

Effects of Incubation Temperature and Estrogen Exposure on Aromatase Activity in the Brain and Gonads of Embryonic Alligators

Matthew R. Milnes, Robert N. Roberts, and Louis J. Guillette Jr.

Department of Zoology, University of Florida, Gainesville, Florida, USA

During embryogenesis, incubation temperature and the hormonal environment influence gonadal differentiation of some reptiles, including all crocodylians. Current evidence suggests that aromatase, the enzyme that converts androgens to estrogens, has a role in sexual differentiation of species that exhibit temperature-dependent sex determination (TSD). During the temperature-sensitive period (TSP) of sex determination, we compared aromatase activity in the brain and gonads of putative male and female alligator embryos to determine if aromatase activity in the embryonic brain could provide the hormonal environment necessary for ovarian development in a TSD species. In addition, we assessed the pattern of aromatase activity in the brain and gonads of embryos treated with estradiol-17 β (E₂) and incubated at male-producing temperatures to compare enzyme activity in E₂ sex-reversed females to control males and females. This has particular significance regarding wildlife species living in areas contaminated with suspected environmental estrogens. Gonadal aromatase activity remained low during the early stages of the TSP in both sexes and increased late in the TSP only in females. Aromatase activity in the brain increased prior to gonadal differentiation in both sexes. These results suggest that aromatase activity in the brain is not directly responsible for mediating differentiation of the gonad. E₂ exposure at male-producing temperatures resulted in sex-reversed females that had intermediate gonad function and masculinized brain activity. This study indicates the need to examine multiple end points and to determine the persistence of developmental alterations in contaminant-exposed wildlife populations. **Key words:** alligators, aromatase, embryos, estrogen, sex determination. *Environ Health Perspect* 110(suppl 3):393–396 (2002).
<http://ehpnet1.niehs.nih.gov/docs/2002/suppl-3/393-396milnes/abstract.html>

During embryonic development in reptiles, steroids influence sexual differentiation of the gonad and the brain. In many vertebrates, the presence or absence of sex-specific chromosomes ultimately determines sexual differentiation. In others, environmental influences such as temperature can be the pivotal factor in determining ovarian or testicular development. Temperature-dependent sex determination (TSD), in which egg incubation temperature determines the sex of the developing embryo, exists in many reptiles, including all crocodylians, most turtles, and some lizards (1). In the American alligator (*Alligator mississippiensis*), incubation temperatures near the low (30°C) and high (34.5°C) end of the viable range produce all females, whereas temperatures near 33°C produce all males (2). Incubating eggs collected from a Louisiana population, Lang and Andrews (2) produced 100% males at 32.5 and 33°C, and 84% males at 33.5°C. Unpublished data from our lab incubating alligator eggs from north central Florida over a 6-year period have resulted in approximately 80% males at 33°C and 100% males at 33.5°C, suggesting the existence of geographic variation in response to incubation temperature. The temperature-sensitive period (TSP) in alligators has been shown to occur during the third quarter of development, in which the bipotential gonad commits to either ovarian or testicular development (2,3).

Unlike mammals, where female differentiation appears to be the default in the absence of androgens (4), estrogens appear to play a key role in the sexual differentiation of nonmammalian vertebrates including birds (5) and reptiles (1). The administration of exogenous estrogens prior to the TSP can override the effects of male incubation temperatures on sexual differentiation in the freshwater turtle *Trachemys scripta* (6) and alligators (7,8). This indicates that the undifferentiated gonad responds either directly to estrogen or indirectly by way of some estrogen-sensitive, extragonadal tissue.

