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Long-term sex reversal by oestradiol in amniotes with heteromorphic sex chromosomes

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Oestradiol application during embryonic development reverses the sex of male embryos and results in normal female differentiation in reptiles lacking heteromorphic sex chromosomes, but fails to do so in birds and mammals with heteromorphic sex chromosomes. It is not clear whether the evolution of heteromorphic sex chromosomes in amniotes is accompanied by insensitivity to oestradiol, or if the association between oestradiol insensitivity and heteromorphic sex chromosomes can be attributable to phylogenetic constraints in these taxa. Turtles provide an ideal system to examine the potential relationship between oestradiol insensitivity and sex chromosome heteromorphy, since there are species with heteromorphic sex chromosomes that are closely related to species lacking heteromorphic sex chromosomes. We investigated this relationship by examining the long-term effects of oestradiol-17β application on sex determination in *Staurotypus triporcatus* and *Staurotypus salvinii*, two turtle species with male heterogamety. After raising the turtles in the lab for 3 years, we found follicular and Müllerian duct morphology in oestradiol-treated turtles that was identical to that of untreated females. The lasting sex reversal suggests that the evolutionary transition between systems lacking heteromorphic sex chromosomes and those with heteromorphic sex chromosomes is not constrained by a fundamental mechanistic difference.

Keywords: sex chromosomes; oestradiol; turtle; *Staurotypus*; sex determination

1. INTRODUCTION

Heteromorphic sex chromosomes determine sex in a variety of vertebrate taxa including mammals, birds and many fish, amphibians and squamate reptiles (Solari 1994). In turtles, heteromorphic sex chromosomes are rare but temperature-dependent sex determination (TSD) and homomorphic (undifferentiated) sex chromosomes are both common (Ewert & Nelson 1991; Janzen & Paukstis 1991), and both appear to function through a similar oestrogen-dependent mechanism. By applying sufficient levels of exogenous oestradiol to the eggs of turtles that have TSD, sex can be reversed at all viable male-producing temperatures (Gutzke & Bull 1986; Wibbels *et al.* 1991). In *Apalone spinifera*, a turtle with genotypic sex determination lacking heteromorphic sex chromosomes, oestradiol application during incubation also results in complete sex reversal, with sex-reversed gonads appearing histologically identical to genetically induced ones (Bull *et al.* 1988).

Among amniotes, the effects of exogenous oestradiol on heteromorphic chromosomal sex determination have been studied in birds and mammals, but not in reptiles. In birds, oestradiol administration has been found to feminize the left gonad of chromosomal males in diverse avian species, although this effect is always transitory (Taber 1964) with follicles and oocytes of sex-reversed individuals degenerating after hatching (Solari 1994). In placental mammals, oestradiol administration does not affect sex determination (Jost 1970). In marsupials, 'ovary-like' gonads have been observed in a small number of males treated with oestradiol (Coveney *et al.* 2001), although the germ cell number of treated gonads is much less than that of control ovaries, and not significantly different from that of normal testes. Further, as these animals were sexed shortly after birth, the partial feminization observed may have been only temporary (as is common in birds; reviewed in Solari 1994).

Thus, there appears to be a fundamental mechanistic difference between amniote groups characterized by heteromorphic sex chromosomes and those that have other mechanisms of sex determination. Specifically, males in species with heteromorphic sex chromosomes cannot be sex-reversed by exogenous oestradiol, whereas males in species with homomorphic sex chromosomes or with environmental sex determination can be completely sex reversed by exogenous oestradiol (Solari 1994). This contrast is of particular interest in light of the fact that the genes involved in sex determination are so highly conserved in amniotes (Crews 2003). It has not been possible to directly address whether a causal relationship exists between sex chromosome heteromorphy and oestradiol insensitivity since heteromorphy tends to be shared among all members of a clade. Turtles provide an unique opportunity to explore the causal nature of the connections between oestradiol insensitivity and sex chromosome heteromorphy, as some species have heteromorphic sex chromosomes, while others have TSD and lack sex chromosomes. Bull *et al.* (1988) predicted that studies of the effects of oestradiol application on reptiles with heteromorphic sex chromosomes could provide insight into the evolutionary patterns of sex determination and sensitivity to exogenous oestradiol in amniotes. They postulated that the evolution of heteromorphic sex chromosomes might be accompanied by insensitivity to sex hormones due to the pleiotropic effects of traits that evolve concurrently with sex chromosome differentiation.

