GENERAL AND COMPARATIVE ENDOCRINOLOGY 99, 316–322 (1995)

Making Males from Females: The Effects of Aromatase Inhibitors on a Parthenogenetic Species of Whiptail Lizard

KIRA L. WENNSTROM AND DAVID CREWS

Institute of Reproductive Biology, Department of Zoology, University of Texas at Austin, Austin, Texas 78712

Accepted May 16, 1995

The parthenogenetic whiptail lizard *Cnemidophorus uniparens* provides a good model for the study of sex determination and sexual differentiation because genetic variation is minimal and all unmanipulated embryos will develop as females. Thus any deviation from the established course of development can be identified as a treatment effect. Previous work has shown that early prenatal treatment with CGS16949A, a nonsteroidal aromatase inhibitor, causes hatchlings to develop as males. The present study explores more fully the effects of dosage and timing of application of CGS16949A and examines the sex-reversing potential of CGS20267, a new and reputedly more potent aromatase inhibitor. Eggs were treated with a range of dosages of the aromatase inhibitors. Hatchlings that received 1 μg or more of either inhibitor were all male, while those that received 0.1 μg or less were all female. No difference in potency between the two compounds was detected. Animals treated with 100 μg of CGS16949A on Day 20 of incubation or later were all female, while those treated on Day 5 were all male. Seven sex-reversed male parthenogens have been raised to sexual maturity. The animals appear similar morphologically and behaviorally to males of the sexually reproducing whiptail species. Spermatogenesis and spermiogenesis have been confirmed by histological examination of the testes and by postcopulatory cloacal swabs. Application of aromatase inhibitors has been shown to sex-reverse both avian and reptilian species. In mammals, the male-determining gene of the Y chromosome (SRY) may code for an intrinsic aromatase inhibitor. Studies show the gene's product has a binding domain which recognizes regulatory elements in the promoter of the aromatase gene. These results suggest that the superficially different sex-determining processes of many amniotes may have underlying mechanisms in common. © 1995 Academic Press, Inc.

The desert–grassland whiptail lizard, *Cnemidophorus uniparens*, reproduces by obligate parthenogenesis. It belongs to a large genus of lizards inhabiting the southwestern United States and South and Central America. Roughly one-third of the species are parthenogenetic and many of these are polyploid, the result of hybridization within the genus (Lowe and Wright, 1966; Wright, 1993). *C. uniparens* arose from the mating of a female *Cnemidophorus inornatus* with a male from another sexual *Cnemidophorus* species, probably *Cnemidophorus burti*. The resulting diploid hybrid mated with a male *C. inornatus*, producing the triploid parthenogen (Wright, 1993).

Because all individuals of the species are female, *C. uniparens* is a good model for the study of sex determination and sexual differentiation. Not only is genetic variation minimal, but knowing in advance that unmanipulated embryos will develop as females means that any deviation from that course of development can be readily identified as an effect of treatment. Initially, as in mammals, the gonads of reptilian embryos are bipotential, having both cortical and medullary tissue. In addition, both sexes have Wolffian and Müllerian duct systems. In males, the medullary portion of the gonad proliferates and the Wolffian duct system develops into the epididymes and vas deferens while the cortical area of the gonad and the Müllerian duct system both regress. In female embryos, the cortical layer of the gonad proliferates and the Müllerian duct system differentiates into oviducts and portions of the cloaca, while the medullary region of the gonad and the Wolffian duct system degenerate (see Raynaud and Pieau, 1985, for review). In the past, attempts

0016-6480/95 $12.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

have been made to alter this differentiation process in individuals of *C. uniparens* by applying exogenous hormones to developing embryos (Billy and Crews, 1986; Crews *et al.,* 1983). While these attempts were successful in producing animals with intact Wolffian ducts, all hatchlings had entirely normal ovaries. It therefore seemed possible that the genes necessary to support male development had been lost or suppressed in the transition from sexual to parthenogenetic reproduction.

