Gonadal Steroidogenesis *in Vitro* from Juvenile Alligators Obtained from Contaminated or Control Lakes

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The ubiquitous distribution of many contaminants and the nonlethal, multigenerational effects of such contaminants on reproductive, endocrine, and immune systems have led to concerns that wildlife worldwide are affected. Although the causal agents and effects are known for some species, the underlying physiological mechanisms associated with contaminant-induced reproductive modifications are still poorly understood and require extensive research. We describe a study examining the steroidogenic activity of gonads removed from juvenile alligators (*Alligator mississippiensis*) obtained from contaminated or control lakes in central Florida. Synthesis of estradiol-17β (E_2) was significantly different when ovaries from the contaminated and control lakes were compared *in vitro*. Additionally, testes from males obtained from the contaminated lake, Lake Apopka, synthesized significantly higher concentrations of E_2 when compared to testes obtained from control males. In contrast, testosterone (T) synthesis from all testes examined in this study displayed a normal pattern and produced concentrations greater than that observed from ovaries obtained from either lake. Interestingly, the pattern of gonadal steroidogenesis differs from previously reported plasma concentrations of these hormones obtained from the same individuals. We suggest that the differences between the *in vivo* and *in vitro* patterns are due to modifications in the hepatic degradation of plasma sex steroid hormones. — Environ Health Perspect 103(Suppl 4):31–36 (1995)

Key words: alligator, gonadal steroidogenesis, estradiol-17β, testosterone

Introduction

Many xenobiotic compounds introduced into the environment by human activity have been shown to modify normal biological function in various wildlife species. The ubiquitous distribution of many contaminants and the nonlethal, multigenerational effects of such contaminants on reproductive, endocrine, and immune systems have led to concerns that wildlife worldwide may be affected (*1,2*).

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The reproductive disorders reported to date in wildlife exposed to xenobiotic compounds involve such factors as reduced fertility, reduced hatchability, reduced viability of offspring, impaired hormone secretion or activity, and modified adult sexual behavior. Although causal agents and effects are known in some cases, the underlying mechanisms associated with contaminant-induced reproductive modifications are still poorly understood and require extensive research (*1,3–5*). All of the abnormalities described above can be caused by disruption of normal endocrine function either prior to or after the hormone interacts with specific cellular receptors (*5*). For example, a number of effects on the reproductive system are associated with decreased circulating levels of sex hormones, as recently reported for juvenile alligators hatched from eggs obtained from a contaminated lake (*6*).

We have documented specific problems associated with eggs and the reproductive system of alligators (*Alligator mississippiensis*) obtained from Lake Apopka, Florida, adjacent to a U.S. Environmental Protection Agency (U.S. EPA) -designated Superfund contaminant site, the former Tower Chemical Company (*6–9*). Lake Apopka is the fourth largest body of freshwater (12,500 hectare) in Florida and is highly polluted (*10,11*). Contaminants and nutrients in the lake derive from extensive agricultural activities around the lake that continue today, a sewage treatment facility associated with the city of Winter Garden, Florida, and a major pesticide spill. The Tower Chemical Company was the site of an extensive spill in 1980 of dicofol (*12*), DDT (and its metabolites DDD, DDE, and chloro-DDT), and sulfuric acid (U.S. EPA, unpublished report). We have previously reported that alligator clutch viability on this lake is significantly depressed compared to viability on other comparable lakes in central and southern Florida (*6,8,9*). A significant decline in the population size of juvenile alligators occurred on Lake Apopka following the Tower Chemical Company spill (*7,9*). The population declined by 90% over the years 1980 to 1984 and remains at a depressed level today.