The aromatase enzyme complex (aromatase cytochrome P450 and the flavoprotein nicotinamide adenine dinucleotide phosphate [NADPH]-cytochrome P450 reductase) is responsible for the conversion of androgens to estrogens. Aromatase activity has been detected in the gonad, brain, liver, and adipose tissue of many vertebrate species. The role of this steroidogenic enzyme is sex- and tissue-dependent, and varies according to the developmental stage of the organism. Most research on aromatase and TSD in reptiles has focused on gonadal aromatase activity. Treatment of eggs with aromatase inhibitors causes male development at female-producing temperatures in *T. scripta* (9) and prevents normal ovarian development in the alligator (10). However, gonadal aromatase, which exhibits increased

mRNA expression and estrogen synthesis only near the end of the TSP in crocodylians (11–13) and turtles (14,15), does not appear to be the primary signal for ovarian development. The question remains, what is the normal signaling mechanism that causes ovarian development and how is this signal duplicated at male-producing temperatures in the presence of exogenous estrogens?

Recent research suggests that the brain plays a role in sexual differentiation in TSD species. Sexually dimorphic transcription of the aromatase gene has been detected in diamondback terrapin embryos (*Malaclemys terrapin*) during the early stages of sex determination, with a greater abundance of aromatase transcripts in the female brain (16). During the second half of the TSP, aromatase activity increases in the male brain to levels greater than those in the female brain (16). Willingham et al. (14) measured aromatase activity in the brains of male and female *T. scripta* embryos and found activity levels in female brains that were higher than those in males at the beginning of the TSP. Aromatase activity of both sexes decreased following the end of the TSP and dropped below detection levels in females prior to hatching (14). In contrast to the sexually dimorphic brain aromatase expression reported in turtles, no significant differences were found in brain mRNA of alligator embryos incubated at male- and female-producing temperatures (13). Although substantial evidence implicating the brain in directing gonadal differentiation is lacking, temperature appears to influence sexual differentiation of the brain during embryonic development in some TSD species.

This article is part of the monograph *Impact of Endocrine Disruptors on Brain Development and Behavior*.

Address correspondence to L.J. Guillette, University of Florida, Dept. of Zoology, 223 Bartram Hall, Box 118525, Gainesville, FL 32611 USA. Telephone: (352) 392-1098. Fax: (352) 392-3704. E-mail: ljjg@zoo.ufl.edu

We thank A. Woodward for assistance with field collections and T. Bryan for assistance with egg incubation and treatments. All lab and field work were conducted in full compliance with the University of Florida Institutional Animal Care and Use Committee. This research was supported by Sigma Xi Grants-in-Aid of Research to M.R.M. and U.S. Environmental Protection Agency grant CR826357-01-1 to L.J.G.

Received 8 January 2002; accepted 26 March 2002.

Like several TSD species of turtles in which low doses of estrogenic compounds cause the development of female offspring at male-producing temperatures, the alligator has become a model for screening environmental contaminants for estrogenicity. Several pesticides and pesticide metabolites that induce ovarian development at environmentally relevant concentrations include *o,p'*-DDE, *p,p'*-DDE (17), *p,p'*-DDD (18), and *trans*-nonachlor (19). Although the mechanism by which these compounds influence sexual differentiation is poorly understood, all show some affinity for the alligator estrogen receptor (aER) (20). The herbicide atrazine shows a weak affinity for the aER (20) and causes testicular aromatase activity uncharacteristic of males or females but does not cause sex reversal (8).

Although the feminizing action of estrogenic compounds has been well documented in terms of gonadal morphology in alligators, little is known about the functional consequences of chemically induced sex reversal. Field studies of several contaminated lakes in Florida have shown a number of functional abnormalities in female alligators, including elevated ovarian synthesis of testosterone, elevated hepatic degradation of testosterone, and reduced ovarian synthesis of estradiol-17 β (E₂) [for review see Guillette (21)]. Because nothing is known concerning the incubation conditions of the animals obtained for these studies, it is unknown if any were sex reversed as a result of embryonic contaminant exposure. The possibility exists that the differences observed in the exposed populations are due in part to altered endocrine function in sex-reversed females.

Given the uncertainty of the mechanisms and consequences of chemically induced sex reversal, we conducted an initial study to examine the timing and levels of aromatase activity in the brain and gonads of putative female, male, and sex-reversed female alligator embryos. Our purpose was to determine if sexual dimorphism in whole-brain aromatase activity could provide a means of directing gonadal differentiation and to compare aromatase activity in the brain and gonads of E₂ sex-reversed females to that in the brain and gonads of untreated males and females.

