Spontaneous sex reversal in *Pleurodeles waltl* (urodele amphibia): analysis of its inheritance

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Summary

Up to now, no case of spontaneous sex reversal has been described in the urodele amphibian *Pleurodeles waltl* reared at ambient temperature. However in offspring reared under laboratory conditions, males, females but also intersex individuals were obtained. The males, some females and the intersexes had the ZZ male genotype identified through a test performed with a sex-linked enzyme. The other females had the ZW female genotype. Using the animals of this particular strain, an offspring analysis was made on crosses between, respectively, ZZ males and ZW females, ZZ males and ZZ females, and ZZ intersexes and ZZ males or females. All these crosses gave ZZ males, ZZ females and ZZ intersexes. The spontaneous complete or partial sex reversal is inheritable, but the genetic mechanism of this inheritance has not yet been elucidated.

1. Introduction

Since the initiation of breeding the salamander *Pleurodeles waltl* at the laboratory about 45 years ago (Gallien, 1952), no case of spontaneous intersexuality or hermaphroditism has been described after the animals were reared at ambient temperature (20 °C ±2°). Under these standard conditions, 49·1% of phenotypic males and 50·9% of phenotypic females were obtained in 1256 progeny from 22 different matings (male ZZ × female ZW), giving a sex ratio of 1:1 (Dournon & Houillon, 1984). It has also demonstrated that, when reared at ambient temperature, all phenotypic males had the ZZ sexual genotype and all phenotypic females the ZW sexual genotype; such a constant correlation demonstrates that a genetic determinism of sexual differentiation is expressed in *Pleurodeles* larvae reared at ambient temperature (Collenot *et al.* 1989; Dournon & Houillon, 1983; Dournon *et al.* 1988).

However, in *Pleurodeles*, sex reversal can be obtained by several experimental means. Estradiol benzoate treatment applied to *Pleurodeles waltl* larvae induces the differentiation of individuals with a ZZ male genotype into phenotypic females i.e. ZZ neofemales (Gallien, 1950, 1951, 1954). Raising the rearing temperature induces a complete or partial sex reversal in *Pleurodeles waltl* and the first case of intersexuality was observed in standard offspring reared at a temperature higher than ambient (Houillon & Dournon, 1978). Later, it was demonstrated that an increase in the rearing temperature from 20 °C to 31–32 °C during a critical period of larval development induces a sexual inversion of ZW females into ZW neomales in *Pleurodeles waltl* (Dournon & Houillon, 1984, 1985), whereas in *Pleurodeles poireti* it induces a sexual inversion of ZZ males into ZZ thermoneofemales (Dournon *et al.* 1984). In offspring reared at ambient temperature, Lacroix (1968) observed an intersexual animal which was an hybrid from a cross between *Pleurodeles waltl* and *Pleurodeles poireti*. In other tests at ambient temperature, several cases of intersexuality have been observed in chimaeric grafts between genotypic male and female embryos (Collenot *et al.* 1977; Houillon & Dournon, 1986; Houillon, 1992).

Up to now, in the absence of any experimental bias, neither complete nor partial sex reversal has been reported in *Pleurodeles sp.* after the larvae were reared at ambient temperature. In 1987, in *Pleurodeles waltl*

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of the A 85 progeny reared at ambient temperature, owing to a *post-mortem* examination, a phenotypic male was identified as intersexual. This individual had symmetrical ovotestes each constituted of a thin ovarian strip with non-pigmented oocytes and a big testicular nodule located at the middle of the ovarian strip. Exploratory laparotomy was then performed on the surviving 13 phenotypic males and 12 phenotypic females. One more intersex (animal no. 7405) was discovered among the phenotypic males.

The discovery of these two intersexes in the A 85 progeny led us to look for complete sex reversal among the phenotypic females of these offspring and to test whether the partial or complete spontaneous sex reversals resulted from inheritable and/or epigenetic factors.