Recently, we reported that juvenile alligators from Lake Apopka exhibited abnormal gonadal morphology and plasma sex steroid concentrations (*6*). Ovaries from alligators 6 months of age, hatched from eggs collected on Lake Apopka, had prominent polyovular follicles, and many of the oocytes were multinucleated. Male alligators from Lake Apopka of the same age had

poorly organized testes with unique, aberrant structures of unknown origin within the seminiferous tubules. Both male and female juvenile alligators exhibited abnormal plasma sex steroid concentrations, with males from Lake Apopka having greatly reduced plasma testosterone (T) concentrations similar to that of females from either the contaminated (Lake Apopka) or control (Lake Woodruff) lakes. In contrast, males from the control lake, Lake Woodruff, had plasma T concentrations 4 times that observed in the juvenile males from Lake Apopka. The females from Lake Apopka had elevated plasma estradiol-17β concentrations compared to the females from the control lake. The data on plasma sex steroid concentrations suggest several hypotheses: *a*) steroidogenesis is abnormal in the juveniles from Lake Apopka due to developmental abnormalities of the gonad; *b*) gonadal steroidogenesis is normal, but degradation of the sex steroids is modified, thus resulting in abnormal plasma concentrations; *c*) the ratio of free to bound hormone has been modified due to varying concentrations of the plasma proteins bound to steroid hormones; or *d*) abnormal stimulation from the pituitary and hypothalamus (abnormal gonadotropin release) could result in abnormal stimulation of the gonad and thus, abnormal plasma sex steroid concentrations. These hypotheses are not mutually exclusive, and the observations reported may be due, to various degrees, to all of these factors. We have performed an initial test of the first hypothesis by incubating the gonads *in vitro* to determine endogenous and gonadotropin-stimulated rates of steroid synthesis. These data can be used to clarify the role of the gonad in producing the reported abnormal plasma hormone concentrations.

Materials and Methods

Animals

Eggs were collected under permit from the Florida Game and Freshwater Fish Commission from alligator nests on Lakes Apopka and Woodruff. Eggs were returned to the University of Florida where they were incubated at 30.7 (±0.4°C) in wet sphagnum moss. After hatching, neonatal alligators were maintained in an insulated building until they were actively feeding. All hatchlings were web-tagged so that clutch number and lake of origin were known for each individual. Within 10 days of hatching, 25 robust neonates from each lake ($n = 50$ total) were transferred to the Sante Fe Teaching Zoo, Gainesville, Florida, where they were housed in an outdoor enclosure (30 × 10 m). This enclosure was wire-mesh covered to prevent predation, but animals experienced natural fluctuations in photoperiod and temperature. Animals were fed *ad libitum* daily with commercial alligator chow (Burris Mill and Feed Inc., Franklinton, LA). Additional information on care and housing of these animals can be obtained in Guillette et al. (*6*). It should be noted that the commercial feed and water were not tested for additional contaminants that might be present, even at low concentrations.

Culture

At 6 months of age, all of the animals used in this study were bled to obtain a plasma sample that was analyzed for the concentration of testosterone and estradiol-17β. The plasma data have been published previously (*6*). The animals were then killed with an overdose of Nembutal. Gonads were surgically removed; the right gonad was fixed in alcoholic Bouin's, whereas the left gonad was placed in short-term tissue culture. Culture vessels were 10 ml Petri dishes filled with 0.5 mg of gonadal tissue slices and 5 ml of 32°C, oxygenated (95% O_2: 5% CO_2) complete Dulbecco's Minimum Essential Medium (DMEM; Gibco). Culture vessels were placed on a rocker in an incubator (32°C) for 12 hr. At the end of this initial incubation period, the culture medium was removed from all plates and flash frozen in liquid nitrogen. Fresh media (5 ml oxygenated DMEM) was introduced to all plates. The gonads were then treated with 5 IU of porcine luteinizing hormone (pLH) per culture; final dose 1.0 IU pLH/ml culture media. Tissue was incubated at 32°C for a further 12 hr, after which the culture medium was flash frozen for radioimmunoassay (RIA) of estradiol-17β and testosterone concentrations.

