species is required for entry into the meiotic stages of spermatogenesis.

Cold treatment

Larvae of *T. c. carnifex* did not survive well at 12–14 °C, and the sex ratios at metamorphosis were too erratic to allow a firm conclusion; but sex determination is probably not affected by cold treatment limited to feeding stages. More prolonged cold treatment from uncleaved eggs to early metamorphosis, however, resulted in a significant excess of females (table 1). XY neofemales isolated from the 13 °C trial shown in table 1 have not yet reached maturity, but show no sign of reverting to their genetic sex.

Several trials of *T. c. cristatus* larvae have produced similar results. One trial at 28 °C yielded an excess of males including XX neomales. Cold treatments from 13–16 °C have consistently caused feminization and yielded XY neofemales, even when the trial began on feeding larvae rather than the unfertilized eggs. This temperature effect probably explains the distorted sex ratios for *T. c. cristatus* recorded previously [19] but has not been pursued, because *T. c. cristatus* is reportedly difficult to breed in captivity [20].

Hormonal sex reversal

Tests of hormonal influences on sex determination have usually involved testosterone and estradiol, either injected in a vegetable oil or dissolved in the water in which larvae are reared. Gallien [21] has reviewed the perplexing array of results obtained from such treatment, which include orthodox responses, paradoxical responses (contrary to expectation) and a few completely refractory species. In general, Ranid and Hylid tadpoles are masculinized by testosterone and by high doses of estradiol, which at low doses sometimes causes a slight or temporary feminization. Most other anurans and all urodeles that have been tested are feminized to some extent by estradiol but are not masculinized by testosterone. Testosterone usually represses the development of gonads in this group, sometimes to such an extent that paradoxical feminization occurs by the recovery of an ovary after the end of the treatment. Relatively few of the apparent sex reversals caused by hormones are known to be permanent, as sex was often diagnosed by autopsy at around the time of metamorphosis. Chang [22] recorded a good example of temporary sex reversal in *Bufo americanus*, where estradiol-treated larvae all appeared to be female at metamorphosis but many of them subsequently reverted to males or intersexes. One common side effect of these steroid hormones is a suppression of oviduct growth, resulting in sterility of feminized specimens. Consequently, there are only a handful of cases where breeding tests have demonstrated the success of the procedure and which sex is heterogametic.

Xenopus laevis tadpoles kept in 50 μg/l of estradiol from feeding to metamorphosis all become female, although some have incomplete oviducts. Breeding such females to normal males resulted in two types of spawnings: those from four genetic females showed both sexes in the progeny, and those from three sex-reversed neofemales had 100% male progeny [23], demonstrating that the male is the homogametic sex in a ZZ/ZW system (fig. 1f). Further tests using the unisexual ZZ progeny from these matings showed the period of sensitivity to estradiol is restricted to about 1 week in the middle of larval life, about 3 weeks after the tadpoles begin to feed, and that subsequent treatment with testosterone prevented the feminization [24]. However, testosterone does not masculinize ZW tadpoles. Gallien [25] provided an independent confirmation of the feminizing influence of estradiol and of the ZZ/ZW system in *Xenopus*.

Pleurodeles waltl larvae kept in 50–100 μg/l of estradiol for at least 4 weeks from the stage with four fingers to metamorphosis almost all become females or intersexes, although only a minority have at least one complete oviduct. Breeding 22 such females to normal males, Gallien [26] obtained six cases where all the offspring were male, demonstrating that *Pleurodeles waltl* has a ZZ/ZW genetic system (fig. 1f). Testosterone has no masculinizing effect on this species, but treatments exceeding 5 μg/l cause paradoxical feminization or intersexes without oviducts. *P. poireti* responds in the same way to estradiol and also has a ZZ/ZW genetic system, according to both lampbrush chromosome markers and breeding tests [11, 27].

Several species of *Ambystoma* have been subjected to androgens or estrogens throughout larval life. *A. opacum*, *tigrinum and mexicanum* all respond in much the same way as *Pleurodeles*, being feminized by estradiol and by testosterone as well as usually lacking complete oviducts [28–30]. Fertile females have only been reported for *A. mexicanum* after larval growth in 100 μg/l of estradiol. Some of these were sex-reversed specimens which produced all-male offspring when mated with normal males, confirming the known ZZ/ZW genetic system of axolotls [31].