2. Materials and methods

(i) *Origin of the experimental animals*

In this work, animals of the A 85 strain were crossed together or crossed with a male issued from the A 86 normal progeny. The A 85 strain came from the A 85 offspring obtained at the 'Laboratoire de Biologie Animale de l'Université de Lille', the embryos of which had been given to one of us (A. C.). The A 86 progeny came from a strain in which all animals were reared under standard conditions (20 °C ± 2°) and in which the sex ratio was 1:1; the animals of this strain were considered as normal. All embryos, larvae, juveniles and adults of the two strains were reared at ambient temperature (20 °C ± 2°). As sexual maturity is late, occurring between 15 and 20 months after laying, the offspring study is long and exacting.

(ii) *Identification of sexual phenotype before sexual maturity*

Before the appearance of secondary sexual characters (cloaca, and nuptial pads), the sexual phenotype of late larvae and juvenile individuals was identified through the observation of the gonads after either autopsy or by bilateral laparotomy on anaesthetized animals. During some weeks before and after metamorphosis, the posterior part of the testis in normal males swells and becomes opaque whereas the fore part is tapered. The ovary of normal females is longer than the testis and looks like a flat translucent strip, its anterior extremity being wide and rounded (Fig. 1).

(iii) *Enzymatic test for the sexual genotype identification*

In *Pleurodeles waltl*, normal males are homogametic (ZZ) and normal females are heterogametic (ZW). An enzymatic test based on the sex-linked peptidase-1 of *Pleurodeles sp.* allows the identification of the sexual genotype. Peptidase-1 is coded by two codominant genes: a peptidase-1A gene linked with the Z chromosome and two alleles, peptidase-1B or -1β, of a gene linked with the W chromosome. The electrophoretic pattern of peptidase-1 from erythrocytes allows the ascertainment of the sexual genotype of any individual tested (Ferrier *et al.* 1980, 1983; Dournon *et al.* 1988).

3. Results

(i) *Gonadal phenotypes*

In the offspring analysis, the phenotypic sex of the gonads was identified for all animals. Most had testes or ovaries with normal appearance but some had intersexual gonads (Fig. 1).

A great variation was noticed in the morphology of these intersexual gonads. Sometimes the gonads appeared as well differentiated testes the apex of which was wrapped by a thin ovarian pellicle containing small translucent oocytes in juvenile individuals, but these oocytes could reach a full-grown state, as was observed from laparotomy in the intersex no. 7405, the mother of the B91 offspring. In other animals, the ovotestes looked like ovarian strips ending with one or two testicular nodules; when there were two nodules the anterior one was separated from the posterior one by a short ovarian strip; few ovotestes bore the testicular nodule anteriorly. However, the testicular nodule was observed predominantly at the posterior end of the ovarian strip (Fig. 1 c).

The aspects of the gonads could be symmetric but asymmetry was not exceptional and its features were diverse; for example, on one side the gonad might be fully differentiated into a testis or an ovary and, on the other side, into an ovotestis comprising an ovarian strip associated with a testicular nodule located in front of or behind that ovary (Fig. 1 b–d).

(ii) *Identification of the sexual genotypes in the initial A 85 offspring*

The peptidase-1 test, applied to the surviving animals (12 males, 1 intersex and 12 females) of the A 85 offspring, showed that the 12 phenotypic males all exhibited the enzymatic pattern AA and therefore had a $Z_A Z_A$ male genotype. All the phenotypic females, except for no. 7462, exhibited the enzymatic pattern AB and so had a $Z_A W_B$ female genotype.