Radioimmunoassays

Plasma samples from gonadal cultures were analyzed for estradiol and testosterone using RIA procedures that were validated for use with culture media. For estradiol determinations, duplicate samples (50 µl) were assayed. Standard curves were prepared in fresh media with known amounts of radioinert estradiol (1, 5, 10, 25, 50, 100, 250, 500, and 1000 pg/ml). The minimum concentration per tube distinguishable from zero was 2.7 pg/ml. Cross-reactivities of the estradiol antiserum (supplied courtesy of RL Butcher, West Virginia University; characterized by TS Gross) were: 11.2% for estrone, 1.7% for estriol, < 1.0% for estradiol-17α, and 0.1% for all other steroids examined. A pooled sample (approximately 105 pg/ml) was assayed serially in 5-, 10-, 20-, 30-, 40-, and 50-µl volumes (final voume of 50 µl with fresh incubation media). This inhibition curve was parallel to the standard curve, and a test for homogeneity of regression indicated that the curves were not different. Further characterization of the assay involved measurement of known amounts (1, 2, 5, 10, 25, 50, 100, 250, and 500 pg) of estradiol in 50 µl of culture media [$y = 0.92 + 0.94x$; y = amount of estradiol measured (pg); x = amount of estradiol added (pg); $r^2 = 0.9134$]. Interassay and intraassay coefficients of variation were 8.7 and 7.8%, respectively.

For testosterone determinations, duplicate samples (50 µl) were assayed directly. The minimum detectable concentration per tube was 4.1 pg/ml. Cross-reactivities of the testosterone antiserum (purchased from ICN Biomedicals, Inc., Wilmington, DE) with other steroids were: 18.75% for 5α-dihydrotestosterone; 3.0% for 5α-androstenediol; < 1.0% for androstenedione; and 0.1% for all other steroids examined. A pooled sample (approximately 211 pg/ml) was assayed serially in 5-, 10-, 20-, 30-, 40-, and 50-µl volumes (final volume of 50 µl with fresh incubation media). The inhibition curve was parallel to the standard curve, and the test for homogeneity of regression indicated that the curves were not different. Further characterization of the assay involved the measurement of known amounts (1, 2, 5, 10, 25, 50, 100, and 250 pg) of testosterone in 50 µl of culture media [$y = 3.12 + 0.92x$; y = amount of testosterone measured (pg): x = amount of testosterone added (pg); $r^2 = 0.8791$]. Interassay and intraassay coefficients of variation were 7.6 and 9.1%, respectively.

Statistics

We tested for differences in hormone concentrations between sexes, lakes, and LH treatment by one- and two-way ANOVA (StatView II, Abacus Concepts, Inc., Berkeley, CA). Where significant ($p < 0.05$) variation existed, Scheffe's F tests were performed. Ratio data (estrogen/testosterone ratios) were log and arcsin transformed prior to significance testing to achieve homogeneity of variance (*13*).

Results

Estradiol-17β Synthesis in Culture

We observed a difference in estradiol-17β (E_2) synthesis *in vitro* when the interaction between lake and sex were examined (F = 12.98; *df* = 1.26; *p* = 0.0013: Figure 1*A*). Ovaries from females hatched from eggs collected on Lake Woodruff (for brevity hereafter designated as Woodruff gonads) synthesized significantly greater quantities of E_2 endogenously when compared to ovaries from Apopka (F = 6.473; *df* = 1.15; *p* = 0.0225: Figure 1*A*). LH stimulation had little effect on *in vitro* synthesis of E_2 from Woodruff ovaries but did on Apopka ovaries. Concentrations of E_2 in culture media having Apopka ovaries after LH stimulation were comparable to those observed in cultures containing either treated or untreated ovaries from Lake Woodruff (Figure 1*A*).

Unstimulated testes obtained from Lake Apopka males synthesized significantly more E_2 than testes from Lake Woodruff males (F = 6.164; *df* = 1.11; *p* = 0.0304). In fact, there was no difference in the synthesis of E_2 when ovaries and testes from Lake Apopka individuals were compared (F = 2.959, *df* = 1.13; *p* = 0.1091). However, Lake Apopka testes exhibited great variation in E_2 synthesis. Interestingly, the two individuals with testes exhibiting femalelike synthesis of E_2 were initially designated as females based on the lack of an enlarged phallus typical of males. LH did not stimulate a significant rise in E_2 synthesis from either Lake Apopka or Lake Woodruff testes (Figure 1*A*).

