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Alligators, Contaminants and Steroid Hormones

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Steroids are essential for successful reproduction in all vertebrate species. Over the last several decades, extensive research has indicated that exposure to various environmental pollutants can disrupt steroidogenesis and steroid signaling. Although steroidogenesis is regulated by the hypothalamic-pituitary axis, it is also modified by various paracrine and autocrine factors. Furthermore, the classical two-cell model of steroidogenesis in the developing ovarian follicle, involving the granulosa and theca cells in mammals, may not be universal. Instead, birds and probably reptiles use the two thecal compartments (theca interna and theca externa) as sites of steroid production. We have documented that embryonic or juvenile exposure to a complex mixture of contaminants from agricultural and storm water runoff leads to altered steroid hormone profiles in American alligators. Our observations suggest that alterations in plasma steroid hormone concentrations are due in part to altered gene expression, modified hepatic biotransformation and altered gonadal steroidogenesis. Future studies must examine the interplay between endocrine and paracrine regulation in the development and expression of gonadal steroidogenesis in individuals exposed to endocrine disrupting contaminants at various life stages if we are to fully understand potential detrimental outcomes.

1. Introduction

Over the last decade, it has become well established that various classes of environmental contaminants alter cellular signaling, particularly that of steroid hormones. Three systems in vertebrates, the nervous, endocrine, and immune systems, coordinate a vast array of external and internal information. Contaminants can alter the production, processing, reception, and interpretation of this information stream.[1,2] For example, various chemical contaminants can act on endocrine receptors, either as ligands or antagonists, or alter receptor gene expression.[3] They can also modify hormone synthesis or degradation.[4,5] This process of disrupting or altering hormone signaling has been termed 'endocrine disruption'.

There is great concern about the role of endocrine active compounds on the developing embryo, as hormonal signals play an important organizational role.[6–9] Furthermore, developmental processes determine the lifelong potential of endocrine tissues to respond to environmental cues, such as season, stress, or nutrition. Therefore, an animal's hormonal milieu at any given time is a function of current environmental and physiological conditions, and developmental history. To understand the full scope of how environmental contaminants affect physiology, it is necessary to appreciate endocrine function and disruption in the context of development.

Developmental windows of vulnerability are not exclusive to embryos. For example, mice undergo ovarian folliculogenesis after birth, and postnatal exposure to

diethylstilbestrol (DES), bisphenol A (BPA), or genistein can result in morphological and functional abnormalities of a mature ovary.[10,11] In alligators, the critical organizational process of folliculogenesis also occurs during the weeks after hatching (Moore, unpublished data). Therefore, the establishment of adult female fertility in alligators is dependent on a postnatal event that is distinctly susceptible to organizational disruption.

In the following short review, we describe the regulation of gonadal steroidogenesis, and summarize a series of recent studies that examine the effect of endocrine disruption on steroid hormones in American alligators from lakes in the state of Florida, USA. We note that, while hypothesis testing in comparative species tends to be informed by more established mammalian models, research continues to uncover important differences between mammals and nonmammals.[12–14]

2. Steroidogenesis and Regulation of Plasma Sex Steroid Concentrations

Plasma steroid concentrations in vertebrates represent an integration of synthesis, storage of hormones on plasma proteins, and clearance, principally via hepatic biotransformation (Fig. 1). To determine how each of these mechanisms is susceptible to disruption by contaminants, one needs to first understand the normal function of these phenomena.

2.1 *Steroid hormone synthesis—overview*

Synthesis of sex steroid hormones occurs primarily in the gonad, either in the Leydig cells of the testis or in the granulosa and theca cells of the ovary. In addition to the gonads, steroidogenesis occurs in the heart, adipose tissue, brain, head kidney (fish), adrenal gland, and placenta.[15]