However, it was recently reported that the nonsteroidal aromatase inhibitor 4-(5,6,7,8,-tetrahydroimidazo[1,5-α]pyridin-5-yl) benzonitrile monohydrochloride (CGS16949A) could sex-reverse genetically female chicken embryos (Elbrecht and Smith, 1991), producing chicks with testes, vasa deferentia, and male-typical external genitalia. Based on this success, Wibbels and Crews (1994) applied the compound to eggs from *C. uniparens,* producing gonadal males in the heretofore all-female species. The purpose of the present study is to expand on the work of Wibbels and Crews (1994) by exploring more fully the effects of CGS16949A on this parthenogenetic species as well as to examine the sex-reversing potential of a new and reputedly more potent aromatase inhibitor, CGS20267.

MATERIALS AND METHODS

Animals. Adult *C. uniparens* were collected near Portal, Arizona, and transported to the laboratory at the University of Texas at Austin. There animals were housed four per cage and maintained as described previously (Gustafson and Crews, 1981). Under our laboratory conditions, whiptails have two reproductive seasons each year, during which each animal lays one to three clutches of two to five eggs. In the reproductive seasons, each animal was examined weekly by abdominal palpation to determine the stage of ovarian development. Animals with oviductal eggs were checked daily for oviposition.

Hatchlings were housed 10 per cage in 10-gallon aquaria under the same environmental conditions as the adults. The cages contained 3–4 cm of sand, a water bowl, and a small pine board placed on the sand as a refuge. A 60-W bulb with a reflector was suspended at one end of the cage to provide a thermal gradient. Hatchlings were fed daily with 2-week-old crickets (Fluker Feeder Insects, Baton Rouge, LA). Once per week the crickets were dusted with vitamin powder containing calcium and vitamin D_3. The diet of older hatchlings (>6 weeks) was supplemented with small mealworms.

Egg collection and treatment. Eggs were collected within 24 hr of oviposition. Each clutch was placed in a 30-ml plastic cup containing 32 g of a 1:1 vermiculite:water mixture. The cups were sealed with plastic sandwich bags secured by a rubber band and placed in an incubator at 28.5°. At this temperature, the hatchlings emerged in an average of 57 days. Two nonsteroidal aromatase inhibitors, CGS16949A and CGS20267 (4-[1-(cyanophenyl)-1-(1,2,4-triazolyl) methyl] benzonitrile) (Ciba-Geigy, Summit, NJ), were dissolved in 95% ethanol at varying concentrations. Lizard eggs (average weight 0.56 ± 0.01 g, range 0.45 to 0.73 g, K. Wennstrom, personal observation) were treated by spotting 5 μl of aromatase inhibitor solution directly onto the eggshell. Previous experiments in this lab using estradiol indicate that this method of drug administration provides a sustained, relatively steady transfer of the administered compound across the eggshell (Crews *et al.,* 1991). Clutches were split between treatment and control and at the time of treatment the incubation cups were assigned codes to prevent bias in sexing the hatchlings.

Sexing of hatchlings. At hatch, the animals were sexed by examination of postanal scale morphology. As in many other lizard species (Smith, 1946), male individuals of *C. inornatus,* the sexual ancestor of *C. uniparens,* have two enlarged scales just posterior to the lip of the cloaca. In females the scales increase in size gradually from the cloacal lip down the tail, with no individual scales prominent. The same pattern exists between normal and sex-reversed *C. uniparens;* sex-reversed individuals have enlarged postanal scales while unmanipulated parthenogens do not. The scales are sexually dimorphic at hatch, permitting accurate sexing without sacrifice of the hatchlings. Subsequent visual and/or histological examination of the gonads of hatchlings sexed by this method indicates accuracy exceeding 98% ($n = 52$). In some cases, full-term embryos that failed to hatch were pulled from eggs and used in the study. These individuals were sexed by visual inspection of the reproductive system through a dissecting microscope. Embryos were counted as male if no oviducts were present and if the gonads were smooth, round, and appeared in a staggered placement along the rostral-caudal axis. Ovaries are somewhat more irregularly shaped, tend to be oval in outline, and are situated very nearly opposite one another across the rostral caudal axis. In many cases, well-developed hemipenes could be seen in the male embryos. This method of sexing is highly accurate; previous experience indicates 100% agreement between this method and subsequent histological evaluation (Wibbels and Crews, 1994; K. Wennstrom, personal observation). Table 1 summarizes the numbers of individuals in each treatment group sexed by each method.