Materials and Methods

Animals and Tissue Collection

Six clutches of alligator eggs were collected from Lake Woodruff National Wildlife Refuge, Volusia County, Florida, within the first 2 weeks postoviposition. Eggs were transported to the University of Florida (Gainesville, Florida, USA) and incubated in damp sphagnum moss at an intermediate

temperature of 32°C until reaching embryonic stage 19. Fifteen eggs from each clutch were systematically assigned to three treatment groups and three dissection stages within each treatment group to avoid clutch effects within the experiment. Treatment groups consisted of control females incubated at 30°C, control males incubated at 33.5°C, and sex-reversed females incubated at 33.5°C and were treated topically with 90 μ g E₂ dissolved in 50 μ L 95% ethanol at stage 19. Previous studies show alligator eggs incubated at male-producing temperatures treated with similar doses of E₂ result in 100% female hatchlings (2,7,8). Additional eggs (three to four per clutch) were incubated at each temperature to verify the appropriate stages for dissection of each clutch.

Ten embryos from each treatment group were selected for dissection at stages 20 (early TSP), 22 (middle TSP), and 24 (late TSP). Upon reaching the appropriate stage, embryos were decapitated immediately upon removal from the egg. Brains and paired gonad–adrenal–mesonephros complexes (GAMs) were removed, flash frozen in liquid nitrogen, and stored at –70°C until assayed. Entire GAMs were used because of the difficulty in separating the three tissues; published research shows that the majority of aromatase activity takes place in the gonad portion of the complex (12).

Aromatase Activity Assay

The tritiated water assay used to measure aromatase activity was a modification of methods described by Lephart and Simpson (22) and Willingham et al. (14). All buffers and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified. Whole brains or paired GAMs were homogenized over ice in 100 μ L homogenate buffer (RPMI-1640 culture medium supplemented with 25 mM Hepes and 1 mM dithiothreitol) in microcentrifuge tubes using a handheld pellet pestle (Kontes, Vineland, NJ, USA). Tissue homogenates were transferred to glass culture tubes along with 400 μ L substrate buffer. Substrate buffer consisted of homogenate buffer supplemented with 1 mM NADPH, 10 mM α -D-glucose 6-dehydrogenase, 1 U/mL glucose 6-dehydrogenase, and 0.8 μ M [1 β -³H] androstenedione (DuPont NEN Research Products, Boston, MA, USA). Culture tubes were covered with parafilm and incubated on a shaker at 32°C.

Following 9 hr of incubation for brains and 6 hr for GAMs, 1.5 mL chloroform was added to halt the reaction. The volume of the aqueous phase was brought up to 900 μ L with the addition of 400 μ L deionized water. Culture tubes were then pulse vortexed and centrifuged at 2,000 \times g for 15 min. A 600- μ L aliquot of the aqueous phase was transferred

to a new tube, and 600 μ L of 5% charcoal/0.5% dextran was added before the tube was vortexed and centrifuged at 2,000 \times g for 15 min. Five milliliters of scintillation fluid (Scintillation BD, Fisher Scientific, Pittsburgh, PA, USA) was added to 300 μ L supernatant, and the tube was counted on a Beckman scintillation counter (Beckman Instruments, Schaumburg, IL, USA).

Aromatase activity is proportional to the amount of tritiated water produced by the cleavage of hydrogen from androstenedione at the 1 β position. Activity was calculated by multiplying the sample decays per minute (dpm) by 3, subtracting the background (blank tube), and dividing by the dpm of substrate originally added. This percentage was then multiplied by the mass of substrate added and expressed as fmol/tissue/hr. Sensitivity of the assay was defined as twice the mean dpm of blank tubes, which corresponded to approximately 8 fmol/tube/hr.

Statistics

Statistical analyses were performed with StatView for Windows (23). Single-classification analysis of variance (ANOVA) was used to test for differences across stages within a treatment group and among treatment groups within a stage. A two-way ANOVA was not performed as part of the analysis because a comparison of all possible combinations of stage and treatment was not consistent with the purpose of this study. Fisher's protected least significant difference was used to make pairwise comparisons, with the level of statistical significance set at $p \leq 0.05$.

