Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with temperature-dependent sex determination

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Abstract

Treatment of developing embryos of two diverse species of reptiles with fadrozole (a potent and specific nonsteroidal inhibitor of aromatase activity in mammals) resulted in the induction of male sex determination. In the first experiment, males were produced in an all-female parthenogenic species of lizard (*Cnemidophorus uniparens*). In the second experiment, male sex determination was induced in a turtle (*Trachemys scripta*) with temperature-dependent sex determination. The results support the hypothesis that the endogenous production of oestrogen may represent a pivotal step in the sex determination cascade of reptiles. Further, the production of male *C uniparens* indicates that the genes required for male sexual differentiation have not been lost in this parthenogenic lizard.

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Introduction

Gonadal sex in reptiles can be determined by sex chromosomes (genotypic sex determination or GSD) or by the temperature at which the egg is incubated (temperature-dependent sex determination or TSD); parthenogenetic species also exist which consist only of female individuals (Bull 1980, Raynaud & Pieau 1985, Ewert & Nelson 1991, Janzen & Paukstis 1991). While these sex determination systems appear diverse, an underlying uniformity may exist. In particular, treatment of developing embryos with exogenous oestrogen induces female sex determination in a variety of reptiles, including some with GSD and others with TSD (Pieau 1974, Raynaud & Pieau 1985, Bull *et al.* 1988). Those studies revealed that testosterone also induced female sex determination. In contrast, dihydrotestosterone (DHT) treatments induced male sex determination in embryonic turtles with TSD (Wibbels *et al.* 1992). These findings are consistent with the hypothesis that conversion of testosterone to its metabolites may represent a pivotal step in the sex determination cascade of reptiles. In the current study, the aromatase inhibitor fadrozole is used in an attempt to block the endogenous production of oestradiol-17β during sex determination in a parthenogenic lizard, *Cnemidophorus uniparens*, and in a turtle with TSD, *Trachemys scripta*.

Materials and Methods

Experiment 1

Cnemidophorus uniparens is a triploid parthenogenic lizard that inhabits regions in southwestern United States and northern Mexico (Dessauer & Cole 1989). It has been hypothesized that *C uniparens* arose during or since the Pleistocene as a result of hybridization between two sexual lineages now identified as *Cnemidophorus inornatus* and *Cnemidophorus burti* (Moritz *et al.* 1989). This parthenogenetic species undergoes premeiotic endomitosis to produce triploid eggs which develop without fertilization into clones of the mother (Dawley 1989). Males have never been reported in *C uniparens*. Freshly laid eggs from individuals (normally 1 to 5 eggs per clutch) in a captive colony of *C uniparens* were assigned to either control or experimental groups. All eggs received a single treatment at time periods ranging from the day of lay to eleven days after lay. Control eggs received 5 μl 95% ethanol, whereas experimental eggs received 100 μg of the aromatase inhibitor fadrozole (CGS 16949A from Ciba-Geigy Co., Summit, New Jersey, USA) dissolved in 5 μl 95% ethanol. Control and experimental treatments were applied topically to the vascularized portion of the eggshell as previously described (Crews *et al.* 1991). The status of hatchlings gonads and genital ducts were determined visually under an operating microscope following dissection and subsequently verified histologically in all cases.

Experiment 2

In a second experiment, eggs from the red-eared slider turtle, *Trachemys scripta*, were used. This turtle exhibits TSD in which warm incubation temperatures (e.g. 31 °C)

