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Studies on Sex Determination in the American Alligator *Alligator mississippiensis*

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ABSTRACT Estradiol-17β was measured in the plasma and urogenital tissues of male and female alligator embryos. Hormone levels were generally very low and there was no significant differences between the sexes. There were no significant changes in estrogen levels during the period of gonadal differentiation in either sex. Alligator embryos incubated at male producing temperatures were feminized by small doses of estrogen applied to the egg shell. The antiestrogen, tamoxifen, masculinizes turtle and bird embryos, but, paradoxically, feminized alligator embryos at male producing temperatures. The contraceptive steroid, norethindrone, a progestin that is claimed to block estrogen synthesis, is the most potent estrogenic steroid tested on alligator embryos. A single dose of norethindrone applied to the egg shell caused massive hypertrophy of the Müllerian duct and feminized embryos at male producing temperatures. The androgen, dihydrotestosterone, had no detectable effect on male or female embryos at the doses tested. Undifferentiated urogenital tracts of embryos were cultured at 30 and 33°C in the presence of steroids, tamoxifen, or antibodies to steroids. None of the treatments had any effect on tissue differentiation. Tissues survived for up to 6 weeks, but there was no evidence of gonadal differential in vitro.

A role for estrogen in vertebrate sex determination and sex differentiation has been postulated since the early 1930s (for a review see Lance and Bogart, '92). In recent years there has been a renewed interest in estrogens and sex determination, particularly in reptiles with temperature dependent sex determination (TSD). A number of workers has shown that it is possible to produce phenotypically normal females by treating reptile eggs, incubated at male-producing temperatures, with estrogen during the temperature sensitive period (TSP), or the period of gonadal differentiation (see Pieau et al. and Wibbels et al. this issue for references). It has not been possible to produce male reptiles by treating embryos incubated at female-producing temperatures with androgens (Lance and Bogart, '92). However, when the non-aromatizable androgen, dihydrotestosterone (DHT), was applied to turtle embryos incubated at the pivotal temperature (a temperature that would produce a 50:50 sex ratio), a significant increase in the number of male hatchlings was observed (Wibbels et al., '92). This treatment, however, has not produced 100% males. The mechanism for this androgen-induced increase in the number of male embryos at pivotal temperature remains unknown.

It has been suggested that the relative amount of cytochrome P450 aromatase, the enzyme that converts androgens to estrogens, is the critical limiting factor in sex determination (Bogart, '87). Support for a role of aromatase activity in reptiles with TSD has been presented by Desvages and Pieau ('92). They showed that during the TSP, aromatase activity in embryonic female turtle gonads increased dramatically, whereas little or no aromatase activity was detected in the gonads of male embryos. A similar increase in aromatase activity during ovarian development was seen in leatherback turtles, *Dermochelys coriacea* (Desvages et al., '93). Smith and Joss ('94a) did find significant aromatase activity in embryonic ovaries of *Crocodylus porosus*, but peak activity occurred well after the TSP. In our previous publications, we demonstrated that estradiol-17β would result in 100% female hatchlings if applied to alligator embryos incubated at a male-producing temperature (Lance and Bogart '91, '92). If estrogen is indeed the primary stimulus for sex determination, it follows that if estrogen synthesis was blocked or an antiestrogen was applied to an embryo, then a male phenotype should be produced. When the

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non-steroidal aromatase inhibitor, Ciba Geigy 16949A (fadrozole), was applied to alligator embryos at a female-producing incubation temperature, the only effect noted was an inhibition of ovarian development (Lance and Bogart, '92). Elbrecht and Smith ('92), however, were able to show that the same aromatose inhibitor could completely sex reverse genetically female chick embryos when injected into the eggs prior to the period of sex determination.

In the American alligator, the role of estrogen remains unclear. We have studied sex determination in the alligator using a number of experimental approaches. The following is a brief summary of our results.