Statistical analysis. Data were analysed with a χ^2 contingency table using Systat version 5.1 on a Macintosh IIci computer. Values of $P \leqslant 0.05$ were considered statistically significant.

Experiment 1: CGS16949A dosage. The purpose of this experiment was to determine the sex-reversing potential of

TABLE 1
ANALYSIS OF TREATED INDIVIDUALS INDICATING DOSAGE AND TIMING OF TREATMENT

Agent	Treatment (μg)	Date (days)	Total	Visual	Scales	Histology
CGS16949A	100	5	21	2	17	2
	100	20	7	7	0	0
	100	27	6	2	4	1
	100	48	8	2	6	0
	10	5	27	2	21	4
	1	5	25	2	18	5
	0.1	5	16	1	11	4
	0.01	5	7	1	2	4
CGS20267	1	5	9	2	7	0
	0.1	5	5	2	3	0
	0.01	5	4	1	3	0
Ethanol only		5	25	3	21	1

Note. Individuals were sexed by visual inspection of the gonads and genital ducts, morphology of postanal scales, and/or histological examination of the gonads. No individuals in this study were sexed by both visual and histological examination.

CGS16949A at a range of dosages. All eggs were treated on Day 5 of incubation as described above with one of the following dosages: 0.01 (n = 7), 0.1 (n = 16), 1.0 (n = 25), 10 (n = 27), and 100 μg (n = 21). These sex ratios were compared to that of a control set of eggs treated with 95% ethanol only (n = 25).

Experiment 2: Comparison of CGS16949A and CGS20267. The purpose of this experiment was to compare the relative potencies of two aromatase inhibitors, CGS16949A and CGS20267. Eggs in this experiment were treated on Day 5 of incubation as described above with one of the following dosages of CGS20267: 0.01 (n = 4), 0.1 (n = 5), or 1 μg (n = 9). The sex ratios obtained from these treatments were then compared to those obtained from eggs treated as part of Experiment 1.

Experiment 3: Timing of administration. The purpose of this experiment was to examine the effect of the timing of application of the aromatase inhibitor. Previous work (Wibbels and Crews, 1994) had shown sex reversal up to and including Day 12 of incubation with a dose of 100 μg of CGS16949A. The same dose of CGS16949A was used in this experiment, applied at 20 (n = 7), 28 (n = 6), or 47 days (n = 8) after oviposition. Only a limited number of eggs are available each season, and these dates were chosen to cover as much of the incubation period as possible. Days 28 and 47 represent the midpoints of the second and third trimesters of development, while Day 20 is the midpoint between the previously established Day 12 reference and the 28-day time point. The sex ratios obtained from these treatments were compared with those obtained at the same dosage in Experiment 1, in which the eggs were treated on Day 5 of incubation.

RESULTS

Experiments 1 and 2: CGS16949A dosage and comparison of CGS16949A and CGS20267. All animals treated with 1 μg or more of either inhibitor were male, while those treated with lower dosages were female (Fig. 1).

Experiment 3: Timing of administration. Animals treated on Day 20 of incubation or later were all female ($P < 0.001$).

Seven sex-reversed male parthenogens representing four different CGS16949A dosages (100, 25, 10, and 1 μg) have thus far been raised to sexual maturity. The animals have maintained their male phenotype and appear similar morphologically and behaviorally to males of their sexual ancestral species. All have secretory femoral pores and bluish ventral coloration, which are secondary sexual characteristics typical of males of the sexual ancestral species *C. inornatus.* All have shown male-typical courtship and mounting behavior when presented with a receptive female conspecific. Spermatogenesis and spermiogenesis have been confirmed both by histological examination of testes from sexually active individuals and by postcopulatory cloacal swabs. The testes of the sex-reversed male parthenogens appear very similar to those of male *C. inornatus* (Fig. 2). However, while the spermatids in the *C. inornatus* testis are uniform in size and shape, with elongated, conical heads, those in the *C. uniparens* testis are sparser and more variable in shape. Many have heads which are bent, twisted, or globular, characteristics which were also evident in the