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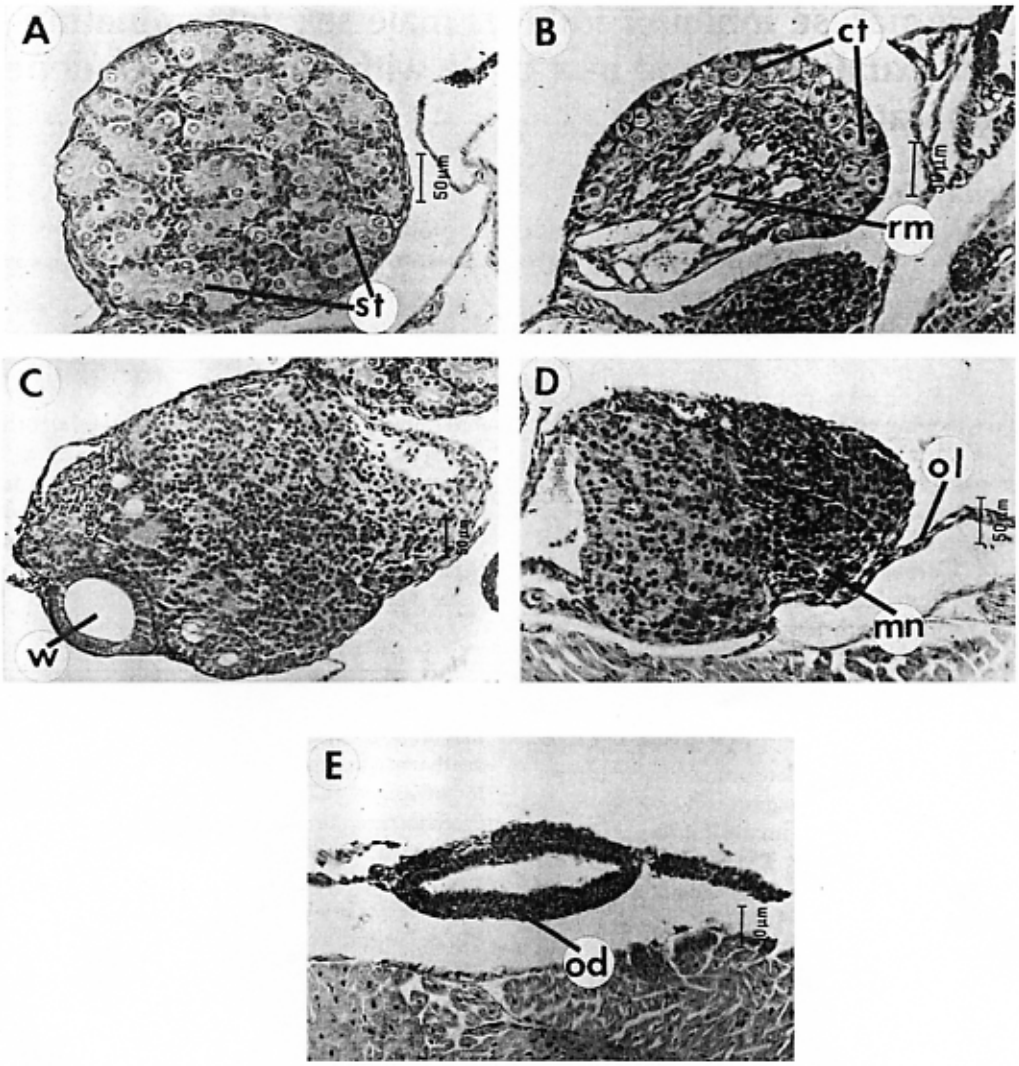

FIGURE 1. (A) Testis and (C) mesonephros from hatchling *Chemidophorus uniparens* that was treated with aromatase inhibitor. (B) Ovary (D) mesonephros and (E) oviduct of hatchling *C. uniparens* treated with ethanol control solution. st=seminiferous tubules, ct=cortex, rm=regressed medulla, w=Wolffian duct, ol=oviducal ligament, mn=mesonephric duct, od=oviduct.

produce all female hatchlings, relatively cool incubation temperatures (e.g. 26 °C) produce all male hatchlings, and incubation temperatures between 29·0 and 30·0 °C produce both males and females in varying ratios (Wibbels *et al.* 1991*a*,*b*). All eggs in the current study were maintained at an incubation temperature of 29·4 ± 0·1 °C, which results in a female-biased sex ratio of approximately 40% male, 60% female in control groups. Embryonic development was monitored by candling eggs and by dissecting two to four eggs approximately twice a week to verify specific developmental stages. At stage 17 of development (i.e. the midportion of the thermosensitive period), eggs were randomly assigned to one control and three treatment groups. Control eggs received 5 μl of 95% ethanol, whereas experimental animals received one of three dosages (1, 10, or 100 μg) of fadrozole dissolved in 5 μl of 95% ethanol. Hatchlings were dissected and the status of gonads and genital ducts determined visually under a dissection microscope as described previously for this species (Wibbels *et al.* 1991*a*,*b*).

Results

Experiment 1

All hatchling *C uniparens* from control eggs (n=6) were female. In contrast, all hatchlings from eggs treated with