The consensus reached from these and similar cases of hormone treatment was expressed most clearly by Witschi et al. [32]. Species with heterogametic males (XY), i.e. Ranid and Hylid frogs, are masculinized by testosterone or by high doses of estrogens, but some species may be feminized by low doses of estrogens. All other tested amphibians were believed to have heterogametic females (ZW), e.g. *Bufo*, *Xenopus* and all urodeles, and these are feminized by estrogens but not masculinized by testosterone, which causes paradoxical feminization in some species but not others. Gallien [21] generally supported this view, but noted that *Bombina variegata* seemed impervious to either hormone.

Later results have destroyed the validity of this consensus, particularly the belief that only the homogametic sex could be sex-reversed by hormone treatment. Consider the results from five anuran species, each of which has been shown to possess an XX/XY genetic system by gynogenetic tests [4]. *Bombina orientalis* is refractory to both estradiol and testosterone, supporting Gallien's conclusions for *B. variegata*. *Rana nigromaculata* and *R. japonica* conform to what we might call 'Witchi's rule': they can be masculinized by testosterone but are unaffected by estradiol. Neomales of both species sire almost entirely female progeny when mated with normal females, confirming the XX/XY system found by gynogenesis (fig. 1c). In contrast, *Hyla japonica* tadpoles do not respond to testosterone but can be feminized by estradiol. Furthermore, feminized XY specimens and albino YY males which have all-male offspring occur naturally [4]. *Rana pipiens* tadpoles can be masculinized by testosterone and feminized by estradiol, but the progeny of neomales or from gynogenetic breeding which should all be XX females usually included a considerable proportion of males, averaging 22% [5]. These were XX males, however, apparently arising by spontaneous sex reversal. At least two other species of *Rana* show the same orthodox response to both hormones as *R. pipiens*. Both *R. catesbiana* [33, 34] and *R. esculenta* [35, 36] can be masculinized or feminized according to the sex ratio found at metamorphosis after hormone treatment, although no breeding tests were performed with sex-reversed specimens. *R. clamitans* larvae are also masculinized by testosterone injections but do not respond to estradiol [37].

The most bizarre case concerns a racophorid frog, *Buergeria buergeri*, which is known to possess a ZZ/ZW system marked by a nucleolar organizer on the Z chromosome. ZW tadpoles selected by nucleolar number are masculinized at a low frequency by immersion in estradiol or by an injection of testosterone [38]. ZZ tadpoles are not affected appreciably by either hormone. Artificial fertilization using the testes of ZW neomales shows that W sperm are fertile, but the WW genotype results in a lethal embryo without nucleoli [39].

The other major fault in Witschi's rule stems from the assumption that all urodeles had heterogametic females (ZZ/ZW), whereas later investigations have shown that the majority of species in three families possess distinguishable X and Y chromosomes [1]. This applies to several members of the genus *Triturus* [17, 40] where sex reversal has been achieved to some extent. Collenot [41] feminized *T. helveticus* and, less completely, *T. alpestris* by rearing larvae in 50–600 μg/l of estradiol, although breeding tests proved impossible or unsuccessful. Pisano [42] suggested that 5–10 μg/l of testosterone partially masculinized *T. cristatus* larvae but found higher doses were toxic. Chieffi [43] could not confirm that result,

finding no sign of masculinization at 20–500 μg/l of testosterone, but recorded 100% females after growing larvae in 100–200 μg/l of estradiol. Thus XY specimens of all three species respond to these hormones in much the same way as ZZ specimens of *Pleurodeles* and *Ambystoma*.

