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Original Article

Sex reversal of the newt *Triturus cristatus* reared at extreme temperatures

HUGH WALLACE and BRIDGET M.N. WALLACE

School of Biosciences, University of Birmingham, United Kingdom

ABSTRACT Crested newt larvae were reared at defined temperatures, either from uncleaved eggs or from early feeding larvae, until metamorphosis when sexual differentiation had occurred. Trials at 18-24ºC showed a 1:1 sex ratio. A higher temperature trial produced more males than females, including some XX neomales. Lower temperatures resulted in a significant excess of females, including XY neofemales. Sex reversal only occurred in about half the possible cases on average. Extreme temperatures must perturb the normal XX/XY system of sex determination, to reveal either an ancestral ZZ/ZW system or a still more primitive environmental control. It is suggested that neofemales (but not neomales) could occur in nature.

KEY WORDS: *Amphibia, sex determination, temperature.*

Introduction

Heterochromosomes, or visibly different sex chromosomes, provide the most direct evidence of a genetic basis for sex determination. Consequently, breeding tests designed to establish whether sex determination is genetic (GSD) or environmental (ESD) have been conducted almost exclusively on species which lack recognisable sex chromosomes. Breeding tests on amphibians have invariably shown GSD, with either male heterogamety (XX/XY) or female heterogamety (ZZ/ZW) in such cases, with the proviso that often the genetic mechanism can be overridden to produce sex reversed specimens (Wallace *et al*., 1999). Probably the best example is the urodele *Pleurodeles waltl*, which has been feminised by rearing larvae in estradiol (Gallien, 1954) or masculinised by rearing larvae at high temperature (Dournon and Houillon, 1984, 1985). Both sex-reversed neofemales and neomales included fertile specimens, permitting breeding tests to establish the ZZ/ZW status of the species.

It is not known how far this example can be generalised to other urodeles, except that *P. poireti* which also has a ZZ/ZW type of GSD is feminised by high temperature treatment (Dournon *et al*., 1984). *Triturus cristatus* is classified in the same family as *Pleurodeles* (Salamandridae) but has cytologically distinct sex chromosomes and male heterogamety XX/XY. *T. c. carnifex*, a mainly Italian subspecies, responds to larval treatments in much the same manner as *P. waltl.* It is feminised by estradiol (Wallace *et al*., 1997) and can be masculinised at high temperature. Subsequent breeding tests did not conform to the expectation based on *Pleurodeles*, however, as the *Triturus* XY neofemales did not produce any YY progeny when mated with normal males and the *Triturus* XX neomales were sterile (Wallace *et al*., 1999). Some evidence was also obtained that low temperature treatment feminised *Triturus* larvae, although only at the limit of tolerance and with high mortality.

The data presented here provide much stronger evidence that cold temperature treatment feminises *T. c cristatus*, the type subspecies which is widespread in northern Europe, and confirms that rearing larvae at high temperature can produce XX neomales. The reservations expressed by Dournon *et al*. (1990) over earlier claimed cases implies that *T. cristatus* may be the only urodele or even the only amphibian known to be susceptible to sex reversal at both extremes of its temperature range, although its GSD system is probably adapted to operate in normal climatic conditions throughout its geographic range.

Results

The eggs obtained from natural spawnings during the main breeding season, April and May, showed more than 95% fertility. Early mortality by extrusion of embryos through weak points in the capsules, fungal infection and occasional developmental abnormalities reduced the yield to about 90% of the collected eggs. The main embryonic mortality of 50% of tailbud embryos, caused by the characteristic balanced lethal system carried on an autosome (Macgregor and Horner, 1980; Wallace, 1991, 1994), should not

Abbreviations used in this paper: ESD, GSD and TSD are Environmental, Genetic and Temperature dependent sex determination.