The phenotypic female no. 7462 and the intersex no. 7405 had the $Z_A Z_A$ male genotype. This was confirmed by analysis of lampbrush chromosomes from their oocytes (Lacroix *et al.* 1990). This result shows that the sex reversal affects genotypic males and that even a complete reversion into phenotypic females is possible. Crosses made between animals of the A 85 strain included $Z_A Z_A \times Z_A W_B$ pair matings and $Z_A Z_A \times Z_A Z_A$ pair matings (Fig. 2).
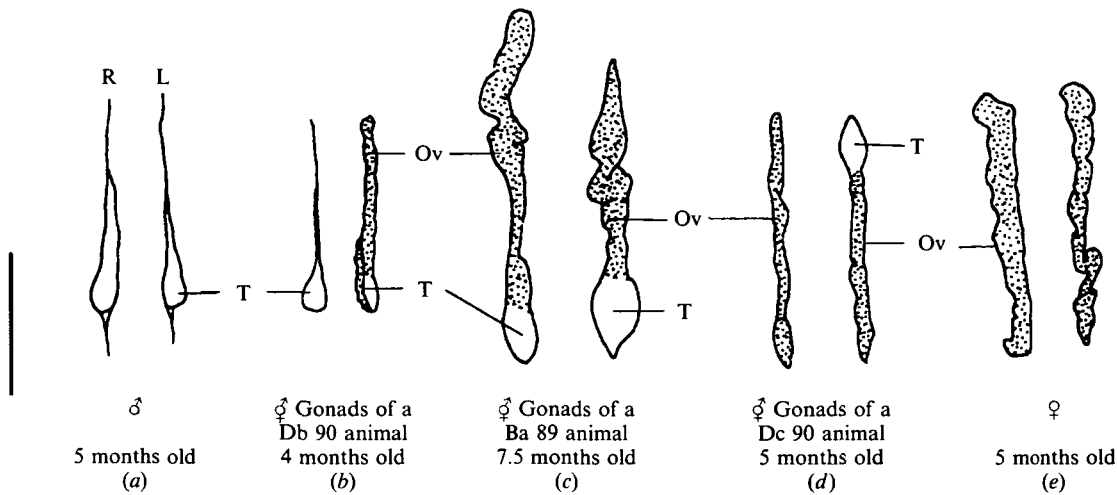
Fig. 1. Morphological aspect of gonads from normal and intersex individuals. – (*a*) and (*e*): gonads of normal *Pleurodeles waltl*, – From *b* to *d*: gonads of A 85 *Pleurodes* strain, Ov: ovary, T: testis, L: left, R: right, scale bar: 5 mm.

(iii) *Crosses between a $Z_A Z_A$ male and $Z_A W_B$ female, both from the A 85 offspring*

A $Z_A W_B$ female and a $Z_A Z_A$ male, both individuals of the A 85 offspring, were crossed twice giving rise to two offspring: Ba 87 and Ba 89 in which theoretical sex-ratio was 1:1. In reality 35 males (31·5%), 10 intersexes (9·0%) and 66 females (59·5%) were numbered in the pooled progeny (Fig. 2). The presence of intersexes shows the occurrence of a sex reversal. Therefore, the abnormality of sexual differentiation detected in the A 85 offspring is inheritable and can be transmitted by individuals with sexual phenotype in accordance with their sexual genotype. The results of χ^2-test applied to the pooled offspring indicated a significant difference from the theoretical ratio. The percentage of sex reversed males was 18% (Table 1).

The results demonstrate that one of the apparently normal parents, or both, issued from the A 85 offspring, transmitted to the progeny an abnormality of sexual differentiation.

(iv) *Crosses between individuals with an ascertained $Z_A Z_A$ genotype*

(*a*) *Crosses between $Z_A Z_A$ males and $Z_A Z_A$ females*
 – *Offspring of the $Z_A Z_A$ female issued from the A 85 strain.*
The $Z_A Z_A$ female no. 7462 issued from the A 85 offspring was crossed with the male no. 74/8 which came from the A 86 normal strain and the sexual genotype of which was enzymatically identified as $Z_A Z_A$ (Fig. 2). This cross gave rise to 159 animals (Bb 87 offspring) which all had a gonadal male phenotype (Table 1). Among them, 70 were randomly tested for peptidase-1 and all had a $Z_A Z_A$ genotype.

Several males of that Bb 87 progeny were later used in further crosses. They were identified in each cross respectively as males: nos 10, 800, 806, 830 and 833.

The $Z_A Z_A$ males no. 800 and no. 806 have been back-crossed with their mother, the $Z_A Z_A$ female no. 7462 (Fig. 2). Two offspring were obtained, Cb 88 with 77 males (74·0%), 6 intersexes (5·8%) and 21 females (20·2%) and Ca 90 with 47 males (51·6%), 29 intersexes (31·9%) and 15 females (16·5%)

The enzymatic peptidase-1 test and laparotomy have been applied in the Cb 88 offspring. All the living animals, including those with ovaries or ovotestes have been characterized as $Z_A Z_A$ genotypic males. The Ca 90 offspring was not tested for enzymatic activity.