Results

GAM Aromatase Activity

No difference in GAM aromatase activity (Figure 1) was detectable among treatment groups at stage 20 ($p = 0.302$), and no differences were detectable between stages 20 and 22 within any treatment group. However, enzyme activity at stage 22 in control females was lower than that in control males ($p = 0.012$) and sex-reversed females ($p = 0.014$). Stage 24 was marked by a dramatic increase in enzyme activity in control females ($p < 0.0001$), whereas control males exhibited a slight decrease in aromatase activity ($p = 0.022$) from stage 22. A moderate increase in aromatase activity was detected in E₂-treated females ($p = 0.0009$) that was higher than that in control males ($p = 0.0001$) and lower than that in control females ($p < 0.0001$) at stage 24.

Brain Aromatase Activity

Brain aromatase activity (Figure 2) increased from stage 20 to 22 in all treatment groups,

and no differences among treatment groups were detectable at these two stages ($p = 0.359$ and 0.806 , respectively). From stage 22 to stage 24, aromatase activity increased in control males ($p = 0.011$) and females ($p = 0.001$); no difference was detected between these two groups at stage 24 ($p = 0.084$). No change in aromatase activity occurred in sex-reversed females from stage 22 to 24 ($p = 0.631$); activity in stage 24 sex-reversed females was lower than that in control females ($p = 0.013$) and was not different from that in control males ($p = 0.363$).

Discussion

Similar to previous studies (11, 12, 14, 15), aromatase activity in the GAM did not increase until the end of the TSP. It is likely that gonadal aromatase activity is associated with ovarian development, as it increased significantly between stages 22 and 24 in both control and sex-reversed females. The proliferation of cortically located germ cells and regression of medullary sex cords occur during these stages in alligators incubated at female-producing temperatures (3). However, temperature-shift experiments by Lang and Andrews (2) show sex determination to occur between stages 20 and 22 when shifting from 30 to 33°C. Aromatase activity alone does not appear to initiate ovarian differentiation, as evidenced by the low activity in both males and females during the early stages of the TSP.

The data presented in this study indicate that the GAM of sex-reversed females is neither malelike nor femalelike with regard to aromatase activity at stage 24. This is especially interesting because E_2 exposure at male-producing temperatures results in ovarian differentiation, as opposed to an intersexed gonad (7,8). That aromatase activity in the sex-reversed females was significantly lower than that in control females and higher than that in control males suggests embryonic exposure to E_2 and incubation temperature affect steroidogenic enzyme levels and/or activity. Apart from directing ovarian differentiation of the gonad, exogenous estrogen could disrupt a number of feedback mechanisms along the hypothalamic–pituitary–gonadal axis, such as gonadotropin release, causing suppression of aromatase synthesis relative to control females (4). Incubation temperature, regardless of sex, influences plasma steroid concentrations. In the red-eared slider turtle, plasma E_2 in females from intermediate incubation temperatures was significantly lower than that in juveniles from the all-female-producing temperature, and no different than that in males from the intermediate temperature (24). This effect could be mediated by the presence of anti-Müllerian hormone (AMH), which decreases

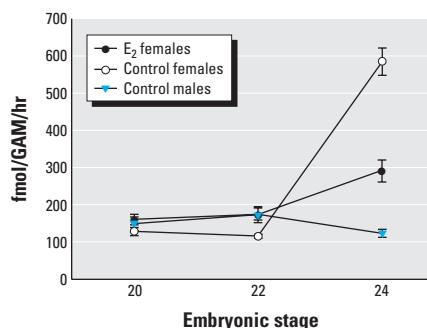


Figure 1. GAM complex aromatase activity (fmol/GAM/hr) in female (30°C), male (33.5°C), and sex-reversed female (33.5°C + E_2) alligator embryos during the early (stage 20), middle (stage 22), and late (stage 24) stages of the TSP.

aromatase synthesis in fetal ovaries of several mammal species (25). Western et al. (26) detected expression of AMH in alligator embryos incubated at male-producing temperatures beginning at stage 22 but not at female-producing temperatures at any stage. AMH expression was limited to the medullary cells of the developing testes (26), indicating a need to examine ovarian differentiation of sex-reversed females on a morphological level (e.g., *in situ* hybridization for AMH mRNA) relative to that in untreated embryos, as well as to measure multiple hormones.