***Address correspondence to:** Dr. H. Wallace, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K. FAX: 0121 414 5925. e-mail: H.Wallace@bham.ac.uk

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affect the sex ratio. All embryonic mortality is discounted in Table 1 by only considering the numbers of larvae from the time they began to feed. All later mortality is shown in Table 1, by including those of unknown sex which died before the gonad had differentiated into a recognisable ovary or testis. Gonad differentiation occurred shortly before metamorphosis, in larvae over 50 mm in length at high temperatures or 60 mm at low temperatures, forming a pear-shaped testis or a tube ovary (see Methods). No intermediate cases of hermaphroditism or intersexuality were observed in specimens examined as postmetamorphic juveniles. The results from larvae reared at 18°C to 24°C showed no significant deviation from the expected 1:1 sex ratio and thus served as a control (Table 1). The sample reared at 28°C contained more males than females, but not significantly so, and an increased mortality of larvae prior to sexual differentiation. Attempts to rear larvae at higher temperatures were abandoned owing to excessive mortality. Four samples of larvae reared at 14°C to 16°C all showed a statistically significant distortion of the sex ratio with an excess of females. The sample reared at 13ºC showed the same trend in the few larvae that survived long enough for diagnosis of their sex. The sex ratios shown in Table 1 suggest that usually only about half of the potential male larvae were feminised at low temperatures, without any clear evidence that 13°C was more effective than 16°C. The single trial showing 100% females could be an aberration resulting from small numbers and 20% early mortality, but a similar result has been obtained previously (Wallace, 1984). The results recorded in Table 1 thus conform to the pattern previously observed in *T. c. carnifex*, masculinisation at high temperature and feminisation at low temperature, both outside an intermediate range where GSD operates to give a 1:1 sex ratio.

Genotypic Sex

The karyotype of *T. c. cristatus* is very similar to that of *T. c. carnifex* (Macgregor and Sessions, 1986) and only the six largest pairs of chromosomes need to be inspected to find the sex chromosomes in C-banded preparations. Chromosomes 1 and 6 are easily identified by their unequal arm-length and patterns of heterochromatin. Chromosomes 2 and 5 are virtually metacentric with little or no heterochromatin. Chromosomes 3 and 4 are submetacentric and carry a subterminal C-band on the long arm. Chromosome 4, the sex chromosome, usually also has a faint C-band in the middle of the short arm which is the only positive characteristic of the X chromosome (Fig. 1). The Y chromosome has an additional C-band on the long arm, so that it either shows a subterminal double band or a single prominent block of heterochromatin (Fig. 1B). The Y chromosome was first recognised by Sims *et al.*(1984) and the faint C-band on the short arm of both X and Y chromosomes has been noticed in *T. c. carnifex* (Macgregor *et al.*, 1990) and in *T. marmoratus* (Sims *et al.*, 1984), but not previously in *T. c cristatus*.

In practice, XY larvae were positively identified by the presence of a Y chromosome in tailtip biopsies but XX larvae often required repeated biopsies to confirm the absence of a Y chromosome. The genotypic sex of larvae identified in this way has consistently predicted the phenotypic sex diagnosed at or after metamorphosis for both *T. c. cristatus* and *T. c. carnifex* (Wallace *et al.*, 1997), but only in specimens reared at 18°C to 24°C. Table 1 indicates the temperature treatments where this prediction failed by underlined numbers. Thirteen of the larvae reared at 28°C were analysed for genotypic sex, two of them proved to be XX neomales, four were XX females and seven were XY males. Similarly, samples of the larvae reared at 13°C and 14°C shown in Table 1 included XY neofemales as well as normal XY males and XX females. These results confirm the evidence for sex reversal provided by distorted sex ratios and demonstrate that most treatments were not completely effective in causing sex reversal.

Temperature Sensitive Period

The time during which the treatment must be maintained was investigated to some extent. Cold treatment could be delayed until first feeding and still caused feminisation (Table 1) but it needed to be prolonged for most of larval life. A 14°C treatment started at first feeding and lasting four months, with tailtip biopsies, yielded 10 XX females and 11 XY males. A similar 14°C trial lasting six months from before hatching also showed no evidence of feminisation according to the sex ratio (13 females, 10 males and 11 of unknown sex). In both of these trials, it seems likely that GSD was able to exert its influence during the later period of larval life spent at 20°C.