As expected, all the progeny of the $Z_A Z_A$ female no. 7462 have the $Z_A Z_A$ male genotype. However, the sexual phenotypes observed in the offspring were different according to the father of the progeny. When the father originated from a normal strain the sexual phenotype was in agreement with the sexual genotype, all animals were phenotypic males. But when males of such a F1 were back-crossed with their mother, partial and complete sex-reversal were observed. The results confirm the inheritance of the abnormality of sexual differentiation which was absent in F1 (Bb 87) and present in the back-crosses Cb 88 and Ca 90.

For the Ca 90 offspring, 52% of the animals were male: one male out of two was feminized whereas, for the Cb 88 offspring, 74% of the animals were male and one male out of four was feminized (Table 1).

Several crosses were made with animals from the Cb 88 offspring.

 – *Offspring of other $Z_A Z_A$ females of the A 85 strain.* Three $Z_A Z_A$ females from the Cb 88 backcross were mated as follows:

$Z_A Z_A$ female no. 25 was crossed with the $Z_A Z_A$ male no. 7409 from the A 85 strain (Fig. 2). This gave rise to the Da 90 offspring consisting of 33 males (50·8%), 18 intersexes (27·7%) and 14 females (22·5%). For this offspring, one male out of two was sex-reversed (Table 1).
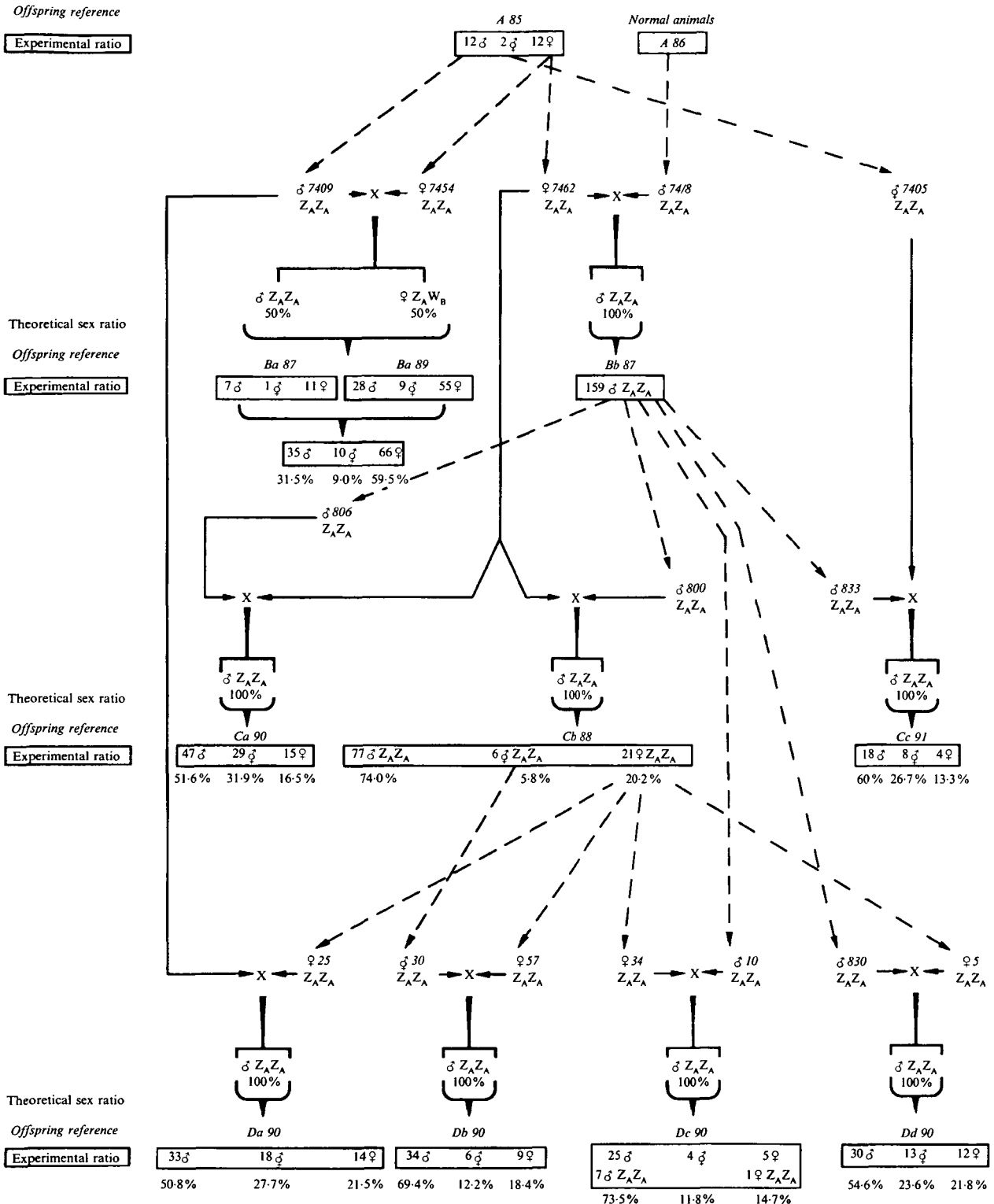
Fig. 2. Sex ratio and genetic analysis in the A 85 strain. The figure gathers the whole of the studied offspring. Characterization of progeny: capital letters indicate successive offspring, small letters specify laying and numbers give birthyear. The number of phenotypic males and/or phenotypic females obtained in each series of crosses, and the theoretical percentages in accordance with genotypic sex determination are indicated.