We have confirmed the feminizing effect of estradiol on *T. c. carnifex* larvae and taken advantage of the identifiable Y chromosome to isolate XY females, in order to characterize their lampbrush chromosomes [44]. The mildest effective treatment found was exposure to 25 μg/l of estradiol for 8–9 weeks, from early feeding larvae with three fingers to beyond the stage when all five toes were present. Nine neofemales from this experiment were reared to maturity and used for attempted matings over two seasons. Most failed to spawn or spawned with difficulty, producing clusters of predominantly damaged or unfertilized eggs. Only one spawning provided enough larvae to test the expected sex ratio, after rearing under standard conditions at 18–20 °C. The expectations with both parents of XY genotype are either a 1:3 ratio including novel YY males (fig. 1e) or a 1:2 ratio if YY is lethal. Neither expectation could be verified, as 24 females and 15 males were scored at metamorphosis and no YY specimen was found among 26 tailtip biopsies, leading to the conclusion that some form of meiotic drive prevents transmission of the Y chromosome into mature eggs. That implies nonrandom segregation of the Y chromosome into a polar body, since the oocytes from this neofemale carried a normal complement of lampbrush chromosomes [44]. Equally unexpectedly, all of the larvae examined cytologically carried a maternally derived copy of chromosome 1A described in [18]. Chromosome 1 is a heteromorphic autosome with a variable C-banding pattern, maintained by a balanced lethal system so that only 1A/1B heterozygotes survive beyond the tailbud stage of development. Previous studies on the inheritance of chromosome 1, including the parentage of this neofemale, have shown a perfectly normal segregation. Consequently, only half of the progeny from the mating were expected to carry this variant of 1A. The results suggest that XY oocytes undergo directed maturation divisions to eliminate both Y and 1B, which were both paternally derived in this neofemale.

The perplexing variety of hormonal effects described here and summarized in table 2 might be interpreted by assuming that amphibians are capable of converting excessive amounts of an administered hormone into another one more suitable for their needs. The conversion of testosterone to estradiol by aromatase has been reported in a wide variety of vertebrates, and in principle it should be a reversible reaction. Aromatase activity has been related to sex determination and masculinization at high temperature in *Pleurodeles waltl* [45]. Steroid hormones are not the basic mechanism of sex determination but are necessary components of the differentiation pathways, as they can reinforce or reverse the initial determination, in addition to coordinating secondary sexual characteristics to the primary sex of the gonad. Additionally, it is almost certain that some unidentified hormonal influence is operating early in the process of sex determination, as deduced from the following cases of surgery and embryonic grafts.

Surgical sex reversal

Bufonid toads resemble undifferentiated races of *Rana* in that all tadpoles develop ovaries which become compressed into Bidder's organ in front of the definitive gonad (ovary or testis) at metamorphosis. Ponse [46] found that castrated adult *Bufo vulgaris* of either sex can convert Bidder's organ into a functional ovary and thus become a fertile female. In the case of castrated males, which presumably change sex in the absence of the normal androgen, repeated matings of the neofemales with normal males over many years allowed Ponse to accumulate 1080 offspring which were all male. In the case of castrated females with regenerated ovaries, four matings to normal males produced 102 female and 106 male offspring—a nearly perfect 1:1 sex ratio. The homogametic sex of these toads must therefore be male, a ZZ/ZW genetic system (fig. 1f).

Grafting a gonad or its embryonic primordium into a host of the opposite genotypic sex has been accomplished in *Ambystoma*, *Pleurodeles* and *Xenopus*. Humphrey pioneered embryonic grafts of the gonad territory, using *A. tigrinum*, *A. punctatum* and colour

Table 2. Summary hormonal sex reversal from references in the text or [41].

Genus	System	Estrogen	Androgen
Urodeles			
Ambystoma	ZZ/ZW	+	fem.
Hynobius	?	+	− or fem.
Pleurodeles	ZZ/ZW	+	fem.
Triturus	XX/XY	+	−
Anurans			
Buergeria	ZZ/ZW	masc.	+
Bombina	XX/XY	−	−
Hyla	XX/XY	+	−
Pelobates	?	+	−
Pseudacris	?	+	+
Rana	XX/XY	+ or −	+
Xenopus	ZZ/ZW	+	−

The responses are classed as effective orthodox (+), minor or none (−) or paradoxical (masc. or fem.).