TABLE 1

SEX RATIO OF *T. C. CRISTATUS* AFTER TEMPERATURE TREATMENTS FROM FEEDING LARVAE WITH 3 FINGERS OR FROM UNCLEAVED EGGS TO EARLY METAMORPHOSIS

CONDITIONS	TOTAL	FEMALE	MALE	UNKNOWN	% FEMALE
28°C for 2 months from larva	70	25	39	6	39
24°C for 2 months from larva	27	13	12	2	52
20°C for 3 months from egg	103	51	43	13	54
18°C for 4 months from larva	67	31	35	1	47
16°C for 4 months from larva	49	33	13	3	72*
15°C for 5 months from larva	46	33	12	1	73*
15°C for 5 months from egg	21	17	0	4	100*
14°C for 9 months from egg	78	52	24	2	68*
13°C for 6 months from egg	33	9	2	22	82

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. The sex ratio (% female) ignores specimens of unknown sex and significant departures from 50% are marked *

Discussion

T. c. cristatus and *T. c. carnifex* are sometimes considered to be distinct species (Macgregor *et al.*, 1990). Only two minor differences between them emerged from our observations. The range of temperatures tolerated by larvae are not exactly the same, as *cristatus* larvae survive better at low temperatures and worse at high temperatures. That might reflect only an adaptation of local populations rather than being a specific characteristic. Sex determination apparently starts before feeding in *carnifex* larvae (Wallace *et al.*, 1999) but at a later stage in *cristatus*. For the present purpose, we generalise the results to conclude that *T. cristatus* (*sensu lato*) has an XX/XY type of GSD that is effective during larval life between 18°C and 26°C, but is perturbed both below and above this temperature range.

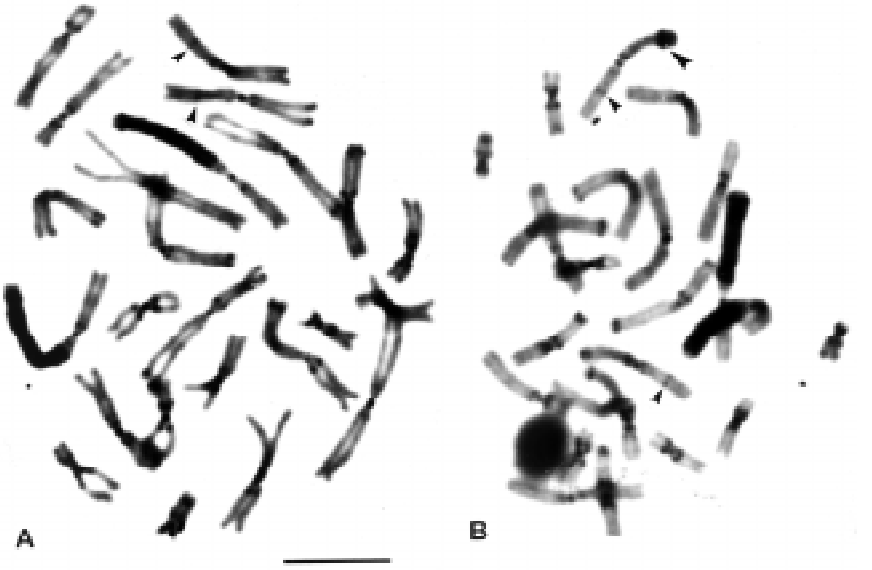

Fig. 1. C-banded chromosome sets from larval tailtip biopsies. (A) *XX,* **(B)** *XY. Small arrowheads indicate the C-band in the middle of the short arm of all sex chromosomes. The large arrowhead indicates the double C-band near the end of the long arm of the Y chromosome. Bar, 10 μm.*