$Z_A Z_A$ female no. 5 was crossed with the $Z_A Z_A$ male no. 830 from the Bb 87 offspring. This gave rise to the Dd 90 offspring which included 30 males (54·6 %), 13 intersexes (23·6 %) and 12 females (21·8 %). For this offspring, one male out of two was feminized (Table 1).

$Z_A Z_A$ female no. 34 was crossed with the $Z_A Z_A$ male no. 10 from the Bb 87 offspring. This gave rise to the Dc 90 offspring which included 25 males (73·5 %), 4 intersexes (11·8 %) and 5 females (14·7 %) (Fig. 2). As a control among these animals, 8 surviving individuals (7 phenotypic males and 1 phenotypic female) were

Table 1. *Comparison of progeny frequencies*

			Progeny					
	Mating		No. ♂	No. ⚥	No. ♀	♂ (%)	⚥ (%)	♀ (rest)	Male sex reversed (%)
ZZ × ZW	Ba 87 + Ba 89		35	10	66	32	9	1:2	18
ZZ × ZZ	Bb 87		159	0	0	100	0	1:0	0
ZZ × ZZ	Ca 90		47	29	15	52	32	1:1	—
	Da 90		33	18	14	51	28	1:1	—
	Dd 90		30	13	12	55	24	1:1	—
		Total	*110*	*60*	*41*	*52*	*28*	*1:1*	*48*
	Cb 88		77	6	21	74	6	3:1	—
	Dc 90		25	4	5	74	12	3:1	—
		Total	*102*	*10*	*26*	*74*	*7*	*3:1*	*26*
	Total		212	70	67	61	20	2:1?	39
ZZ × ZZ intersexes	Cc 91		18	8	4	60	27	2:1	—
	Db 90		34	6	9	69	12	3:1	—
	Total		52	14	13	66	18	2:1	34

tested for the peptidase-1. The test indicated that all had the $Z_A Z_A$ genotype. For this Dc 90 offspring, one male out of four was feminized (Table 1).

The presence of both numerous intersexes and phenotypical females in each offspring proves that the parents are vectors of the abnormality of the sexual differentiation.

(b) Crosses between $Z_A Z_A$ intersexes and either a $Z_A Z_A$ male or a $Z_A Z_A$ female

– Cross between a $Z_A Z_A$ intersex and a $Z_A Z_A$ phenotypic male.
The $Z_A Z_A$ intersex no. 7405 issued from the A 85 strain and used as female, has been crossed with the $Z_A Z_A$ male no. 833 issued from the Bb 87 offspring. They gave rise to the Cc 91 offspring which included 18 males (60·0 %), 8 intersexes (26·7 %) and 4 females (13·3 %) (Fig. 2). For this offspring, one male out of three was sex-reversed (Table 1).

– Cross between a $Z_A Z_A$ intersex and a $Z_A Z_A$ phenotypic female.
The $Z_A Z_A$ female no. 57 has been crossed with the $Z_A Z_A$ intersex no. 30 used as male; they belong to the same progeny Cb 88 (Fig. 2). They gave rise to the Db 90 offspring which included 34 males (69·4 %), 6 intersexes (12·2 %) and 9 females (18·4 %). For this offspring, one male out of four was feminized (Table 1).