mutants of *A. mexicanum*. He found that a male gonad, whether host or graft, was usually dominant in determining the sex of the chimaera (except when grafting between species with different rates of development) and so presumably released an androgen. The best example is of an *A. tigrinum* flank region, including the gonad primordium, grafted orthotopically on to the flank of a white axolotl embryo which developed into a fertile male [47]. When mated to normal white females, however, this specimen sired white offspring with a sex ratio of 77 females to 26 males—a 3:1 ratio indicating the operated specimen was a ZW genetic female (fig. 1d). Humphrey [48] isolated WW females from these offspring as those which produced all-female progeny when mated to normal males, and eventually showed that WW specimens could also be masculinized by virtually the same grafting procedure. His tests using unmarked grafts suggested that *A. tigrinum* also employs a ZZ/ZW genetic system of sex determination.

Humphrey [49] later employed very small flank grafts which contained primordial germ cells but not enough gonad mesoderm to cause sex reversal of the host embryo. He retrospectively selected cases involving dark male donors and white female recipient axolotls, and recorded four white females which produced some dark offspring when mated with normal white males. These exceptional offspring, which must have been derived from the grafted germ cells, all proved to be male (fig. 1f). This experiment thus confirmed that male axolotls are the homogametic sex and demonstrated that ZZ germ cells can differentiate into mature oocytes and fertile eggs. Collenot [50] employed the same grafting technique on *Pleurodeles waltl* embryos, using triploid hosts whose own gametes rarely result in viable descendants. He obtained masculinized ZW germ cells, as determined by an enhanced proportion of female offspring including one proven WW female specimen (fig. 1d). Further grafts using all-male spawnings from ZZ neofemales and all-female spawnings from WW females are described by Collenot et al. [51, 52]. A grafted testis primordium or differentiated testis regularly inhibited the host's ovarian development and occasionally converted part of the host gonad into a testis. The difference between these results and the effect of testosterone treatment is a strong argument for the action of another testicular hormone, perhaps equivalent to the mammalian antimullerian hormone.

Mikamo and Witschi [53] implanted a testis from a recently metamorphosed *Xenopus laevis* into 3-week-old tadpoles of the same species. Successful grafts were removed about 1 year after metamorphosis to allow the host gonads to mature. Sex-reversed neomales were identified by two breeding tests. They produced offspring with a 3:1 sex ratio of females to males when mated to normal ZW females (fig. 1d) and 1:1 ratios when mated to ZZ neofemales (obtained previously by estradiol treatment). The former breeding test produced WW specimens which became fertile females or, in one case, a WW neomale which had been masculinized by the same grafting procedure [54].

General implications

Ambystoma, *Pleurodeles* and *Xenopus* show a remarkable conformity in their genetic control of sex determination. All possess a ZZ/ZW switch mechanism, and all possible combinations of the sex chromosomes ZZ, ZW and WW are viable. After experimental sex reversal, germ cells of any of these genetic constitutions seem capable of forming either eggs or sperm. The common underlying feature here is probably that there is very little difference between Z and W chromosomes in these species, although the short heteromorphic segment detected in axolotls [55] and *Pleurodeles* [12] suggests that the difference is more than a single switch gene. No equivalent generalization can be applied to amphibians which possess XX/XY chromosomes, simply for lack of evidence. It probably applies to *Hyla japonica* where fertile XY females and YY males have been recorded [4] and to *Rana rugosa* where XX females are present in some populations and ZW females in others [56]. It could apply generally to Ranid frogs where sex chromosomes have rarely been distinguished, except in the hybrid *R. esculenta* where males are XY [57] and *R. tigrina* where females are reported to be ZW [58]. Although XX neomales have been demonstrated in several other *Rana* species, the unpredictability of sexual differentiation makes them unreliable material for further tests. A more substantial genetic disparity between the sexes would be expected in those amphibians with easily visible differences between the sex chromosomes. *Triturus* species, however, mostly show only a small difference of heterochromatin, and even that is not visible in *T. helveticus* [40]. Since *T. helveticus* and *T. alpestris* can be feminized by estradiol but no fertile adults resulted from the treatment, the only available information comes from sex reversal of *T. cristatus*. That led to the surprising results reported here: XX neomales are sterile, and XY neofemales seem incapable of transmitting the Y chromosome to their progeny. So even breeding tests have limitations.