Testosterone Synthesis in Culture

Testes obtained from animals from Lakes Apopka and Woodruff exhibited no significant difference in their ability to synthesize testosterone (T) when unstimulated *in vitro* (F = 0.389; *df* = 1.11; *p* = 0.5454: Figure 1*B*). No significant increase in T synthesis was observed following LH stimulation. Following culture with LH, cultures containing testes still had significantly more T than those cultures containing ovaries (F = 10.371; *df* = 1.26; *p* = 0.0034). All cultures exhibited T concentrations at the end of the second 12-hr incubation period that were similar to those seen after the first 12 hr of incubation (Figure 1*B*), suggesting that the tissue was viable and that the lack of a response to LH stimulation was not due to tissue degradation.

Ovaries obtained from females from both lakes showed significantly less T synthesis than that observed from cultures containing testes (F = 24.968; *df* = 1.26; *p* < 0.0001: Figure 1*B*). Ovaries did not exhibit increased synthesis of T when exposed to LH.

E/T Ratio

We observed that cultures of testes obtained from Lake Woodruff males exhibited very low E/T ratios (Figure 2), whereas testes from Lake Apopka had significantly elevated E/T ratios (F = 8.86; *df* = 1.11; *p* = 0.013) due to elevated E_2 synthesis. The ratio for Lake Woodruff males was significantly below that reported for females from either lake (F = 41.49; *df* = 1.29; *p* < 0.0001), but the E/T ratio for males from Lake Apopka was not significantly different than the E/T ratios generated from cultures having ovaries taken from either lake. The only cultured gonads showing a significant change in E/T ratio in response to LH treatment were those having ovaries obtained from Lake Apopka females (F = 11.86; *df* = 1.29; *p* = 0.004; Figure 2).