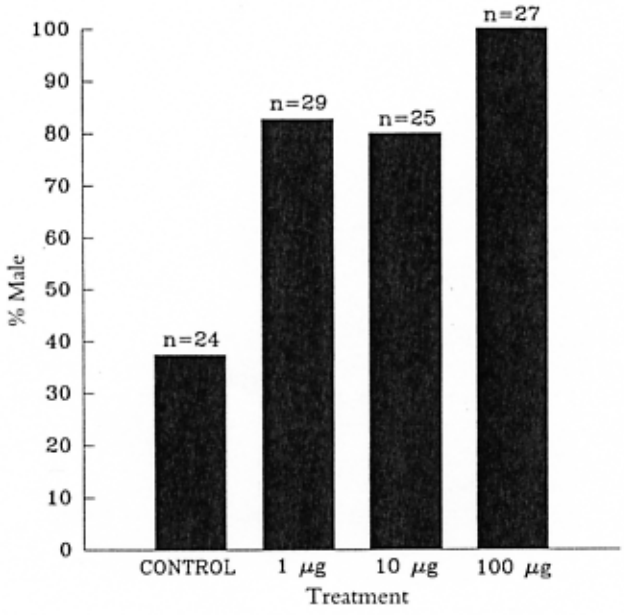

FIGURE 2. Percentage of hatchling *T. scripta* which developed as males following treatment with aromatase inhibitor or with a control solution. Eggs were incubated at 29·4 °C. Actual sex ratios from each treatment group (males:females): control 9:15, 1 μg 24:5, 10 μg 20:5, 100 μg 27:0. Sex ratios of all groups treated with aromatase inhibitor were significantly different from the sex ratio of the control group (Fisher's exact tests, $P<0·001$).

the aromatase inhibitor ($n=10$) were male, with well developed testes, distinct Wolffian ducts, and no oviducts (Fig. 1). Additionally, one individual from this study was reared to adulthood and cloacal washes revealed the presence of sperm.

Experiment 2

Comparisons of the resulting sex ratios reveal that all three groups of *T. scripta* receiving the aromatase inhibitor produced significantly more males than the control group ($P \leq ·001$, Fisher's exact test), with the 100 μg dosage producing all males (Fig. 2). All females in control and experimental groups had oviducts which appeared similar to those in nontreated hatchlings. All control males lacked oviducts, as did all but two of the treated males: one male from the 1·0 μg group and one male from the 100 μg group had regressed, but visible oviducts.

Discussion

In the current study, treatment of developing embryos with a nonsteroidal aromatase inhibitor (i.e. fadrozole) induced male sex determination in two diverse species of reptiles. While the specificity of fadrozole has not been reported in reptiles, it is a potent and specific aromatase inhibitor which lacks oestrogenic or androgenic activities in mammals (Steele *et al.* 1987). If the current results are due to aromatase inhibition, then these findings provide strong support for the hypothesis that oestrogen may be naturally involved in sex determination and gonadal differentiation (Raynaud and Pieau 1985, Crews *et al.* 1989). However, such a conclusion will require verification of its mechanism of action (e.g. future studies of aromatase activity, oestrogenic activity, androgenic activity, etc.). Regardless, the ability of fadrozole to induce male sex determination indicates that it could prove to be an effective tool for studying the physiology of the reptilian sex determination cascade. For example, it will be of interest to examine its effects on sex determination in combination with various steroid hormones or their agonists (e.g. aromatizable androgens, nonaromatizable androgens, oestrogens, etc.) as well as steroidogenic enzyme inhibitors (e.g. 5α-reductase inhibitors, etc.).

In addition to the current findings, several lines of evidence from previous studies suggest that the production of steroid hormones (in particular oestradiol-17β and possibly the 5α-reduced androgen, DHT) may play a pivotal role in reptilian sex determination: (i) treatment of developing reptile embryos with oestrogen induces female sex determination (Pieau 1974, Raynaud & Pieau 1985, Bull *et al.* 1988, Crews *et al.* 1991, Wibbels & Crews 1992), (ii) the actions of oestrogen appear similar to those of temperature in species with TSD (Raynaud & Pieau 1985, Wibbels *et al.* 1991*a*, 1993), (iii) steroid hormones and steroidogenic enzymes are present during the sex determination period in many reptiles (Pieau 1973 1974, Merchant-Larios *et al.* 1989, Thomas *et al.* 1992, White and Thomas 1992*a*,*b*,*c*), (iv) higher aromatase activity and greater amounts of oestradiol-17β have been detected in turtle gonads differentiating at female-producing temperatures in comparison to those at male-producing temperatures (Desvages & Pieau 1991, 1992*a*,*b*, Dorizzi *et al.* 1991), (v) aromatase inhibitors have been shown to disrupt ovarian development in the alligator (Lance & Bogart 1992), (vi) there is a greater metabolism of precursor to 5α-reduced androgens at male-producing temperatures (Desvages & Pieau 1991), and (vii) treatment of turtle embryos with exogenous DHT can induce male sex determination (Wibbels *et al.* 1992).