In the present study, we examine the effects of oestradiol-17β application during embryogenesis on sex determination in *Stauroypus triporcatus* and *Staurotypus salvinii*, two turtles characterized by male heterogamety (Bull *et al.* 1974; Sites *et al* 1979). To ensure that any observed effects of the steroid treatment were not

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transient, as is common in other taxa, hatchlings were maintained in the laboratory for 3 years. Histological examination of the gonads was performed to analyse the extent to which oestradiol application could feminize the reproductive morphology of chromosomally male turtles. Complete, lasting gonadal feminization would indicate a mechanistic dissimilarity between heteromorphic chromosomal sex determination in turtles and that in other amniotes. The absence of gonadal feminization would suggest that a fundamental mechanistic difference exists between systems with heteromorphic sex chromosomes and systems lacking heteromorphic sex chromosomes in amniotes.

2. MATERIAL AND METHODS

In January of 1998, recently laid eggs of *S. triporcatus* and *S. salvinii* were acquired from the Columbus (Ohio) zoo and were distributed among three temperature-controlled incubators maintained at 24, 26 and 28 °C. A 5 µl aliquot of either 10, 50 or 100 µg/5 µl of oestradiol-17β (Sigma 8875) in 95% ethanol was applied to each egg at the onset of embryonic stage 15 (Yntema 1968), concurrent with the period of sensitivity to oestradiol in studies of sex reversal in turtles with TSD (Gutzke & Chymiy 1988). These applications were repeated 1–2 times per egg at one week intervals. Since it was not known what dose of oestradiol would be needed to induce sex reversal, a total dose ranging from 20 to 110 µg oestradiol per egg was used. In all, 21 *S. triporcatus* and 15 *S. salvinii* were treated with oestradiol, while seven *S. triporcatus* and three *S. salvinii* were left untreated. As a 1 : 1 sex ratio has been previously confirmed in *Staurotypus* from this breeding pool (Ewert & Nelson 1991; M. A. Ewert, unpublished data) we used a minimal number of untreated turtles as controls for normal gonadal development in this part of the experiment to avoid needlessly sacrificing animals.

One week after hatching, turtles were placed in individually marked plastic containers filled with approximately 6 cm water. At approximately 3 years from the average hatching date, 24 oestradiol-treated turtles and 10 control turtles were arbitrarily selected to be sacrificed for macroscopic sexing and histological analysis. After 3 years, the *Staurotypus* were still sexually immature and did not exhibit secondary sexual characteristics.

After the gonads had been grossly inspected for sex determination under a dissecting microscope, they were removed and placed in Bouins' fixative for 48 h, and then stored in formalin until they were embedded in Paraplast (Monoject Scientific, St Louis, MO). Histological slides were generated to allow us to compare cell structure and composition between treated and control turtles. Slides were developed for 12 *S. triporcatus*: eight oestradiol-treated females, three untreated females and one untreated male. Embedded tissue was serially sectioned at 10 µm and stained using a modified Masson's trichrome stain (Presnell & Schreibman 1997). Digital light micrographs were obtained using an MDS 290 digital camera system (Eastman Kodak Company, Rochester, NY).