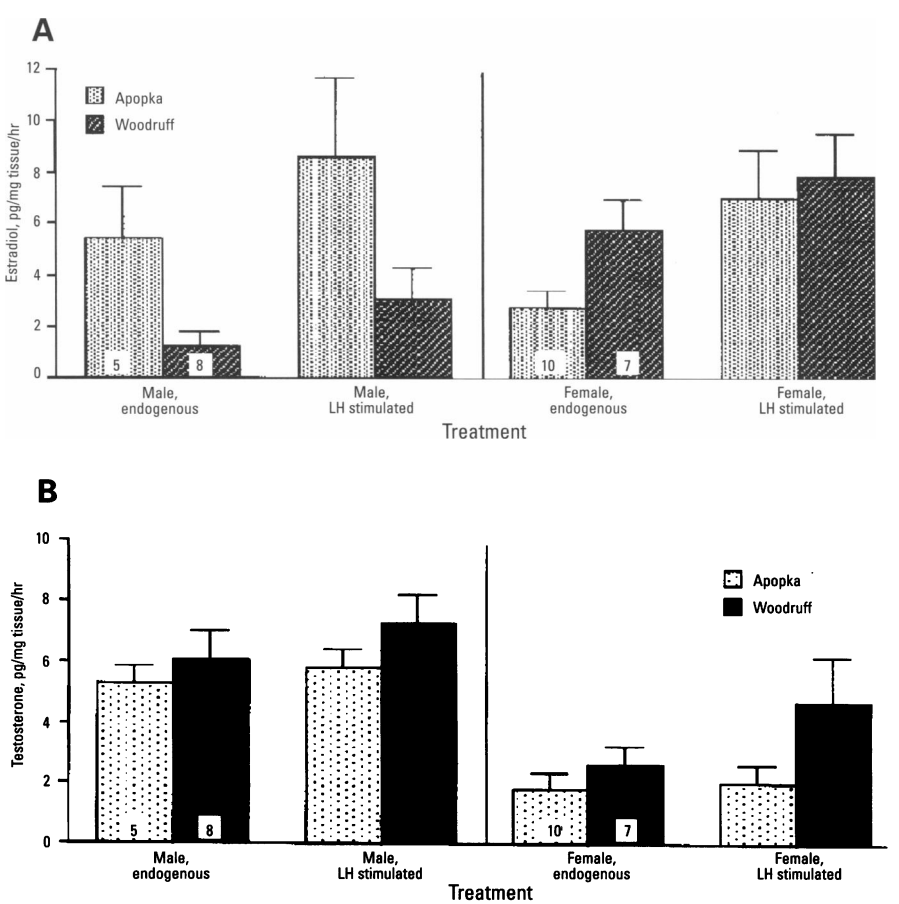

Figure 1. Concentrations (mean ± 1 SE) of estradiol (*A*) and testosterone (*B*) released by gonads *in vitro* following 12-hr incubation with or without LH stimulation. The N for all groups is given at the base of the bars in *A*. See discussion for indications of significance.

Discussion

Synthesis of estradiol-17β (E_2) was significantly different *in vitro* when ovaries from alligators hatched from the contaminated and control lakes were compared. Additionally, testes from males obtained from the contaminated lake, Lake Apopka, synthesized significantly higher concentrations of E_2 when compared to testes obtained from control males. In contrast, testosterone (T) synthesis from all testes examined in this study displayed an apparently normal pattern (normality is defined in this study as the pattern seen in the animals from the control lake) and produced concentrations greater than those observed from ovaries obtained from females from either lake. Interestingly, the pattern of gonadal steroidogenesis differs (Table 1) from that previously reported for plasma concentrations of these hormones obtained from the same individuals as used in this experiment (*6*). Patterns for plasma concentrations were as follows: males from Lake Apopka, the contaminated

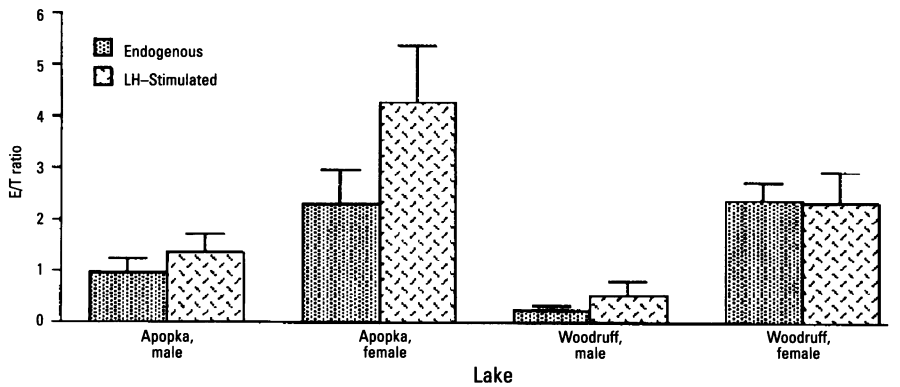

Figure 2. The hormonal milieu secreted by the gonads *in vitro* is represented as an E/T ratio which is calculated by dividing the estradiol concentration by the testosterone concentration in individual culture vessels.

lake, had lower than normal plasma T and normal low E_2 concentrations, whereas females had low T but elevated plasma E_2 concentrations (Table 1).

The data presented above for gonadal steroidogenesis *in vitro* suggest that the differences in plasma steroid concentrations we have previously reported between alligators from contaminated and control lakes are due in part to modifications of gonadal steroidogenesis with additional changes in degradation pathways and response to the stimulation of gonadotropins from the hypothalamo-hypophysial system. Although we have no data on the activity of various enzymes associated with gonadal steroidogenesis nor data on degradation pathways in alligators exposed to various contaminants, it is clear that changes in these systems have occurred and future studies should examine these systems. Data, however, are available from other species that allow hypotheses to be developed to explain our observations.