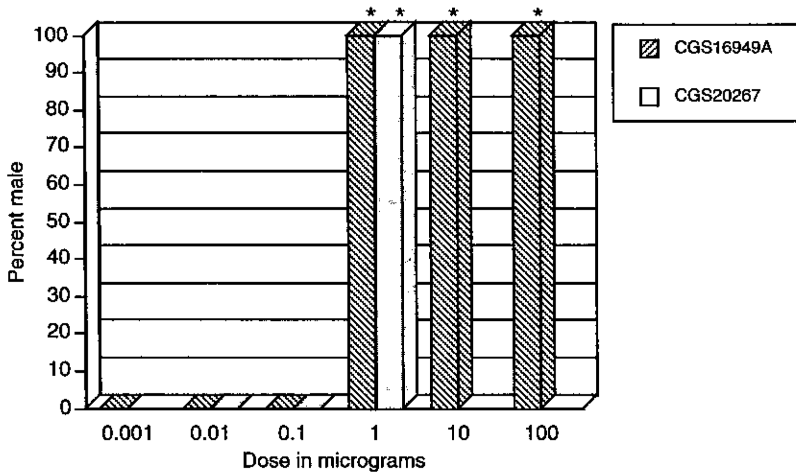

FIG. 1. Percentage of male *Cnemidophorus uniparens* hatchlings produced with a range of dosages of two aromatase inhibitors, CGS16949A and CGS20267. Compound CGS20267 was tested only at .01, 0.1, and 1 μg. The asterisks indicate significance at $P \leq .001$ in comparison to the 0.0-μg dose.

spermatazoa obtained from cloacal swabs. It is unknown whether these sperm are viable, though many are motile. The cells of the triploid *C. uniparens* testis, including the spermatids, are visibly larger than those of the diploid *C. inornatus*. When the nuclei of a random sample of 25 cells from the walls of the seminiferous tubules of each of the two testes were measured using a computer imaging system, the *C. uniparens* nuclei averaged 1.45 times the size of those of *C. inornatus* (cross-sectional area 3.50 $\times 10^{-5}$ mm^2 vs 2.41 $\times 10^{-5}$ mm^2), a value that closely corresponds to the 50% greater amount of DNA the parthenogen carries.

DISCUSSION

The results of the present study indicate that parthenogenetic whiptails can be sex-reversed by the application *in ovo* of two nonsteroidal aromatase inhibitors, CGS16949A and CGS20267. The two inhibitors appear to have similar potency in this system, and doses as low as 1.8 μg per gram of egg produce 100% male