Gonadal synthesis of steroids is classically regulated by the hypothalamic-pituitary axis, via gonadotropin releasing hormones (GnRHs), which modulate the secretion of the pituitary gonadotropin, luteinizing hormone (LH) (Fig. 1). Gonadal steroidogenesis is initiated in the mitochondria following LH stimulation; free cholesterol is acted upon by a P450 side chain cleavage enzyme (P450 scc) to generate pregnenolone (Fig. 2). Pregnenolone can be further modified in the mitochondria by 3β-hydroxy steroid dehydrogenase (3β-HSD) to produce progesterone, or it can be transported to the cytoplasm where it is converted to various steroids, including estrogens and androgens, by interaction with enzymes associated with cytoplasmic microsomes. The initiation of this process is thought to be regulated largely by the availability of a steroid acute regulatory (StAR) protein, which serves to transfer free cholesterol from cytoplasmic pools to the inner membrane of the mitochondria where P450scc is located[16] (Fig. 2). The expression of gonadal StAR is regulated by steroidogenic factor-1 (SF-1) in mice.[17,18] SF-1 is expressed in steroidogenic tissues where it also regulates the expression of several steroidogenic enzymes.

2.2 *Regulation of testicular steroidogenesis—the mammalian model*

The regulation of steroidogenesis in the testes is usually via LH. However, the autocrine/paracrine roles of StAR and SF-1 in the gonadal regulation of steroidogenesis indicate that the reality is more complex. In addition, there are significant regulatory differences between developing and mature testes. Unlike adult mouse testicular steroidogenesis, which is LH-dependent, the initiation of fetal testicular steroidogenesis, along with Leydig cell differentiation, is LH-independent, although still LH-responsive.[19–21] This is evident in LH-receptor (LH-R)-knockout mice that show normal steroidogenesis as fetuses and newborns, but dramatically reduced steroidogenesis as adults.[22] Although LH is not necessary for fetal mouse testicular steroidogenesis, an intact pituitary is required. That is, adrenocorticotrophic hor-

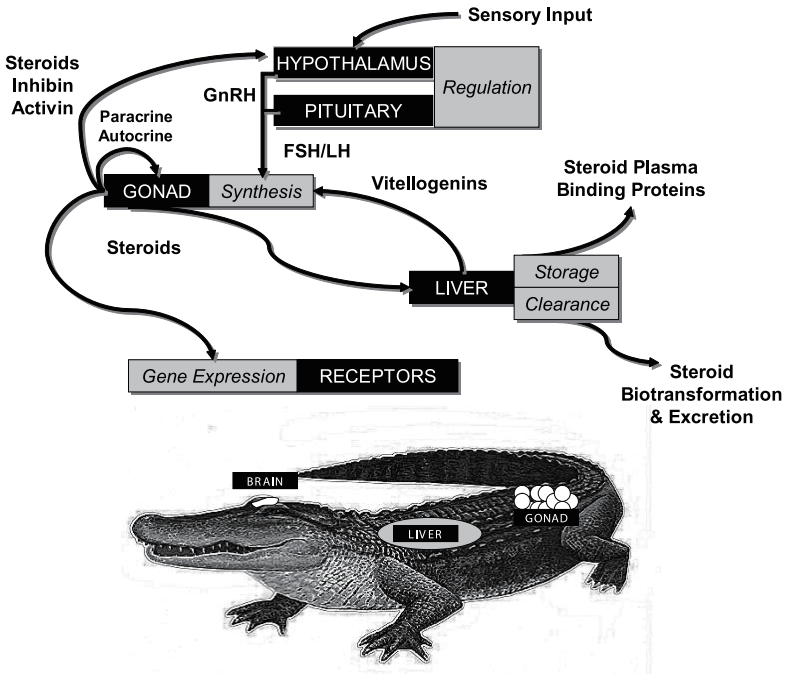


Fig. 1. Steroid hormone production from the gonad is regulated, in part, by the hypothalamo-pituitary axis. Circulating concentrations of hormones interact with steroid receptors in tissues throughout the body, altering gene expression profiles. Plasma steroid hormone concentrations are also affected by plasma binding proteins, synthesized by the liver as well as hepatic biotransformation and clearance.