4. Discussion

The results of several crosses showed that in the A 85 strain the anomaly of the male sexual differentiation is inheritable but they do not enable us to explain its genetic basis.

The progeny of the $Z_A Z_A$ female no. 7462 exhibit strikingly different sex ratios according to the male partner. When this female was outbred with a standard male all the descendants (Bb 87) were phenotypic males but when it was backcrossed with males issued from that former cross there were phenotypic males and females and intersexes (Cb 88 and Ca 90). In the A 85 strain, the ZZ × ZZ offspring can be pooled in three groups: a first group formed by Ca 90, Da 90 and Dd 90, where 48 % of males were sex reversed, a second group formed by Cb 88 and Dc 90, where 26 % of males were feminized, and a third group (ZZ × intersexes) formed by Cc 91 and Db 90, where 34 % of males were sex reversed (Table 1). Moreover, the percentage of sex reversed males in the pooled first and second groups (ZZ × ZZ) and in the third group (ZZ × intersexes) was respectively 39 % and 34 % (Table 1). Such results lead us to hypothesize that the cross with a male of the normal strain had combined a dominant gene(s), borne by the Z chromosome or an autosome, with the corresponding recessive gene(s) of the A 85 strain and the crosses between animals of the A 85 strain had combined recessive genes inducing ovary differentiation. This hypothesis cannot be led further because the analysis of the sex ratios in the different offspring shows a not yet elucidated discrepancy: one out of two up to one out of four males is reversed into a female or an intersex animal.

Another drawback to arguing about the numerical values results from the interpretation of the various features of the gonadal phenotypes. These were identified in late larvae, in metamorphosing and juvenile individuals and, a few months later, in adults. There was no difficulty in the identification of ovaries. As regards the testes, although their identification was easy, their appearance was not the same in each case. In some males, the testes did not extend into thin filaments fastened to the lungs as in standard males

but they were directly attached to the lungs. These individuals exhibiting a male gonadal phenotype previously could have been intersexes during the juvenile period. So the sex ratio might be modified according to the age at which the sexual phenotype of a dead animal was established.

The appearance of the intersex gonads differs among individuals. However, it is possible to note an antero-posterior organization which could be correlated with the processes of the gonad organogenesis. During normal development, the anterior part of the gonad differentiates first, before the middle and posterior parts. In intersex gonads an ovarian strip or a differentiated ovary was often observed anterior to small or large testicular nodules. Sometimes, the ovarian structures are located before and behind a testicular nodule which is located in the middle part of the ovaries; an ovary was rarely entirely located behind a testicular structure.

These particular features of the gonads could be related to the timing and the spatial progress of gonadogenesis. They could correspond to the successive steps in the progressive determination of the phenotype of the gonads during their sexual organogenesis. So, according to these observations, the sex reversal phenomenon could have worked differently at different times during the phase of sexual differentiation.

In adult intersexes, ovaries can become functional whereas testes stay juvenile and conversely. So, such intersexes can be used as functional females (for instance intersex no. 7405) or as functional males (intersex no. 30). In A85 strain, we did not observe intersexes which, after being functional female or male, became functional male or female respectively, because such an evolution requires many years (Gallien, 1962; Houillon & Dournon, 1986).

Unknown epigenetic factor(s) might be involved in sex-reversal and could account for both the variability of the gonadal phenotypes of the sex reversed individuals and the differences in the numerical values established from the sex ratio of different spawnings. It is noteworthy that even a cross between two sex-reversed $Z_A Z_A$ individuals (Db 90) did not produce a greater number of intersexes and females than other crosses (Fig. 2). In that spawning as in others, some $Z_A Z_A$ male individuals could have been potentially qualified for sex reversal.