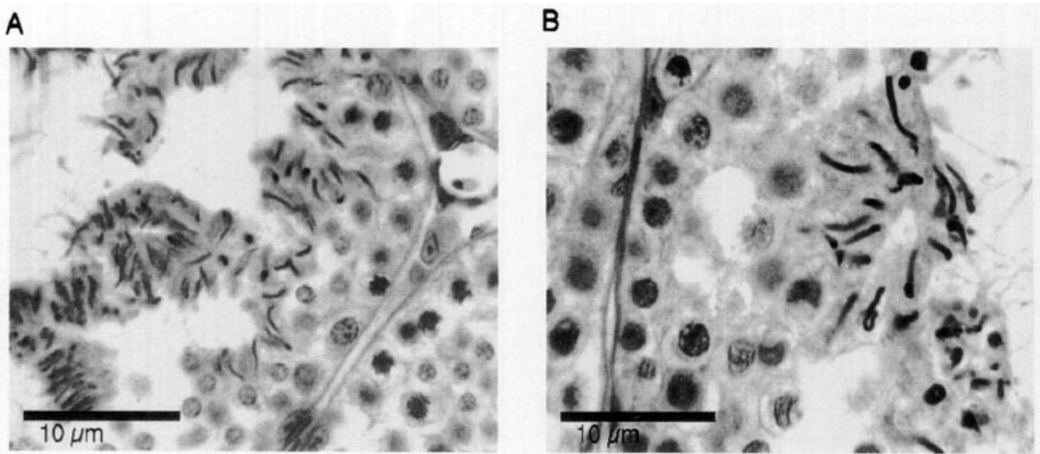

FIG. 2. Cross-section of seminiferous tubules in the testes of a normal male *Cnemidophorus inornatus* (A) and a sex-reversed, genetically female *C. uniparens* (B). Both individuals were reproductively active.

hatchlings. In this respect, whiptails appear to be more sensitive to the effects of the inhibitors than the other reptile species in which they have been tried. At a dose of 10 μg/g, just 30% male red-eared slider turtles are produced at a normally female-producing temperature (Crews and Bergeron, 1994). In the alligator, treatment with 2 μg/g of CGS16949A was unable to induce male development in eggs incubated at a female-producing temperature, although ovarian development was disrupted (Lance and Bogart, 1992). However, in the chicken, a dose of 2 μg/g produced 100% males (Elbrecht and Smith, 1991). This difference in sensitivity may be related to the fact that while chickens and whiptails have genetic sex determination (GSD), the turtle and alligator have temperature-dependent sex determination (TSD). In TSD species, it has consistently been more difficult to produce males than to produce females (Crews *et al.,* 1989; Gutzke and Bull, 1986; Raynaud and Pieau, 1985; Wibbels *et al.,* 1992).

Treatment of eggs on or after Day 20 of incubation produced 100% female hatchlings. Based on these results and those of Wibbels and Crews (1994), the sensitive period for sex reversal in this species appears to end between Days 12 and 20 of incubation. Knowledge of the embryology of this species is incomplete at best, and intraspecific variation in stage of development at the time of oviposition appears to be large. However, the best evidence indicates that the gonadal ridge forms at stage 10 (Billy, 1986), 58 pairs of somites, which corresponds approximately to Days 2 or 3 of incubation. The ovary first appears at stage 11, 62 pairs of somites, which was reported to occur as early as Day 9 or as late as Day 22 of incubation. A large portion of this variability may be attributed to the inconstant egg incubation temperatures in this study. The temperature at which the eggs are incubated greatly affects the rate of development (Crews, 1989). Based on these data, sex reversal is possible throughout the period in which the gonad is bipotential and possibly for a short time after ovarian development is initiated.

While the sex determination of individuals treated on or after Day 20 was unaffected, it remains possible that some aspect of sexual differentiation was altered, e.g., sexual differentiation of the brain. Gonadal steroids have long been known to play important roles in this process in many species (Harris and Levine, 1965; Young, 1964; Young *et al.,* 1964). In particular, the aromatization of testicular androgens is necessary to the process of brain masculinization in several mammalian and avian species (Carroll *et al.,* 1988; McEwen *et al.,* 1977; Naftolin and MacLusky, 1984; Olsen, 1988; Sayag *et al.,* 1989).

Since this parthenogenetic species still has the capacity not only to form testes but to support the formation and release of motile sperm, it is apparent that the genes required for these processes have been retained, even though no male of the species develops naturally. All that is lacking is a developmental trigger. The fact that an exogenous aromatase inhibitor can provide that trigger raises questions about the role of steroids and steroidogenic enzymes in the sex determination process (Crews, 1994). Although sex steroids have long been known to play pivotal roles in sexual differentiation (Jost, 1953), there is little evidence that they affect sex determination in amniotes with GSD. However, recent experiments have shown that the putative DNA binding domain of the murine SRY gene product recognizes upstream regulatory elements in the promoters of the genes for both aromatase and anti-Müllerian hormone (AMH) (Haqq *et al.,* 1993). This indicates that the control of aromatase activity may be a key step in mammalian sex determination as well. Further indication that this may be the case comes from evidence that AMH itself can act as an inhibitor of aromatase biosynthesis, blocking the production of estrogen while concomitantly increasing the relative amount of androgens, in effect producing an endocrinological sex reversal of the fetal ovaries of several mammalian species (Di Clemente *et al.,* 1992; Vigier *et al.,* 1989). Estrogen has been shown to inhibit AMH in the chick (Hutson *et al.,* 1982; Newbold *et al.,* 1984). While it has not yet been demonstrated, it is possible that a similar process may act in reptiles. If this is the case, feedback mecha-