HORMONES IN THE EMBRYO

Tissue and circulating steroids have been measured in embryos of three species of turtle during sex differentiation and the results have proven contradictory. Pieau and colleagues showed that in the European pond turtle, *Emys orbicularis*, and the leatherback turtle, *Dermochelys coriacea*, tissue estrogen content increased during ovarian differentiation, but was very low or undetectable in male gonadal tissue (Dorizzi et al., '91; Desvages and Pieau, '91; Desvages et al., '93). In contrast, While and Thomas have been unable to document significant sex differences in either circulating or gonadal tissue levels of steroids in the turtle *Trachemys picta* (White and Thomas, '92a). Furthermore, White and Thomas ('92b,c) showed that adrenal-kidney tissue, but not gonadal tissue, was the major source of sex steroids in *Trachemys* embryos. Histochemical studies on steroidogenic enzymes in reptile embryos have also yielded contradictory results. Pieau ('73) demonstrated the presence of 3β-hydroxysteroid dehydrogenase (3β-HSD) in embryonic *Emys* testes. Joss ('89) reported the presence of 3β-HSD in the testes, but not in the ovaries, of alligator embryos. Thomas et al. ('92), however, were unable to demonstrate 3β-HSD in embryonic *Trachemys* gonads, but did find intense 3β-HSD activity in adrenal tissue. In *Crocodylus porosus* embryos, Smith and Joss ('94a) also found 3β-HSD activity in the adrenal gland, but not in the gonad (see Pieau et al. and Wibbels et al. in this issue).

We collected blood samples from the peripheral circulation of alligator embryos from eggs incubated at 30°C (female producing temperature) and from eggs incubated at 33°C (male producing temperature) immediately prior to the TSP and at 2–3 day intervals until hatching. The urogenital ridge, consisting of mesonephros, adrenal, and gonad, was dissected, frozen, and later homogenized and extracted for steroid analysis (Medler, '92). Estrogens, progesterone, testosterone, and corticosterone were measured in the extracts by radioimmunoassay using highly specific antibodies. No sex differences in plasma estradiol, testosterone, or progesterone were detected. Estradiol levels in the plasma were highly variable, but in general were very low. No clear association of estradiol with gonadal differentiation could be detected (Fig. 1). A similar lack of sex difference in tissue steroid levels was observed (Medler, '92).

Deeming and Ferguson ('88) suggested that the signal for sex determination in reptiles with TSD might originate in the hypothalamus, as this area of the brain is known to be temperature sensitive. If this hypothesis is correct then we would expect to see changes in the content of gonadotropin-releasing hormone (GnRH) in the hypothalamus during the period of sex differentiation as is seen in birds (Li et al., '91; Millam et al., '93). Whole brain and hypothalamic tissues were collected from alligator embryos at male-inducing and female-inducing temperatures, frozen immediately in liquid nitrogen, and assayed for chicken-1 GnRH using a highly sensitive ELISA assay (Ottinger and Lance, unpublished data). Levels of GnRH were extremely low in extrahypothalamic tissue with no apparent sex differences. Tissue content increased gradually during development. GnRH levels in the hypothalamus were an order of magnitude higher than in the whole brain, but again, no clear sex difference or change in concentration during the period of sex differentiation

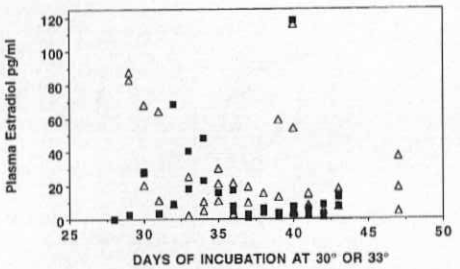

Fig. 1. Plasma estradiol in male and female alligator embryos during the period of sex determination. Open triangles represent individual samples from male embryos (33°C) and solid squares individual samples from female embryos (30°C).

was observed. Hypothalamic tissue levels of GnRH also increased throughout development (Ottinger and Lance, unpublished data).