Results of recent studies on aromatase in the brain of TSD species have differed according to species and end points measured. In the diamondback terrapin, transcripts of the aromatase gene were in greater abundance in females during the first half of the TSP, then higher in males during the second half (16). When aromatase enzyme activity was measured in the brain of red-eared slider turtle embryos, females exhibited an increase early in the TSP, whereas males showed no significant increase throughout the same period (14). In the alligator, transcripts of the aromatase gene did not differ between sexes and showed no significant increase for any stage of development (13). In contrast, our data indicate an increase in enzymatic activity throughout the TSP in both sexes, with slightly higher activity in putative females at stage 24, indicating that gene expression does not necessarily reflect enzyme activity. Furthermore, E_2 -induced sex reversal resulted in brain activity similar to that in control males, suggesting that sex-reversed females do not function as normal females on all levels.

Because an increase in aromatase activity occurred early in the TSP but did not differ between sexes, it is difficult to determine the role of brain aromatase activity with regard to sex determination. If the increase in brain aromatase activity is sufficient to increase circulating E_2

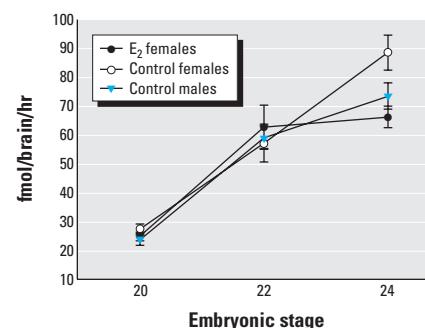


Figure 2. Brain aromatase activity (fmol/brain/hr) in female (30°C), male (33.5°C), and sex-reversed female (33.5°C + E_2) alligator embryos during the early (stage 20), middle (stage 22), and late (stage 24) stages of the TSP.

concentrations, temperature-dependent expression of the estrogen receptor (ER) could be the key to gonadal differentiation. That is, a slight increase in circulating E_2 resulting from aromatase activity in the brain early in the TSP, coinciding with an increase in ER expression in the gonad, could lead to ovarian differentiation. Bergeron et al. (27) measured ER transcripts in the gonads of red-eared slider turtle embryos and found higher concentrations in the gonads of embryos incubated at female-producing temperatures at the beginning of the TSP. However, it is not known if the estrogen produced locally in the brain is capable of crossing the blood–brain barrier to an extent great enough to affect circulating steroid concentrations. Furthermore, translation of ER transcripts to functional receptor proteins should be confirmed before strong inferences are made regarding the interplay of aromatase activity and ER expression in sex determination.

In contrast to the results from the gonads, levels of aromatase activity in the brain of sex-reversed females are not different from those of control males, indicating E_2 exposure did not override the effects of incubation temperature on enzyme activity in the brain. Studies on eutherian mammals have shown that the presence of α -fetoprotein, which binds to circulating E_2 , prevents maternal E_2 from crossing the blood–brain barrier of embryos developing *in utero* (28). Although α -fetoprotein has not been reported in any reptile, cytosolic-binding proteins have been described in the alligator that show an affinity for E_2 and, to a lesser extent, synthetic steroids and contaminants (29). Conley et al. (30) reported high concentrations of steroids (E_2 , testosterone, androstenedione) in alligator egg yolks that decline significantly during the TSP. The presence of steroid-binding proteins in developing embryos could function as a means to protect the embryo from high

concentrations of maternal steroids deposited in the yolk during vitellogenesis and prevent feminization of the brain following embryonic exposure to exogenous E₂.