nisms could explain the ability of the aromatase inhibitors to produce complete sex reversal of the genital ducts. In this paradigm, during normal female development aromatase activity would result in the production of estrogens, inhibiting AMH and allowing the preservation of the Müllerian ducts. In sex-reversed animals, the lack of estrogen may disinhibit AMH production, resulting in regressed Müllerian ducts.

Taken together, the information presented here indicates that the sex-determining processes of reptiles, birds, and mammals—though there are important differences—may have underlying mechanisms in common. Since both birds and mammals arose from reptilian ancestors, the study of reptilian sex determination and sexual differentiation can shed light on the same processes in mammals.

ACKNOWLEDGMENTS

The authors thank A. Alexander and A. Lillie for technical assistance. This research was supported by NSF predoctoral (K.L.W.) and by NIMH Research Scientist Award 00135 and NIMH MERIT Award 41770 (both to D.C.).

REFERENCES

Billy, A. J. (1986). Sexual differentiation in a unisexual reptile, *Cnemidophorus uniparens* (Teiidae). Doctoral dissertation. University of Texas at Austin.

Billy, A. J., and Crews, D. (1986). The effects of sex steroid treatments on sexual differentiation in a unisexual lizard, *Cnemidophorus uniparens* (Teiidae). *J. Morphol.* **187,** 129–142.

Carroll, R. S., Weaver, C. E., and Baum, M. J. (1988). Evidence implicating aromatization of testosterone in the regulation of male ferret sexual behavior. *Physiol. Behav.* **42,** 457–460.

Crews, D. (1989). Absence of temperature-dependent sex determination in congeneric sexual and parthenogenetic *Cnemidophorus* lizards. *J. Exp. Zool.* **252,** 318–320.

Crews, D. (1994). Temperature, steroids, and sex determination. *J. Endocrinol.* **142,** 1–8.

Crews, D., and Bergeron, J. M. (1994). Role of reductase and aromatase in sex determination in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. *J. Endocrinol.* **143,** 279–289.

Crews, D., Bull, J. J., and Wibbels, T. (1991). Estrogen and sex reversal in turtles: A dose-dependent phenomenon. *Gen. Comp. Endocrinol.* **81,** 357–364.

Crews, D., Gustafson, J. E., and Tokarz, R. (1983). Psychobiology of parthenogenesis in reptiles. *In* ''Lizard Ecology'' (R. B. Huey, E. R. Pianka, and T. W. Schoener, Eds.), pp. 205–231. Harvard Univ. Press, Cambridge, MA.

Crews, D., Wibbels, T., and Gutzke, W. H. N. (1989). Action of sex steroid hormones on temperature-induced sex determination in the snapping turtle (*Chelydra serpentina*). *Gen. Comp. Endocrinol.* **76,** 159–166.

Di Clemente, N., Ghaffari, S., Pepinsky, R. B., Pieau, C., Josso, N., Cate, R. L., and Vigier, B. (1992). A quantitative and interspecific test for biological activity of anti-Müllerian hormone: The fetal ovary aromatase assay. *Development* **114,** 721–727.

Elbrecht, A., and Smith, R. G. (1991). Aromatase enzyme activity and sex determination in chickens. *Science* **255,** 467–470.

Gustafson, J. E., and Crews, D. (1981). Effect of group size and physiological state of a cagemate on reproduction in the parthenogenetic lizard *Cnemidophorus uniparens* (Teiidae). *Behav. Ecol. Sociobiol.* **8,** 267–272.

Gutzke, W. H. N., and Bull, J. J. (1986). Steroid hormones reverse sex in turtles. *Gen. Comp. Endocrinol.* **71,** 265–267.