IN VITRO CULTURE OF UROGENITAL TISSUE

Merchant-Larios and Villalpando ('90) cultured embryonic gonads from the sea turtle *Lepidochelys olivacea* for 10 days at 28°C (male producing temperature) and 32°C (female producing temperature). They observed that the temperature at which the gonads were cultured had no effect on sexual differentiation. The gonads differentiated as ovaries if they came from embryos that had been incubated at female-producing temperature and as testes if they came from embryos that had been incubated at male-producing temperature prior to the culture experiments. The authors concluded that an extragonadal mechanism mediated the effect of temperature on sex determination.

To determine whether similar results could be obtained using alligators, we dissected the urogenital tracts from 60 embryos incubated at 30°C until stage 19 and randomly assigned the left and right tract to a culture treatment at 30 or 33°C. Differentiation of the gonad does not begin until stage 20–21 (see Smith and Joss, this issue). Tissues were cultured as described by Merchant-Larios and Villalpando ('90) and the media changed daily. Tissues were subjected to the following treatments: estradiol-17β, 20 μg/ml; DHT, 20 μg/ml; fadrazole, 20 μg/ml; tamoxifen, 6 μg/ml; antisera to estradiol and antisera to corticosterone at a final dilution of 1:10,000. Tissues were sampled at 2-week intervals to assess viability, fixed in Bouin's and examined histologically. The experiment was terminated at 6 weeks. Media from 4 weeks and the final day in culture were assayed for estradiol by RIA. There was considerable necrosis by 6 weeks, but most of the tissues survived for the duration of the experiment. There was no evidence of gonadal differentiation (Fig. 2). Estradiol was undetectable in the media from either sampling period. None of the treatments had any measurable effect on tissue differentiation, and there was significantly more necrosis in the tissues treated with steroids than in any of the other treatments. Since the steroids were given in what could be considered pharmacological doses, such a result was not too surprising. From these results we can see that although alligator urogenital tissues can survive in culture for up to 6 weeks, no differentiation occurs, and none of the treatments known to effect embryos in ovo has any effect on gonadal differentiation in vitro. These negative results also suggest an extragonadal site for temperature and hormones on alligator sex differentiation.

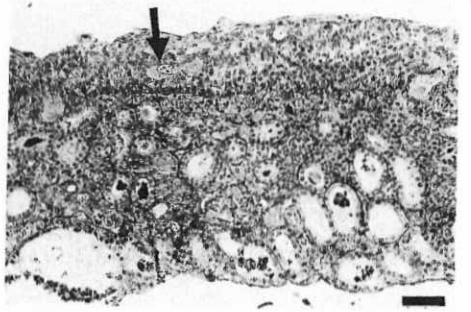

Fig. 2. Histological section of embryonic alligator urogenital tract after 6 weeks in culture. Gonadal tissue in the top 30% of section. Arrow indicates germ cell. Mesonephric tubules with cellular debris can be seen at the bottom of the section. Mallory's trichrome stain. Bar = 60 μM.

DRUG AND HORMONAL TREATMENT OF EMBRYOS

Our results with estradiol have already been referred to and are no different from what has been seen in other reptiles (Table 1). In earlier studies, test substances injected into eggs resulted in high embryonic mortality (Lance and Bogart, '92). All of the treatments listed in Table 1 were applied directly to the alligator egg shell in 95% ethanol. Mortality was less than 5%.

Tamoxifen is an effective antiestrogen, but has weak estrogenic activity in rat uterus. In the bird embryo it also acts as an antiestrogen and causes masculinization of the gonad (Weniger, '91). In turtle embryos it also causes partial masculinization of the gonad (Dorizzi et al., '91). In alligator embryos however, tamoxifen is consistently estrogenic, in low doses (Lance and Bogart, '91) or repeated high doses, whether given early in development or just prior to the TSP (Table 1).