Despite the accumulated examples of genetic sex determination in amphibians, there is little direct information about how it operates. The W chromosome certainly has a feminizing influence and appears to be dominant, as shown by triploid ZZW females [12], but ZZ gonad grafts can masculinize a ZW host [47, 53]. One laboratory strain of *Pleurodeles waltl* has an inherited tendency to produce partially or completely femi-

nized ZZ specimens [59]. Extensive breeding tests on the neofemales and intersexes of this strain show that roughly 50% or 25% of their ZZ progeny are feminized to some exent, but outcrossing to another stock results in 100% male progeny. These data suggest that the strain must carry at least two unlinked recessive alleles affecting sex determination. One of them might be a defective version of the initial gene in the masculinization pathway, but their linkage to a Z chromosome or an autosome has not yet been established. The alternative pathways of female and male differentiation are much the same in all vertebrates, however, including the secretion of steroid hormones which act early enough to allow experimental sex reversal in many of the cases reviewed here. The pathways have been traced in most detail in mammals [60–62], to provide the following salient features. Male differentiation depends on expression of the *Sry* gene in prospective Sertoli cells of the gonad. The Sertoli cells probably direct the differentiation of other gonad cells, so that their secretion of an antimullerian hormone is followed by androgen production in Leydig cells. The testis can develop in the absence of germ cells, but fertility obviously requires them and the expression of autosomal and Y-linked fertility factors in the germ cells themselves [63, 64]. Female differentiation normally occurs in the absence of Sry and requires both the expression of an autosomal or X-linked gene such as *Dax-1* and the presence of germ cells prior to the estrogen production by the thecal cells of the ovarian follicle. The early expression of *Sox-9* in testicular development of both XY mammals and ZZ birds [65] indicates that this scheme probably applies generally to vertebrates, apart from the initial genetic switch and the Y-linked fertility factors. The conversion of testosterone to estradiol by aromatase is also characteristic of vertebrates generally, and a temperature-dependent aromatase synthesis apparently accounts for the masculinization of *Pleurodeles waltl* at high temperature [66]. Secondary sexual characterisics are largely dependent on steroid hormones in vertebrates and may show even wider genetic conservation [67, 68].

Hillis and Green [3] have accumulated impressive evidence that the basic pattern of genetic sex determination (GSD) in amphibians involves ZW sex chromosomes. At least seven groups of amphibians have subsequently evolved an XY system, and one has probably reverted to a ZW system. Another likely case of evolution from XY to ZW in *Rana rugosa* has been discovered more recently [56]. Such flexibility of the genetic switches surely implies that they are imposed on a uniform mechanism of sexual differentiation. We envisage the Z chromosome as the carrier of a recessive male determinant (or testis determining factor, TDF) which could be an inactive mutation of the corresponding W-linked gene needed for female differentiation. A novel recessive TDF would impose a ZZ/ZW form of GSD with a stable sex ratio on a population which previously employed temperature sex determination (TSD) and hence a fluctuating sex ratio, like that found in several groups of reptiles [13, 69]. Any subsequent mutation that created a dominant male determinant such as Sry would override the existing ZW form of GSD, and eventually supplant it as a typical XY system but only when the Z chromosome had been lost from the population. Hillis and Green [3] advocated an equivalent scheme where the W chromosome carries a TDF suppressor which is absent from the Z chromosome. They pointed out that the sex ratio is inherently unstable for the period when both W and Y chromosomes coexist in the population, but argued that the most probable outcome would be fixation of male heterogamety (XX/XY) on a neoautosomal ZZ background. There are hidden assumptions in both schemes, particularly that the recessive and dominant forms of TDF are carried on different chromosomes and that neither of them is linked to any recessive lethal factor. The balanced lethal system carried by chromosome 1 of *T. cristatus* has previously been interpreted as a relic of its former status as a sex chromosome [18, 44]. Consequently, the evolution of an XY form of GSD in this group of newts has apparently reached fixation on a neoautosomal ZW background.

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