The possible mechanisms by which environmental contaminants alter gonadal steroidogenesis include a reduction in synthesis of gonadotropin releasing hormone (GnRH) from the hypothalamus, a reduction in luteinizing hormone (LH) release from the pituitary, a reduction in the availability of the precursor cholesterol, a modification of the enzymes required for steroidogenesis (e.g., aromatase, cytochrome $P450_{scc}$), and modifications of the cellular receptor numbers and function (*14*). A decrease in plasma testosterone concentration, as well as a 95% reduction in plasma LH concentration, was reported in male rats following exposure *in utero* to small concentrations (0.064 µg/kg/bw) of dioxin (*15*). One mechanism by which dioxin modifies plasma androgen concentrations in adult male rodents is to inhibit testicular cholesterol mobilization while leaving cellular concentrations of the enzyme cytochrome $P450_{scc}$ unaltered; this enzyme is responsible for the conversion of cholesterol to pregnenolone, the initial step in gonadal steroidogenesis (*14*). The effect of dioxin is apparently organ specific as adrenal steroidogenesis is altered following dioxin exposure by reduction of the enzyme cytochrome $P450_{scc}$ (*16,17*).

Table 1. Comparison of plasma steroid concentrations versus *in vitro* gonadal steroidogenesis for juvenile alligators obtained from two central Florida lakes.

Lake	Sex	Hormone	Plasma[a]	Culture
L. Apopka	Male	E_2	Normal[b]	Greater than normal
		T	Less than normal	Normal
	Female	E_2	Greater than normal	Less than normal
		T	Normal	Normal
L. Woodruff	Male	E_2	Normal	Normal
		T	Normal	Normal
	Female	E_2	Normal	Normal
		T	Normal	Normal

[a]Data for plasma values are taken from Guillette et al. (*6*). [b]Normal is defined as the plasma or culture hormone concentrations exhibited by the Lake Woodruff control animals. Indications of abnormality (greater than normal, less than normal) are based on statistical difference between Lake Woodruff and Lake Apopka values at the $p<0.05$ level.

A decrease in gonadotropin release and steroidogenesis are not responses only to dioxin exposure, as male rodents exposed to diethylstilbestrol (DES) or *o′,p′*-DDT exhibit significantly reduced plasma LH concentrations in response to a GnRH challenge (*18*). Nor are abnormal gonadotropin synthesis and steroidogenesis specific to males exposed to various contaminants or synthetic estrogens. Exposure of female mice to DES, a synthetic estrogen that was once used in humans as a therapeutic agent, or various other contaminants (e.g., DDE, PCBs) can modify steroidogenesis (*4*). Additionally, exposure to an estrogenic compound during development has more subtle effects at the cellular level. The cellular effects of DES exposure in female mice include changes in the number of receptors for estrogens (ER), progestins (PR), and epidermal growth factor (EGFR) in the vagina and in the ER and PR in the uterus and mammary gland (studies summarized in *3*). Similar modifications in receptor number are seen in the prostate and seminal vesicle of male mice (*4*). Additionally, there are numerous changes in the protein secretion patterns of the reproductive tract of the mouse following neonatal exposure to DES that are indicative of changes in gene expression (*4,19,20*). These studies in adult and neonatal rodents provide clues to possible loci affected by *in utero* exposure, but mechanisms by which steroidogenesis is altered following embryonic or neonatal exposure to endocrine-disrupting xenobiotic chemicals are still under study. No data are available concerning the activities of various enzymes associated with steroidogenesis in alligators, nor are there data on circulating gonadotropin concentrations. Further studies must examine the biochemistry of gonadal steroidogenesis in alligators as well as other wildlife models, as our data suggest that a modification of gonadal estrogen synthesis has occurred.

Interestingly, we reported that male juvenile alligators from Lake Apopka with suspected organochlorine contamination (e.g., DDE) had low plasma T concentrations similar to those of females from either the contaminated or control lakes (*6*). Previous studies had shown that alligator eggs obtained from Lake Apopka had as their major contaminant *p,p′*-DDE, with concentrations up to 5.8 ppm wet weight (*21*). We hypothesized that the testes from animals obtained from the contaminated lake were feminized due to the endocrine-disrupting activity of DDE, and thus they synthesized estrogens instead of androgens.