Norethindrone is a contraceptive steroid classed as a progestin, but has subsequently been shown to have an inhibitory effect on the aromatase complex in mammals (Osawa et al., '82). It has been shown to have weak estrogenic effects in bird embryos, but is generally antiestrogenic (Hutson et al., '85). Our results with norethindrone were simi-

TABLE 1. Drug treatment effects on alligator embryos

Compound	Class	Dose (μg)	33°C	30°C
Estradiol	Estrogen	100	Feminize+++	Feminize+
ICI-M164, 384	Antiestrogen	100	No effect	No effect
Tamoxifen	Antiestrogen	200	Feminize+++	No effect
Norethindrone	Antiestrogen	200	Feminize+++++	Feminize+++++
DHT	Androgen	200	No effect	No effect
Cyproterone acetate	Antiandrogen	100	Feminize+	No effect
CG-16949A	Arom-inhib	200	No effect	Inhibits ovary+++
Eli Lilly-LY043578	Arom-inhib	200	No effect	No effect
Hydroxyandrostenedione	Arom-inhib	200	No effect	Inhibits ovary+
Aminoglutethimide	Arom-inhib	200	No effect	Inhibits ovary+

Arom-inhib, aromatase inhibitor.

lar to those of Austin ('91) who applied the drug to the chorioallantoic membrane of female alligator embryos very late in development. The degree of Müllerian duct hypertrophy was much greater in our study (Figs. 3, 4, 5). We applied the drug to embryos at male-producing and female-producing temperatures prior to the TSP. In all instances, embryos from male-inducing temperature treated with norethindrone were phenotypic females with massively hypertrophied Müllerian ducts. No detectable change in ovarian structure could be detected. Wibbels and Crews ('92) also showed that low doses of norethindrone were estrogenic in turtle embryos at male-producing temperatures. The weak estrogenic effect of norethindrone in

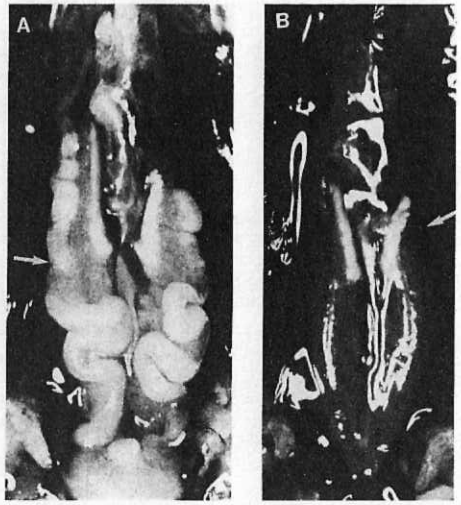

Fig. 3. Gross morphology of the Müllerian duct of alligator hatchlings after treatment with norethindrone (**A**) or ethanol (**B**). Anterior is to the top of the page. Note the massive hypertrophy of the duct in the treated embryos, arrow in **A**. The thread-like Müllerian duct in the control, arrow in **B** can be barely made out.

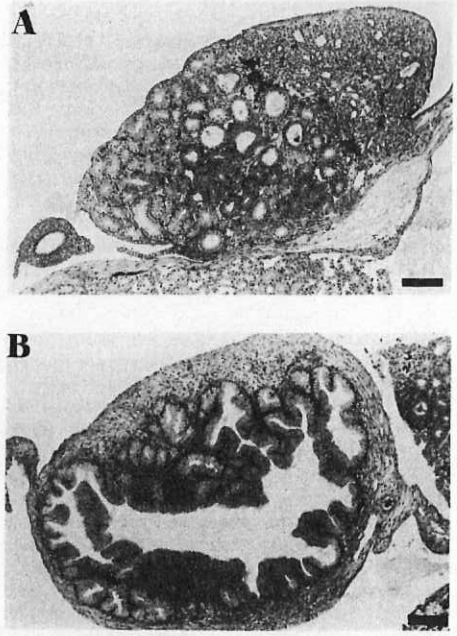

Fig. 4. Histological section of control ovary (**A**) and the Müllerian duct of a norethindrone-treated embryo (**B**) at the same magnification. In section **A** the Müllerian duct is to the lower left and the ovary to the upper right. Bar = 80 μM.