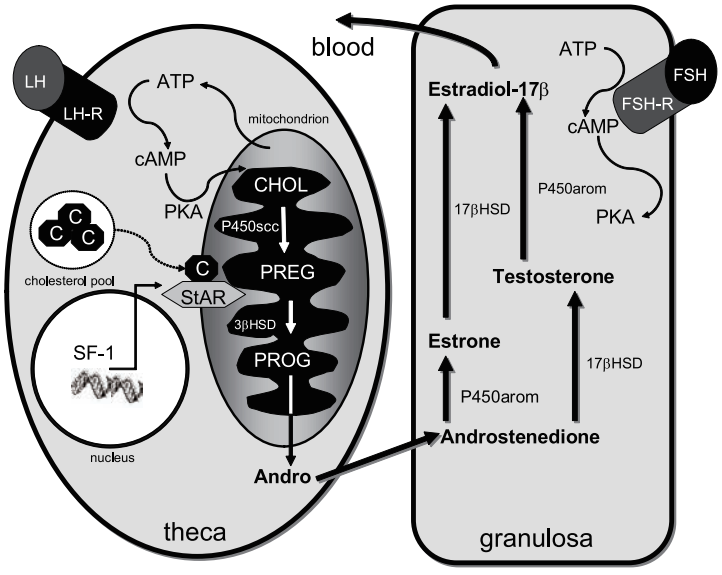


Fig. 2. Representative common model of ovarian follicular steroidogenesis involving granulosa and theca interna cells in mammals. The transfer of cholesterol into the mitochondrion by steroid acute regulatory (StAR) protein is considered a major rate-limiting step. Pregnenolone or progesterone is released into the cytoplasm/smooth endoplasmic reticulum to be further modified to androstenedione which is transferred to the granulosa where it can be converted to various estrogens.

mone (ACTH) also stimulates Leydig cells and maintains their steroidogenic function in a possibly redundant relationship with LH.[21]

Several paracrine factors, some from Sertoli cells, have been demonstrated to modulate Leydig cell differentiation and steroidogenic capacity. These include interleukin-1α, transforming growth factor-β, inhibin, insulin-like growth factors I and II, vascular endothelial growth factor, and relaxin-like growth factor.[23] In addition, fetal Leydig cell differentiation and the initial up-regulation of SF-1 and P450scc expressions are regulated through the expression of desert hedgehog (Dhh) produced in Sertoli cells.[24] Furthermore, developmental steroid signaling itself has the ability to modulate steroidogenesis. For example, follicle stimulating hormone (FSH) from the pituitary increases aromatase expression in fetal Sertoli cells, stimulating them to produce estradiol.[25] The estradiol binds estrogen receptors (ERα) expressed by fetal Leydig cells, and inhibits both Leydig cell hypertrophy and expression of StAR and steroidogenic enzymes.[26] This natural regulatory mechanism represents one pathway by which environmental estrogens can alter testicular development and function. Loss of androgen signaling in mouse testes, with androgen-receptor-knockout Sertoli cells, results in decreased number of Leydig cells in the adult, putatively through changes in downstream paracrine signaling.[27] Again, these results indicate that naturally occurring mechanisms are present through which anti-androgenic contaminants can alter testicular steroidogenesis.[28]

2.3 *Regulation of ovarian steroidogenesis—the mammalian model*

Hypothalamic-pituitary effects are important in ovarian follicular steroidogenesis, as they are in the testis. And, like the testis, paracrine ovarian signals also affect many aspects of the steroidogenic capabilities of the ovary. For example, paracrine factors can stimulate theca differentiation in LH-R-deficient thecal precursor cells.[29] Later in follicular development, paracrine ovarian signals stimulate androgen production by inducing steroidogenic enzyme expression in the presence of only basal levels of LH, which, alone, could not induce androgen production.[30–32]

Inhibin, activin and IGF-1, produced by granulosa cells, modulate ovarian steroidogenesis.[33,34] The transforming growth factor inhibin is a heterodimer of the alpha and beta subunits. Activin, a homodimer of two beta subunits, has been demonstrated to increase the proliferation of thecal cells,[35] but decreases androgen production and CYP17 expression in the presence of forskolin, a pharmaceutical cAMP stimulator, compared with forskolin alone[34] (Fig. 3). In contrast, inhibin, also in the presence of forskolin, up-regulates thecal androgen production by increasing the expression of CYP17.[34] FSH induces granulosa cell production of inhibin which, in turn, has been shown to increase theca androgen production *in vitro*[36] and *in vivo*[37] in a way unrelated to thecal LH-R expression. Therefore, the gonadotropin regulation of granulosa activin/inhibin production affects theca proliferation and function (Fig. 3).