3. RESULTS

All 24 oestradiol-treated turtles examined exhibited well-formed Müllerian ducts and all but two had normal follicular ovaries indistinguishable from those of the control females (table 1). The two exceptions were both treated with the lowest dose of oestradiol used (20 µg total). One of the two, an *S. salvinii*, had smaller-than-average highly follicular ovary-like gonads that exhibited a male-like component in the centre of each gonad. The other individual, an *S. triporcatus*, had almost no visible gonads. Of the seven untreated *S. triporcatus* examined, three were males which lacked Müllerian ducts and had testes, while four were females that had normal Müllerian-duct and ovarian morphology. Of the three untreated *S. salvinii* examined, one was a normal male, and two were normal females. The sex ratio of the oestradiol-treated group with clearly distinguishable gonads differed significantly from 1 : 1 (0 of 22 male observed versus 11 of 22 male expected without sex reversal; $\chi^2_1 = 14.7$, $p < 0.0001$). Even if both turtles with ambiguous gonadal morphology were conservatively classified as male, the sex ratio still differed significantly from 1 : 1 ($\chi^2_1 = 10.8$, $p = 0.0015$).

Table 1. Turtle specimens examined.

ID	species[a]	oestradiol (µg)[b]	expressed sex[c]
IC2	T	0	M
IL6	T	0	F
1J2	T	0	F
1J6	T	0	F
9B5	T	0	M
9B6	T	0	F
4B8	T	0	M
A1	T	20	F/N[d]
A5	T	20	F
B4	T	20	F
C3	T	20	F
C6	T	20	F
B3	T	20	F
A2	T	20	F
B1	T	20	F
B7	T	20	F
C2	T	20	F
C5	T	20	F
C7	T	20	F
D1	T	110	F
D3	T	110	F
4D5	S	0	F
4D6	S	0	F
I45	S	0	M
U2	S	20	F
U3	S	20	F
Z3	S	20	F
X1	S	20	F/I[e]
Z1	S	30	F
T2	S	100	F
T3	S	100	F
T7	S	100	F
T5	S	100	F
T6	S	100	F

[a] S, *Staurotypus salvinii*; T, *Staurotypus triporcatus*.
[b] total oestradiol application from 2–3 doses.
[c] the phenotypic sex as determined gross gonadal and Müllerian duct examination.
[d] one turtle had large Müllerian ducts, but almost no gonads.
[e] one turtle had large Müllerian ducts and a male-like component in the middle of each ovary.

Examination of histological sections revealed that the gonads of oestradiol-treated females appeared identical to the gonads of control females. Both untreated and treated females had numerous enlarged follicles (figure 1*a*,*b*). Further, the gonads of treated females lacked obvious testes-typical characters such as testicular cords or tubes, whereas a well-formed medullary region was found in the gonads of control males (figure 1*c*). The histological comparisons coupled with the strong statistical result indicate that at least some of the oestradiol-treated females were sex-reversed chromosomal males.