The importance of the conversion of testosterone to oestrogen or 5α-reduced androgens may not be restricted to reptilian sex determination. It has long been known that application of exogenous oestrogen or androgen is capable of altering sex determination in fishes and amphibians (Witschi 1939, Witschi & Dale 1962, Yamamoto 1962). Further, application of the aromatase inhibitor fadrozole to chicken embryos has been shown to induce male sex determination in genetic females (Elbrecht & Smith 1992). Thus, the production of estrogen or 5α-reduced androgens

could represent conservative components in vertebrate sex determination. It will be of particular interest to determine if mammalian sex determination is sensitive to fadrozole.

The production of male *C uniparens* addresses the subject of dispensable genes. It has been suggested that in unisexual species the genes associated with male sexual differentiation would be dispensable (Ohno 1985). However, treatment of their eggs with aromatase inhibitor yielded males. Similar results have been obtained with all-female gynogenetic fish treated with androgens (Turner & Steeves 1989, Schartl *et al.* 1991). These results indicate that genes involved in the formation of the male phenotype are retained in these all-female species, although the genetic trigger for male sex determination may be absent, nonfunctional, or suppressed. Currently, the best candidate for a genetic trigger in vertebrate sex determination is the SRY putative testis determining gene in mammals (Sinclair *et al.* 1990). Of particular interest, it has recently been suggested that SRY down-regulates P450 aromatase activity (Haqq *et al.* 1993). If a similar system exists in reptiles, it is plausible that fadrozole may be compensating for decreased or absent SRY-like regulatory activity in *C uniparens*.

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References

Bull JJ 1980 Sex determination in reptiles. *Quarterly Review of Biology* **55** 3–21.

Bull JJ, Gutzke WHN & Crews D 1988 Sex reversal in three reptilian orders. *General and Comparative Endocrinology* **70** 425–428.

Crews D, Bull JJ & Wibbels T 1991 Estrogen and sex reversal in turtles: A dose-dependent phenomenon. *General and Comparative Endocrinology* **81** 357–364.

Crews D, Wibbels T & Gutzke WHN 1989 Action of sex steroid hormones on temperature-induced sex determination in the snapping turtle (*Chelydra serpentina*). *General and Comparative Endocrinology* **76** 159–166.

Dawley R 1989 An introduction to unisexual vertebrates. In *Evolution and Ecology of Unisexual Vertebrates* pp 1–18 Eds RM Dawley & JP Bogart. Albany, New York: The New York State Museum, Bulletin 466.

Dessauer HC & Cole CJ 1989 Diversity between and within nominal forms of unisexual Teiid lizards. In *Evolution and Ecology of Unisexual Vertebrates* pp 49–71. Eds RM Dawley & JP Bogart. Albany, New York: The New York State Museum, Bulletin 466.

Desvages G & Pieau C 1991 Steroid metabolism in gonads of turtle embryos as a function of the incubation temperature of eggs. *Journal of Steroid Biochemistry and Molecular Biology* **39** 203–215.

Desvages G & Pieau C 1992*a* Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of eggs. *Journal of Steroid Biochemistry and Molecular Biology* **41** 851–853.

Desvages G & Pieau C 1992*b* Time required for temperature-induced changes in gonadal aromatase activity and related gonadal structures in turtle embryos. *Journal of Steroid Biochemistry and Molecular Biology* **41** 851–853.

Dorizzi M, Mignot T-M, Guichard A, Desvages G & Pieau C 1991 Involvement of oestrogen in sexual differentiation of gonads as a function of temperature in turtles. *Differentiation* **47** 9–17.

Elbrecht A & Smith RG 1992 Aromatase enzyme activity and sex determination in chickens. *Science* **255** 467–470.

Ewert MA & Nelson CE 1991 Sex determination in turtles: Diverse patterns and some possible adaptive values. *Copeia* **1991** 50–69.

Haqq CM, King C-Y, Donahoe PK & Weiss MA 1993 SRY recognizes conserved DNA sites in sex-specific promoters. *Proceedings of the National Academy of Sciences of the USA* **90** 1097–1101.

Janzen F & Paukstis GL 1991 Environmental sex determination in reptiles: Ecology, evolution, and experimental design. *Quarterly Review of Biology* **66** 149–179.

Lance VA & Bogart MH 1992 Disruption of ovarian development in alligator embryos treated with an aromatase inhibitor. *General and Comparative Endocrinology* **86** 59–71.

Merchant-Larios H, Fierro IV & Urruiza BC 1989 Gonadal morphogenesis under controlled temperature in the sea turtle *Lepidochelys olivacea*. *Herpetological Monographs* **1989** 43–61.