IGF-1 from granulosa cells alone can up-regulate LH-R and 3β-HSD expression, but not CYP17 or StAR, in thecal cells independent of LH[38–40] (Fig. 3). However, IGF-1 also works synergistically with LH signaling to promote steroidogenesis.[41] Additionally, IGF-1 interacts with activin to promote the proliferation of thecal cells[35] and with forskolin to promote the expression of androgenic enzymes.[42]

In addition to the paracrine androgenic actions of molecules originating from granulosa cells, recent research has focused on oocyte-derived factors capable of regulating thecal cell steroidogenic function through granulosa-oocyte paracrine feedback that, in turn, modulates thecal steroidogenic activity (Fig. 3). Kit ligand (KL), produced by granulosa cells, also known as stem cell factor, communicates with both oocytes and theca, promoting thecal cell recruitment and regulation of androgen production[43,44] (Fig. 3). In immature follicles, the expression of Bone Morphogenic Protein -15 (BMP-15) from follicles stimulates KL expression from

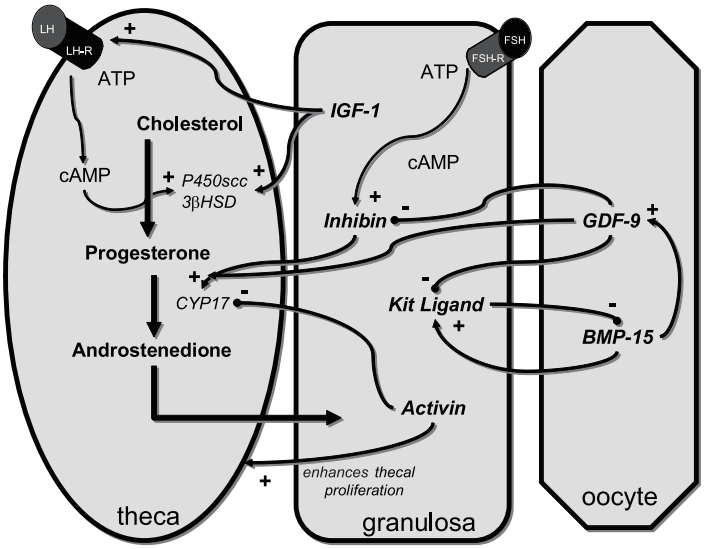


Fig. 3. Partial representation of many paracrine factors involved in regulation of steroidogenesis in mammalian ovarian follicle. This model begins to categorize the many possible mechanisms that can be disrupted by endocrine-active contaminants. See text for further explanation.

granulosa cells (Fig. 3). KL then down-regulates oocyte BMP-15 expression and, in turn, oocytes increase the expression of Growth and Differentiation Factor-9 (GDF-9) that down-regulates granulosa KL expression.[45] Although some research studies have demonstrated that oocyte-derived GDF-9 directly stimulates androgen production and CYP17 expression in theca cell lines,[46] other studies show that GDF-9 does not directly regulate theca development[47] (Fig. 3). Rather, GDF-9 has an indirect effect by suppressing granulosa expression of the pro-theca factors, inhibin and KL. Ovaries deficient in GDF-9 overexpress inhibin and KL[48] but do not develop theca.[49] Thus, contrary to the pro-theca/steroidogenic actions of both inhibin and KL, the lack of thecal cells in ovaries that do not express GDF-9 implies more complexity to this mechanism than currently understood. Additionally, ovaries lacking both GDF-9 and inhibin expression produce a theca layer that does not express appropriate markers such as CYP17 and LH-R.[47]

While the full paracrine interactions of IGF-1, inhibin, activin, KL, GDF-9, and BMP-15 in recruiting theca and modulating later steroidogenesis need to be elucidated, extensive research demonstrates that oocytes and granulosa cells function in both LH independent and LH synergistic ways to regulate thecal steroid production in mammals. Moreover, FSH plays an indirect role in androgen steroidogenesis through the regulation of many of the paracrine factors produced by the granulosa.