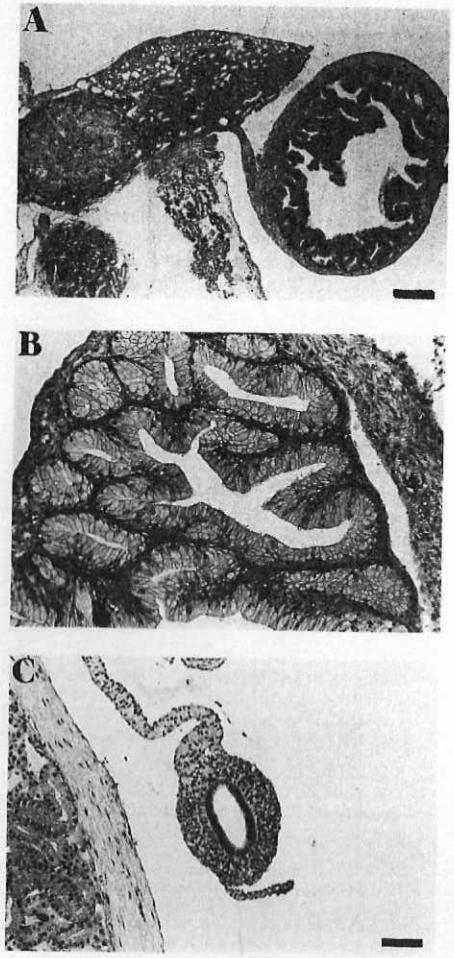

Fig. 5. Details of glandular hypertrophy in Müllerian duct of norethindrone-treated alligator embryo (**A**, **B**). Bar = 300 μM in **A**. Control duct in **C** at the same magnification as **B**, bar = 60 μM.

mammals has been attributed to its extremely low conversion rate to ethynylestradiol (1%) by aromatization in the liver (Yamamoto et al., '86). It is possible that a similar, or even greater conversion occurs in reptile embryos.

The antiandrogen, cyproterone acetate, was generally ineffective in alligator embryos at the dose tested, but in a few instances it resulted in feminized gonads in embryos at a male-producing incubation temperature (Table 1). The steroidal antiestrogen from ICI showed no effect at the dose tested.

DISCUSSION

From this brief overview it is clear that despite their common evolutionary origin the embryos of birds and alligators differ in many respects. Bird embryos show a sex-specific increase in circulating estrogen around the period of sex differentiation (Weniger, '91). Alligators show no sex differences in steroid levels or any changes associated with the period of gonadal differentiation. All of the treatments applied to bird and alligator embryos gave qualitatively different responses. Tamoxifen partially masculinizes bird embryos, but feminizes alligator embryos (for references, see Lance and Bogart, '92). Norethindrone, which causes Müllerian duct agenesis or regression in birds (Hutson et al., '85; Stoll et al., '90), is the most potent estrogenic substance tested in alligator embryos. The aromatase inhibitor, fadrozole, is able to completely sex reverse genetically female chickens (Elbrecht and Smith, '92; Wartenburg, et al., '92), but causes only moderate ovarian inhibition in alligators. It is also apparent that crocodilians differ from other reptiles in that tamoxifen and aromatase inhibitors cause masculinization of turtle embryos at female-producing incubation temperatures (Dorizzi et al., '91). Some of these differences in response to drugs could be due to species differences in the structure of the estrogen receptors and the aromatase enzyme. Drugs that completely block hormone effects in mammals may have only weak effects in reptiles due to sequence differences in the site at which the drug binds. Based on anomalous results with the antiandrogen, cyproterone, on amphibians (the drug *masculinized* larvae), Rastogi and Chieffi ('75) suggested that hormone receptors in vertebrate embryos may differ from those of the adult. There are no data available at present on any hormone receptors in reptile embryos.