Estradiol-exposure studies serve as valuable models but cannot always predict the effects of estrogenic contaminants because the pathways through which these compounds work vary widely. Although many of the environmental estrogenic compounds that cause sex reversal are capable of binding to the aER (20), many differ from natural estrogens in hepatic degradation rates, binding affinity for plasma proteins, and binding affinity for other nuclear and membrane-bound receptors. As the results of E₂ exposure differed between the brain and gonad in this study, special consideration should be given to which end points are monitored in organisms exposed to estrogenic compounds. Estrogens and aromatizable androgens have been shown to override the effect of incubation temperature on sex determination (7–9), but few studies have looked beyond gross morphology of the gonad. Egg-dosing studies in which embryos are exposed to environmentally relevant concentrations of contaminants have produced alterations similar to those reported in exposed wildlife populations. For example, alligator embryos exposed to ecologically relevant concentrations of various pesticide metabolites exhibit sex reversal but also have altered gonadal steroidogenesis and gonadal enzyme activity (18,19).

In the present study, E₂ exposure at male-producing temperatures resulted in intersexed gonadal and malelike brain aromatase activity in female embryos. Although our study did not examine specific brain regions, this initial study demonstrates that the gonad and brain respond to differing degrees after exogenous estrogen treatment. This is extraordinary, considering that the response in any given region of the brain would be tempered by the fact the entire brain was homogenized. Future studies should examine aromatase activity in distinct regions of the brain associated with sexual differentiation, such as the preoptic area and the hypothalamus. Further research is warranted to determine if alterations in enzyme activity occur following contaminant-induced sex reversal, and whether they persist in light of the endocrine alterations reported in field studies of exposed alligator populations. These data clearly demonstrate that environmental contaminants could

alter the differentiation of the gonad morphologically while having only partial influence on the differentiation of gonadal physiology. Moreover, gonadal differentiation could be affected differently from the response seen in the brain. Such differences could be associated with the timing of exposure or exposure dosage as modified by physiological phenomena such as the transfer of chemicals across the blood–brain barrier. Because of the pervasive influence of the hypothalamic–pituitary–gonadal axis in numerous endocrine activities including reproduction, growth, and metabolism, understanding the effects of environmental estrogens and antiestrogens is essential if we are to determine the impact these compounds have on development and reproduction of many wildlife species. Only a thorough assessment at the tissue, cellular, and molecular levels can determine the full impact of a chemically altered embryonic environment.