Moritz C, Brown WM, Densmore LD, Wright JW, Vyas D, Donnellan S, Adams M & Baverstock P 1989 Genetic diversity and the dynamics of hybrid parthenogenesis in *Cnemidophorus* (Teiidae) and *Heteronotia* (Gekkonidae) In *Evolution and Ecology of Unisexual Vertebrates* pp 87–112. Eds RM Dawley & JP Bogart. Albany, New York: The New York State Museum, Bulletin 466.

Ohno S 1985 Dispensable genes. *Trends in Genetics* **1** 160–164.

Pieau C 1973 Variation de l'activite enzymatique Δ5–3β-hydroxystéroide déshydrogénasique dans les glandes génitales d'embryons d'*Emys orbicularis* L (Chélonien), en fonction de la temperature d'incubation. *Comptes rendus hebdomadaires des Séances de l' Academie Sciences, Paris, Série D* **276** 197–200.

Pieau C 1974 Différenciation du sexe en fonction de la température chez les embryons d'*Emys orbicularis* L (Chélonien) Effets des hormones sexuelles. *Annales d' Embryologie et de Morphogenese* **7** 365–394.

Raynaud A & Pieau C 1985 Embryonic development of the genital system. In *Biology of the Reptilia* pp 149–300. Eds C Gans & F Billet New York: Wiley.

Schartl M, Schlupp I, Schartl A, Meyer MK, Nanda I, Schmid M, Epplen JT & Parzefall J 1991 On the stability of dispensable constituents of the eukaryotic genome: stability of coding sequences versus truly hypervariable sequences in a clonal vertebrate, the Amazon molly *Poecilia formosa*. *Proceedings of the National Academy of Sciences of the USA* **88** 8759–8763.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf Badge, RL & Goodfellow PN 1990 A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346** 240–244.

Steele RE, Mellor LB, Sawyer WK, Wavary JM & Browne LJ 1987 *In vitro* and *in vivo* studies demonstrating potent and selective estrogen inhibition with nonsteroidal aromatase inhibitor CGS 16949A. *Steroids* **50** 1–3.

Thomas EO, Licht P, Wibbels T & Crews D 1992 Development of hydroxysteroid dehydrogenase activity in sexually undifferentiated and differentiated embryos of the turtle. *Trachemys (Pseudemys) scripta*. *Biology of Reproduction* **46** 140–145.

Turner B & Steeves HR 1989 Induction of spermatogenesis in an all-female fish species by treatment with an exogenous androgen. In *Evolution and Ecology of Unisexual Vertebrates* pp 113–122. Eds RM Dawley & JP Bogart. Albany, New York: The New York State Museum, Bulletin 466.

White RB & Thomas P 1992*a* Stimulation of *in vitro* steroidogenesis by pituitary hormones in a turtle (*Trachemys scripta*) within the temperature-sensitive period for sex determination. *Biology of Reproduction* **47** 952–959.

White RB & Thomas P 1992*b* Adrenal-kidney and gonadal steroidogenesis during sexual differentiation of a reptile with temperature-dependent sex determination. *General and Comparative Endocrinology* **88** 10–19.

White RB & Thomas P 1992*c* Whole-body and plasma concentrations of steroids in the turtle, *Trachemys scripta*, before, during and after the temperature-sensitive period for sex determination. *Journal of Experimental Zoology* **264** 159–166.

Wibbels T, Bull JJ & Crews D 1991*a* Synergism of temperature and estradiol: A common pathway in sex determination? *Journal of Experimental Zoology* **260** 130–134.

Wibbels T, Bull JJ & Crews D 1991*b* Chronology and morphology of temperature-dependent sex determination. *Journal of Experimental Zoology* **260** 371.

Wibbels T, Bull JJ & Crews D 1992 Steroid hormone-induced male sex determination in an amniotic vertebrate. *Journal of Experimental Zoology* **262** 454–457.

Wibbels T & Crews D 1992 Specificity of steroid hormone-induced sex determination in a turtle. *Journal of Endocrinology* **133** 121–129.

Wibbels T, Gideon P, Bull JJ & Crews D 1993 Estrogen- and temperature-induced medullary cord regression during gonadal differentiation in a turtle. *Differentiation* **53** 149–154.

Witschi E & Dale E 1962 Steroid hormones at early developmental stages of vertebrates. *General and Comparative Endocrinology* (Suppl) **1** 356–361.

Witschi E 1939 Modification of the development of sex in lower vertebrates. In *Sex and Internal Secretions* pp 145–226. Eds CH Danforth & EA Doisy. Philadelphia: WB Saunders Co.

Yamamoto T 1962 Hormonic factors affecting sex differentiation in fish. *General and Comparative Endocrinology* (Suppl) **1** 341–345 1962.

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