There have been a number of studies in mice in which it has been shown that the mesonephros plays an important role in gonadogenesis. The results, however, remain difficult to interpret. Taketo-Hosotani and Sinclair-Thompson ('87) showed that the presence of the mesonephros influenced sex differentiation of fetal mouse ovarian grafts. Ovary plus mesonephros grafts would dif-

ferentiate as ovotestis when transplanted into male hosts but not when transplanted into female hosts. If ovarian tissue alone was transplanted, ovotestis would develop regardless of the sex of the host (Taketo-Hosotani and Sinclair-Thompson, '87). The authors concluded that the mesonephros inhibited testicular differentiation in fetal ovaries transplanted into female hosts. Buehr et al. ('93), on the other hand, showed that fetal gonads from male mice failed to develop as testes in culture unless the mesonephros was attached. The authors showed that cells from the mesonephros migrated into the fetal testis and formed components of the peritubular region. The role of the mesonephros in reptilian gonadal differentiation remains unknown. Gahr et al. ('92) showed that the mesonephros, but not the gonad, of embryonic *Trachemys* accumulated radioactive estradiol. The authors suggested that the feminizing effect of exogenous estrogen might act via the mesonephros or adrenal, which also showed some estrogen accumulation (Gahr et al., '92). The gonads of *Lepidochelys* that differentiated in vitro in the experiments of Merchant-Larios and Villalpando ('90) appeared to be without the mesonephros attached. The alligator gonadal tissue we cultured had both mesonephros and adrenal tissue attached which may have affected the results.

Pieau et al. and Wibbels et al. (this issue) present convincing arguments for a central role for estrogen (or aromatase) in sex determination: estrogen can induce a female phenotype in embryos at a male-producing incubation temperature; aromatase activity is present in embryonic female gonadal tissue and absent or very low in male gonadal tissue; and blocking estrogen action with antiestrogens or blocking estrogen synthesis with aromatase inhibitors masculinizes embryonic gonads. In the alligator, however, it has not been possible to duplicate these findings. The antiestrogens, tamoxifen and norethindrone, *feminize* alligator embryos, aromatase inhibitors slow ovarian development but fail to masculinize, and circulating and tissue estrogens show no correlation with sex or stage of differentiation. The one study in which aromatase activity was measured in crocodile gonads showed increased activity *after* sex differentiation had taken place (Smith and Joss, '94a). It could be argued that very low concentrations of estrogen acting in a paracrine fashion could be sufficient to feminize embryos, and that gross measurements of circulating or tissue estrogens might fail to detect these subtle changes.

If estrogen, or the synthesis of estrogen, in response to a particular temperature regime is what drives sex differentiation in TSD reptiles, there are still a number of perplexing questions remaining. Two recent papers in particular suggest that female sex differentiation in mammals can proceed in the absence of estrogen. An 18-year-old 46,XX human female with primary amenorrhea, sexual infantilism, polycystic ovaries, and no detectable estrogens was found to have a mutated form of the aromatase gene, and was thus diagnosed as exhibiting aromatase deficiency syndrome (Ito et al., '93). A gene targeting experiment in which a defective estrogen receptor gene was inserted into mice resulted in female mice that were infertile, had polycystic ovaries and were totally unresponsive to estrogen. The males appeared phenotypically normal but had low testis weight and sperm count (Lubahn et al., '93). Although in both instances reproductive function was impaired, embryonic sex determination and gonadal differentiation was apparently normal despite, in the human case, there being no measurable estradiol and the mouse being completely unresponsive to estradiol. If female sex differentiation and ovarian development can proceed in the absence of estrogens in mammals, we are faced with the problem of explaining how genetically female chicks differentiated as phenotypically normal males when estrogen synthesis was blocked (Elbrecht and Smith, '92). It is possible that the basic mechanism of sex determination in mammals is different from that of reptiles and birds. The model of Jost ('53) in which a female phenotype develops in the absence of embryonic testicular hormones (and apparently in the absence of ovarian hormones), may not apply to birds and reptiles.

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