2.4 *Regulation of ovarian steroidogenesis—a comparative view*

Although extensive work has been carried out to understand the paracrine and endocrine factors associated with steroidogenesis in the ovarian follicles of mammals, the body of literature in other species is less developed. However, variation from the mammalian model is coming to light with studies of various avian and reptilian species. Unlike that described in mammals, steroidogenesis in the preovulatory ovarian follicles of the chicken employs a three-cell system: granulosa cells produce progestins, theca interna cells synthesize androgens, and theca externa cells synthesize estrogens[50–52] (Fig. 4). However, in small follicles with only a single layer of thecal

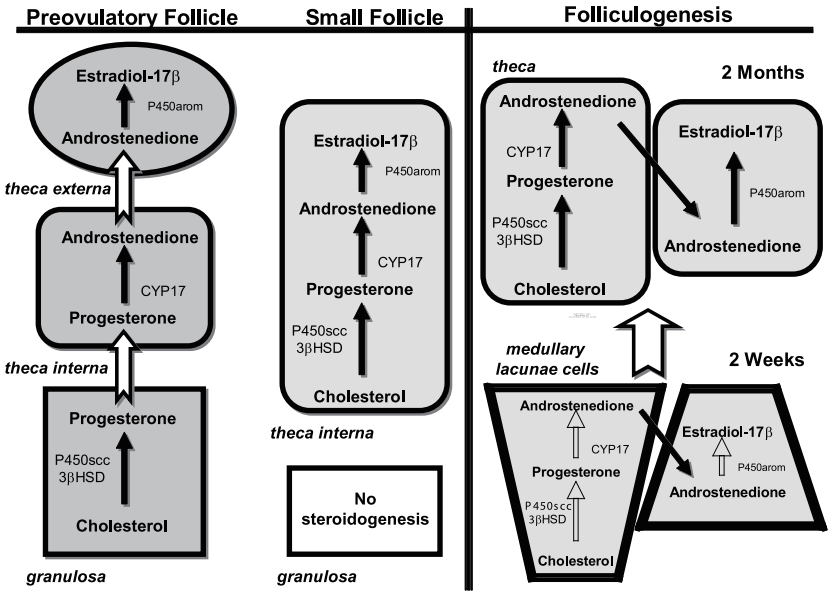


Fig. 4. Development of steroidogenic pathway in ovarian follicle of birds. Large preovulatory follicles use three cell types to complete the steroidogenic pathway from cholesterol to estadiol-17β, whereas steroidogenesis occurs in the thecal cells (no layers distinguished at this stage) of the small follicle only. In the developing ovary, thecal cells are derived from two populations of steroidogenically active medullary lacunae cells: the interstitial cells that synthesize androgen and a second cell type, the aromatase cells. Both cell types migrate to the cortex and surround the granulosa cells of the primary follicle, becoming the steroidogenically active thecal cells. Thus, the cellular source of ovarian steroids varies significantly in the bird, depending of the stage of folliculogenesis.

cells, the granulosa cells are steroidogenically inactive and progestins, androgens, and estrogens are all produced solely in the theca.[53–55] This relationship may also hold true for developing follicles of snakes and lizards in which only the theca, not the granulosa, store lipids and possess organelles needed for steroid synthesis.[56]

In the hatchling chick ovary, two types of steroidogenic cells are derived from the primary sex cords and are localized in the medulla.[57–59] One population of cells secretes androgens, whereas the other synthesizes estrogens[60] (Fig. 4). From two weeks on, concomitant with the expansion of the cortex, these steroidogenic cells move toward the cortex in the connective tissue of the trabecula and enter the intrafollicular spaces. By two months after hatching, these steroidogenic cells are recruited into the theca layer of the developing ovarian follicles.[58,60] A similar morphological rearrangement/recruitment in steroidogenic cells is also observed in the lizard *Calotes versicolor*. Histochemistry shows 3β-HSD activity beginning after sexual differentiation only in the medulla until one month after hatching when activity was also observed in the cortex concomitant with robust folliculogenesis.[61]