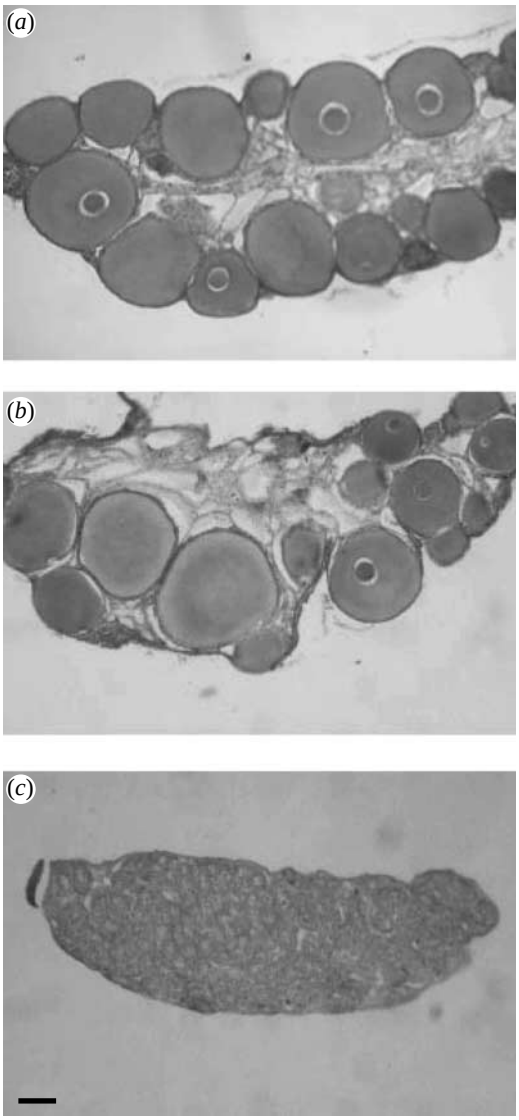

Figure 1. Histological cross-section of gonads from 3 year old *S. triporcatus*. (*a*) Normal follicular development in an untreated female and (*b*) a representative oestradiol-treated turtle. Well-formed follicles were similarly seen in 23 of 24 oestradiol-treated turtles. (*c*) Conversely, follicles were completely absent from all untreated males. Scale bar represents 250 μm.

4. DISCUSSION

In turtles of the genus *Staurotypus*, oestradiol-17β application during embryonic development causes normal and lasting female gonadal development despite the presence of male-determining heteromorphic sex chromosomes. These results are in direct contrast to all other studies of amniotic vertebrates with heteromorphic sex chromosomes, where oestradiol administration has failed to override chromosomal sex and induce persistent normal ovarian differentiation.

Only females were found among the 22 treated individuals with clear gonadal morphology. Importantly, the gonads of all oestradiol-treated females appear identical to those of normal chromosomal females of similar age. The gross gonadal morphology and histology of all oestradiol-treated females is indistinguishable from that of normal chromosomal females, with no evident difference in cell structure or composition between oestradiol-treated and chromosomal females (figure 1*a*,*b*). The normal female gonadal histology (especially follicle development) of 3 year old oestradiol-treated turtles, comparable to that of untreated chromosomal females of similar age, strongly suggests that the oestradiol-induced sex reversal is both permanent and functional. The observation of one 'incomplete' female is consistent with the few intersexuals seen at low oestradiol dosages in studies of turtles with TSD (Crews *et al.* 1991). This individual was treated with the lowest dose of oestradiol used in our study. The turtle with well-formed Müllerian ducts but almost no gonads may represent a developmental defect.

While Bull *et al.* (1988) hypothesized that pleiotropic effects of traits associated with heteromorphic sex chromosomes may result in insensitivity to sex steroids, we have shown that this is not the case. Our findings instead point to a difference between the mechanisms controlling sex determination in birds and mammals versus other vertebrates: oestradiol apparently cannot override male sex determination in birds and mammals, but can in fish, amphibians and reptiles (Wallace *et al.* 1997; Devlin & Nagahama 2002; this study). Mammals have evolved regulatory genes that control the initiation of sex determination (i.e. the *SRY* gene), apparently reducing the role of sex steroids in the early phases of the sex-determining cascade (Crews 1994). Thus, while oestradiol is important in later stages of female differentiation in mammals, it does not play a primary role in initial sexual differentiation. Analogous regulatory genes associated with the sex chromosomes may have also replaced oestradiol in the early stages of sexual differentiation in birds, as female-specific oestradiol expression cannot be detected in hatchling chicks (Clinton & Haines 1998).

Heteromorphic chromosomal sex determination is notably rare in turtles. Despite the fact that sex chromosome heteromorphy is widespread in higher animals (Bull 1983), heteromorphic sex chromosomes are known for only four turtle species (Bull *et al.* 1974; Sharma *et al.* 1975; Carr & Bickham 1981). Our findings suggest that the rarity of heteromorphic sex chromosomes in turtles cannot be attributed to a constraint in the transition between the mechanisms underlying TSD and heteromorphic sex chromosomes: both appear to operate via an estrogen-dependent system.

Animal care at Indiana University was approved by the Bloomington institutional animal care and use committee (BIACUC). Acquisition of eggs for this study was approved by the Animal Research Committee of the Columbus Zoo. We thank Zoo staff members J. M. Goode, R. E. Hatcher and E. G. Burke for their early encouragement and assistance. We are grateful to J. P. Bogart for his help throughout the study and J. J. Bull for comments on an earlier draft of the manuscript.

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