The data obtained from culturing these testes support the first prediction; that is, the testes from Lake Apopka juvenile male alligators make significantly more E_2 than testes obtained from the control males. However, we also observed that the testes from Lake Apopka males released T into the culture media at a level similar to the control testes. Thus, the differences in plasma T concentrations previously reported in these animals (*6*) are apparently due to a factor other than modified gonadal steroidogenesis. Variation in plasma T concentrations can be due to a modification in the degradation rate of this steroid either by biochemical changes in the liver or variation in the plasma proteins that bind steroids.

Typically, steroid hormones are found in either a bound or free form in the plasma (*22*). The ratio of free to bound hormone is highly variable, and is dependent on sex, species, and many other physiological attributes. The plasma proteins most effective in binding steroids include plasma albumins and sex steroid binding globulin (SBG) (*23*). The source of the SBG is not fully established, but a human hepatoblastoma cell line synthesizes a protein indistinguishable from SBG, suggesting that the liver may be the source of the sex steroid binding protein (*24*). It is known that elevated plasma E_2 concentrations stimulate a rise in plasma SBG concentration in humans whereas elevated plasma T stimulates a decline (*22*). In reptiles, steroid binding proteins have been identified in the plasma. An SBG-like protein has been reported in several snakes (*25*), a freshwater turtle (*26,27*), and in a lizard (*28,29*). The SBP-like protein identified in a turtle and a lizard had high affinity for T as well as E_2. The source of the SBGs in reptiles is unknown as is the stimulus for their production.

The binding of sex steroid hormones to plasma proteins affects not only the amount of hormone available to the target cells but also influences the degradation rate of these hormones. The hepatocytes of the liver are the major site of steroid metabolism. The amount of hormone available to these cells is dependent upon the capillary transit time, the dissociation time of the hormone from the protein carrier, the amount of hormone bound to various plasma proteins, and the membrane permeability of the hepatocytes. Hormones bound to SBG are thought to be protected from hepatic metabolism.

Modifications in the ratio of SBG to plasma albumins can have dramatic effects on the circulating concentrations of various steroid hormones and their function at the cellular level. It has been suggested that high levels of SBG produce a feminizing influence as large amounts of T are removed from the free pool in the plasma, whereas masculinization occurs when SBG concentrations drop (*30*). Our data suggest that the abnormally low concentrations of free T found in the blood of male juvenile alligators from Lake Apopka is not due to depressed synthesis of this hormone from the gonad but is due instead to either elevated levels of degradation (presumably in the liver) or binding to plasma proteins, so that plasma concentrations are comparable to those seen in normal females.

Elevated metabolism of plasma T may be due to modifications of the liver or changes in the concentrations of plasma carrier proteins. If SBG levels are reduced due to exposure to contaminants, then we could hypothesize that the majority of the testosterone would be bound to plasma albumins and readily metabolized. In contrast, estrogens have been shown to raise SBG concentrations in the plasma, and many contaminants have been shown to be estrogenic (*1,31*). Elevated concentrations of SBG, induced by estrogenic or anti-androgenic contaminants, could significantly decrease the free T in the plasma pool. Future research needs to investigate the role of hormone-mimicking contaminants in modifying the composition and concentrations of various steroid binding plasma proteins. There is ample evidence that exposure to various hormones or contaminants modifies hepatic enzymes and hepatic function during embryonic or neonatal development (*32,33*). As the liver is suggested as the source of the steroid binding plasma proteins, it is not unlikely that changes in hepatic functioning would modify steroid metabolism in contaminant-exposed wildlife as reported for laboratory rodents (*34*).

We hypothesize that xenobiotic compounds are modifying reproductive and endocrine development and function in alligators exposed *in ovo*. We suggest that the changes in the reproductive and endocrine systems we have reported here and previously are the result of modifications in gonadal steroidogenic activity, hepatic degradation of steroids, and synthesis of plasma sex steroid binding proteins. These predictions need further study at the cellular and biochemical level.

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