Haqq, C. M., King, C. Y., Donhoe, P. K., and Weiss, M. A. (1993). SRY recognizes conserved DNA sites in sex-specific promoters. *Proc. Natl. Acad. Sci. USA* **90,** 1097–1101.

Harris, G. W., and Levine, S. (1965). Sexual differentiation of the brain and its experimental control. *J. Physiol.* **181,** 379–400.

Hutson, J., Ikawa, H., and Donahoe, P. K. (1982). Estrogen inhibition of Müllerian inhibiting substance in the chick embryo. *J. Pediatr. Surg.* **17,** 953–959.

Jost, A. (1953). Problems of fetal endocrinology: The gonadal and hypophyseal hormones. *Recent Prog. Horm. Res.* **8,** 379–418.

Lance, V. A., and Bogart, M. H. (1992). Disruption of ovarian development in alligator embryos treated with an aromatase inhibitor. *Gen. Comp. Endocrinol.* **86,** 59–71.

Lowe, C. H., Jr., and Wright, J. W. (1966). Evolution of parthenogenetic species of *Cnemidophorus* (whiptail lizards) in western North America. *J. Ariz. Acad. Sci.* **4,** 81–87.

McEwen, B. S., Lieberburg, I., Chaptal, C., and Krey, L. C. (1977). Aromatization: Important for sexual differentiation of the neonatal rat brain. *Horm. Behav.* **9,** 249–263.

Naftolin, F., and MacLusky, N. (1984). Aromatization hypothesis revisited. *In* ''Sexual Differentiation: Basic and Clinical Aspects'' (M. Serio, M. Motta, M. Zanisi, and L. Martini, Eds.), pp. 79–91. Raven Press, New York.

Newbold, R. R., Suzuki, Y., and MacLachlan, J. A. (1984). Müllerian duct maintenance in heterotypic organ culture after in vivo exposure to diethylstilbestrol. *Endocrinology* **115,** 1863–1868.

Olsen, K. L. (1988). A comparison of the effects of three androgens on sexual differentiation in female hamsters. *Physiol. Behav.* **42,** 569–573.

Raynaud, A., and Pieau, C. (1985). Embryonic development of the genital system. *In* "Biology of the Reptilia" (C. Gans, Ed.), Vol. 15, pp. 149–300. Wiley, New York.

Sayag, N., Snapir, N., Robinzon, B., Arnon, E., El Halawani, M. E., and Grimm, V. E. (1989). Embryonic sex steroids affect mating behavior and plasma LH in adult chickens. *Physiol. Behav.* **45,** 1107–1112.

Smith, H. M. (1946). "Handbook of Lizards." Comstock, Ithaca, NY.

Vigier, B., Forest, M. G., Eychenne, B., Bezard, J., Garrigou, O., Robel, P., and Josso, N. (1989). Anti-Müllerian hormone produces endocrine sex reversal of fetal ovaries. *Proc. Natl. Acad. Sci. USA* **86,** 3684–3688.

Wibbels, T., and Crews, D. (1994). Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with temperature-dependent sex determination. *J. Endocrinol.* **141,** 295–299.

Wibbels, T., Bull, J. J., and Crews, D. (1992). Steroid-hormone induced male sex determination in an amniotic vertebrate. *J. Exp. Zool.* **262,** 454–457.

Wright, J. W. (1993). Evolution of the lizards of the genus *Cnemidophorus*. *In* "Biology of Whiptail Lizards (Genus *Cnemidophorus*)" (J. W. Wright and L. J. Witt, Eds.), pp. 27–81. Oklahoma Museum of Natural History, Norman, OK.

Young, W. C. (1964). The hormones and behavior. *In* "Comparative Biochemistry: A Comprehensive Treatise" (M. Florkin and H. S. Mason, Eds.), Vol. VII, pp. 203–239. Academic Press, New York.

Young, W. C., Goy, R. W., and Phoenix, C. H. (1964). Hormones and sexual behavior. *Science* **143,** 212–218.