REFERENCES AND NOTES

- Pieau C. Temperature variation and sex determination in reptiles. *BioEssays* 18:19–26 (1996).
- Lang JW, Andrews HV. Temperature-dependent sex determination in crocodylians. *J Exp Zool* 270:28–44 (1994).
- Smith CA, Joss JMP. Gonadal sex differentiation in *Alligator mississippiensis*, a species with temperature-dependent sex determination. *Cell Tissue Res* 273:149–162 (1993).
- Norris DO. *Vertebrate Endocrinology*. San Diego, CA:Academic Press, 1997.
- Andrews JE, Smith CA, Sinclair AH. Sites of estrogen receptor and aromatase expression in the chicken embryo. *Gen Comp Endocrinol* 108:182–190 (1997).
- Sheehan DM, Willingham E, Gaylor D, Bergeron JM, Crews D. No threshold dose for estradiol-induced sex reversal of turtle embryos: how little is too much? *Environ Health Perspect* 107:155–159 (1999).
- Lance VA, Bogart MH. Studies on sex determination in the American alligator *Alligator mississippiensis*. *J Exp Zool* 270:79–85 (1994).
- Crain DA, Guillette LJ Jr, Rooney AA, Pickford DB. Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. *Environ Health Perspect* 105:528–533 (1997).
- Crews D, Bergeron JM, Bull JJ, Flores D, Tousignant A, Skipper JK, Wibbels T. Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. *Dev Genet* 15:297–312 (1994).
- Lance VA, Bogart MH. Disruption of ovarian development in alligator embryos treated with an aromatase inhibitor. *Gen Comp Endocrinol* 86:59–71 (1992).
- Smith CA, Joss JMP. Steroidogenic enzyme activity and ovarian differentiation in the saltwater crocodile, *Crocodylus porosus*. *Gen Comp Endocrinol* 93:232–245 (1994).
- Smith CA. Aromatase enzyme activity during gonadal sex differentiation in alligator embryos. *Differentiation* 58:281–290 (1995).
- Gabriel WN, Blumberg B, Sutton S, Place AR, Lance VA. Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures. *J Expl Zool* 290:439–448 (2001).
- Willingham E, Baldwin R, Skipper JK, Crews D. Aromatase activity during embryogenesis in the brain and adrenal-kidney-gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. *Gen Comp Endocrinol* 119:202–207 (2000).
- Desvages G, Pieau C. Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of eggs. *J Steroid Biochem Mol Biol* 41:851–853 (1992).
- Jeyasuria P, Place AR. Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: a role for P450 aromatase (CYP19). *J Exp Zool* 281:428–449 (1998).
- Matter JM, Crain DA, Sills-McMurry C, Pickford DB, Rainwater TR, Reynolds KD, Rooney AA, Dickerson RL, Guillette LJ Jr. Effects of endocrine-disrupting contaminants in reptiles: alligators. In: *Principles and Processes for Evaluating Endocrine Disruption* (Kendall RJ, Dickerson RL, Giesy JP, Suk WA, eds). Pensacola, FL:SETAC Press, 1998;267–289.
- Crain DA. Effects of Endocrine-Disrupting Contaminants on Reproduction in the American Alligator, *Alligator mississippiensis* [PhD Thesis]. Gainesville, FL:University of Florida, 1997.
- Rooney AR. Variation in the Endocrine and Immune System of Juvenile Alligators: Environmental Influence on Physiology [PhD Thesis]. Gainesville, FL:University of Florida, 1998.
- Vonier PM, Crain DA, McLachlan JA, Guillette LJ Jr, Arnold SF. Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator. *Environ Health Perspect* 104:1318–1322 (1996).
- Guillette LJ Jr, Crain DA, Gunderson MP, Kools SA, Milnes MR, Orlando EF, Rooney AA, Woodward AR. Alligators and endocrine disrupting contaminants: a current perspective. *Am Zool* 40:438–452 (2000).
- Lephart ED, Simpson ER. Assay of aromatase activity. *Methods Enzymol* 206:477–483 (1991).
- SAS Institute Inc. *StatView for Windows, Version 5.0*, Cary, NC:SAS Institute Inc, 1998.
- Rhen T, Willingham E, Sakata JT, Crews D. Incubation temperature influences sex-steroid levels in juvenile red-eared slider turtles, *Trachemys scripta*, a species with temperature dependent sex determination. *Biol Reprod* 61:1275–1280 (1999).
- Vigier B, Forest MG, Eychenne B, Bezard J, Garrigou O, Robel P, Josso N. Anti-Müllerian hormone produces endocrine sex reversal of fetal ovaries. *Proc Natl Acad Sci U S A* 86:3684–3688 (1989).
- Western PS, Harry JL, Graves JAM, Sinclair AH. Temperature-dependent sex determination in the American alligator: AMH precedes SOX9 expression. *Dev Dyn* 216:411–419 (1999).
- Bergeron JM, Gahr M, Horan K, Wibbels T, Crews D. Cloning and *in situ* hybridization analysis of estrogen receptor in the developing gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. *Dev Growth Differ* 40:243–254 (1998).
- Milligan SR, Khan O, Nash M. Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (*Oncorhynchus mykiss*) plasma. *Gen Comp Endocrinol* 112:89–95 (1998).
- Crain DA, Noriega N, Vonier PM, Arnold SF, McLachlan JA, Guillette LJ Jr. Cellular bioavailability of natural hormones and environmental contaminants as a function of serum and cytosolic binding factors. *Toxicol Ind Health* 14:261–273 (1998).
- Conley AJ, Elf P, Corbin CJ, Dubowsky S, Fivizzani A, Lang JW. Yolk steroids decline during sexual differentiation in the alligator. *Gen Comp Endocrinol* 107:191–200 (1997).