The high temperature sex reversal is probably explicable in terms of the conversion of androgens to estrogens by aromatase, as investigated in *Pleurodeles* (Chardard *et al.*, 1995; Chardard and Dournon, 1999). We suppose that aromatase activity is reduced indirectly by expression of a sex-determining gene on the Y chromosome of normal males and by high temperature in XX neomales. The low temperature sex reversal demands a more ingenious explanation. If the postulated Y-linked sex determinant is inactivated at low temperature, its failure or the single X of XY larvae is not always sufficient to feminise them. Inactivation of the XX/XY sex determinant could reveal the presence of an ancestral ZZ/ZW system, which Hillis and Green (1990) considered to be characteristic of all amphibians. The balanced lethal system of chromosome 1 in *T. cristatus* has been interpreted as the cause of a permanent ZW heterozygosity (Wallace *et al.*, 1997, 1999), which should promote feminisation if released from suppression. Alternatively, inactivation of the XX/XY sex determinant could reveal a temperature dependent sex determination (TSD) like that found in many reptiles and some fish. That is surely the original means of sex determination in vertebrates where GSD systems have evolved repeatedly, essentially as homeostatic mechanisms providing independence from climatic fluctuations (Bull, 1983).

A well-adapted GSD should operate over the complete temperature range normally experienced by a particular species, yet unusual temperatures are the most likely explanation for the few recorded cases of spontaneous sex reversal in amphibians (Wallace *et al.*, 1999). The possibility of natural sex reversal in *T. cristatus* can now be addressed, by considering if *cristatus* larvae ever exist at water temperatures above 28°C for about 2 months or below 16°C for at least 4 months. Local populations of *cristatus* spawn as the water temperature rises from about 8°C to 18°C. Both embryonic development and larval growth are very slow at the lower temperatures, so normally all larvae are subject to the maximum summer water temperature before metamorphosis. That never rises enough to masculinise larvae, but might occasionally remain cool for long enough to feminise them. The present investigation began with *cristatus* eggs collected in April 1982 and kept in an unheated laboratory at an estimated temperature close to 16°C (Wallace, 1984). The larvae reached metamorphosis in 3-4 months and nearly 90% of them were female. Eggs collected a month later and kept in the same conditions developed into larvae with a 1:1 sex ratio. The rare combination of a mild spring and an English summer might duplicate these conditions and result in spontaneous neofemales.

Material and Methods

Eggs were collected from ribbons placed in a garden pond overnight and cultured in batches of up to 20 in each Petri dish in an incubator (Wallace, 1991). Some batches were maintained at a constant temperature from the uncleaved egg to metamorphosis, up to 9 months later. Others were reared at 20ºC until starting a temperature regime when the larvae began to feed, about three weeks after egg collection. All larvae were fed successively on *Artemia*, *Daphnia* and *Tubifex* worms, and allowed increasing space as they grew – from 30 ml to 300 ml water per larva.

The genetic sex of some larvae was established by taking repeated biopsies of regenerating tailtips for culture in colchicine and C-banded cytological preparations. The isolated tailtips were placed in drops of a 2:2:1 mixture of 1 mg/ml colchicine, Steinberg's amphibian saline and Flow F10 mammalian cell medium, with antibiotics in a Petri dish at 20°C for 24-36 hours. Hypotonic treatment, fixation, slide preparation and C-banding were adapted from Sims *et al.* (1984), modified by ageing the slides for 3 days followed by hydrolysis in 2.2% barium hydroxide at 40°C for 12 minutes.

The actual sex of all late larvae or postmetamorphic juveniles was established from the appearance of the gonads at autopsy, as described by Gallien (1954). In males, the strip gonad lateral to each fat body enlarges posteriorly to form first a pear-shaped and then a globular solid testis. In females, the gonad widens uniformly as a translucent tube ovary containing growing oocytes. The oocytes are usually visible under a dissecting microscope or can be detected in orcein-stained squash preparations (Wallace and Wallace, 1995). A conflict between gonadal sex and genetic sex of a specimen or a distortion from the expected 1:1 sex ratio of an experimental group are each considered to be evidence of sex reversal.

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