As yet, neither the genetic background of the sex reversal process nor a possible epigenetic factor is identified. As for the former at least two hypotheses can be considered. First, all the individuals with the AA peptidase-1 genotype have, conflicting proof excepted, the ZZ male genotype. In the A85 strain, the *Pleurodeles* individuals can be classified as *Pleurodeles waltl* in reference to their morphology, their length, their pigmentation and the $Z_A Z_A$ genotype of the males and the $Z_A W_B$ genotype of some females. So it is interesting to compare this spontaneous sex reversal

to the sex reversal obtained after a hormonal or heat treatment. Estradiol benzoate treatment induced the conversion of ZZ males into ZZ females in *Pleurodeles waltl* (Gallien, 1950, 1951, 1954) as in *Pleurodeles poireti* (Lacroix, 1968). 30 °C–32° heat treatment induced the reversal of ZW individuals into ZW neomales in *Pleurodeles waltl* (Dournon & Houillon, 1984) but in *Pleurodeles poireti*, the same treatment induced the reversal of ZZ individuals into ZZ neofemales (Dournon *et al.* 1984). Given the opposite effects of the thermal treatment in *Pleurodeles waltl* and *Pleurodeles poireti* and the fact that spontaneous sex reversal was observed in *P. waltl–P. poireti* hybrids (Lacroix, 1968) a first hypothesis might be proposed that the individuals of the A85 strain, although *Pleurodeles waltl*, are carrying one or several genes involved in the sex-determination mechanism in *Pleurodeles poireti* species. These genes would have been introduced into the *Pleurodeles waltl* genome through an uncontrolled hybridization in the donor laboratory. This hypothesis cannot be tested because no evidence of such a cross could be obtained, even through the examination of the lampbrush chromosomes in the oocytes of A85 $Z_A Z_A$ females.

A second and more plausible hypothesis is to suppose a possible alteration, i.e. a mutation, of the genome in a *Pleurodeles waltl* standard strain, borne either by the Z chromosome or by one autosome, which leads to an alteration of the male differentiation of the gonads. In Vertebrates, such alterations of sex differentiation have been described which are based on alterations of the genome. Spontaneous complete or partial sex reversal was reported in the mouse (Eicher, 1988; Washburn & Eicher, 1989). The interpretation was based on the introduction of the Y chromosome from a particular strain (C57BL/6J) into the wild strain, associated with the presence, at the T locus located on the 17 autosome, of the T^{hp} mutation or of the T^{Orl} allele. For instance, Eicher (1988) obtained, among 95 foetuses from a particular cross, 43 XX foetuses in which 23 were XX, $+/+$ and 20 were XX, $T^{hp}/+$, all possessing two ovaries; and 52 XY foetuses in which 25 were XY, $+/+$ with testes, and 27 were XY, $T^{hp}/+$ in which 15 had two ovaries, 7 one ovary and one ovotestis, 5 two ovotestes. Allelic variability of the Y chromosome in the different mouse strains could explain a timing variation between the expression of the masculinisation genes (probably more precocious) and of the feminisation genes. A later activation of the masculinisation genes controlled by autosomal genes located near the locus T on chromosome 17 could be the initial cause of the intersexuality and of the complete sex reversal.

Sex reversal was reported in a natural population of the male species *Talpa occidentalis* (Jiménez *et al.* 1988). It was characterized by complete or partial masculinization of the gonads of XX individuals. A race of hermaphrodite-producing pigeons has been described by Riddle *et al.* (1945) and the process of

sex-differentiation of the gonads in the late stages of the embryonic development has been studied (Lahr & Riddle, 1945). They reported that in normal races of pigeons the left testis exhibited a transient inter-sexuality characterized by the temporary development of ovarian cortical tissue. Recently, a recessive mutation was described in the common carp (*Cyprinus carpio* L.). It induces testicular development in XX individuals leading to the differentiation of either testes or ovotestes (Komen *et al.* 1992). In amphibians, the so-called semi-differentiated and undifferentiated sex races exhibit a spontaneous and transient inter-sexuality of the male gonads (review in Foote, 1964). Well studied in anurans, local sex races exist also in urodeles (Witschi, 1933). However, it is noteworthy that such spontaneous intersexuality was never observed in *Pleurodeles* species bred in the laboratory, an urodele which belongs to the differentiated sex race.

This analysis has clearly shown that complete or partial sex reversal is controlled by a genetic process. However, the expression of the gene(s) implicated in the feminisation process of ZZ male individuals does not correspond to a simple and regular rule. So such a sex reversal is probably dependent on several genes and perhaps on epigenetic factors.

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