These observations suggest that, similarly to the mammalian model, the regulation of steroidogenesis in birds and reptiles is likely very complex, with paracrine and endocrine mechanisms. Furthermore, dramatic developmental modifications in the relationships among the precursor cells of the granulosa and thecal cells are likely to play an important role in establishing how various endocrine disruptive chemicals alter steroidogenesis early in the development of the ovary.

2.5 *Regulation of plasma hormone concentrations by steroid binding proteins*

Sex steroid concentrations in the plasma are mainly a result of synthesis from the gonads, but the concentration measured in circulation is also due to the 'storage' of sex steroid on plasma proteins such as albumin and sex steroids binding proteins. Alligators have a specific sex hormone binding protein and its presence in the blood varies seasonally.[62] Plasma proteins in alligator blood bind to sex steroids, such as E_2 or various contaminants, and limit availability to induce cellular responses.[63,64] Plasma proteins play an essential role in regulating sex steroid actions during pregnancy in mammals and yet their role in developmental biology of most species is poorly understood.[65,66]

2.6 *Regulation of plasma hormone concentrations by hepatic biotransformation*

Hepatic biotransformation plays an important role in maintaining circulating steroid concentrations in all vertebrates. The liver plays a key role in metabolizing toxins, and peptide and steroid hormones. Hepatic metabolism of steroids and contaminants can exhibit a sexually dimorphic pattern that has been used as a biomarker of exposure to naturally occurring or synthetic hormones and contaminants with hormonal activity.[67–69] Several methods are used for hepatic hormone biotransformation including direct conjugation, in which a steroid is conjugated to glucuronic acid or sulphate, producing a water-soluble product that is excreted in urine (for review, see ref. 4). Steroid hydroxylation can also produce a water soluble metabolite by stereoselectively and regiospecifically attaching hydroxyl groups to a steroid. Oxidoreduction of testosterone to androstenedione, dihydrotestosterone and androstanediols is yet another hepatic pathway by which circulating concentrations of testosterone and other androgens can be regulated.

2.7 *Gonadotropins in alligators*

Two forms of GnRH have been identified in alligator brains, and are similar to the chicken variants cGnRH-I and cGnRH-II.[70] Additionally, bioactive FSH and LH have been isolated from alligator pituitary glands.[71] The amino acid composition of alligator LH is similar to chicken and turkey LH;[72] however, alligators also respond *in vivo* to mammalian LH with increased testosterone secretion.[73] Additionally, studies have shown that exogenous ovine FSH stimulates a marked increase in circulating sex steroids in juvenile, subadult, or adult alligators (alligator gonadotropin peptides are not available and ovine FSH has been shown to give the most robust steroid response).[74] It is not known if ovine FSH enhances steroidogenesis through downstream paracrine signaling similarly to that observed with endogenous LH or FSH. A receptor with high sequential similarity to chicken FSH-R has been sequenced from alligator gonad cDNA; however, its receptor dynamics remain to be characterized (Moore, Zhang and Guillette, unpublished data). Given these observations, the regulation of steroidogenesis in the alligator by gonadotropins is likely to be similar to that reported in other vertebrates.

2.8 *Steroid receptors in alligators*

Steroids act on tissues throughout the body by interaction with their specific receptors. In vertebrates, the steroid receptors belong to a superfamily of nuclear transcription factors that include the steroid hormone receptors for progestogens, androgens, glucocorticoids, mineralocorticoids, as well as the vitamin D and retinoic acid receptors.[75] Three types of ER have been isolated in vertebrates. Fish have ERα, ERβ and ERγ but the teleost ERγ form appears to be closely related to teleost ERβ indicative of gene duplication that occurs within the teleosts.[76] The ancestral condition for the vertebrates appears to be the presence of two forms of ER, ERα

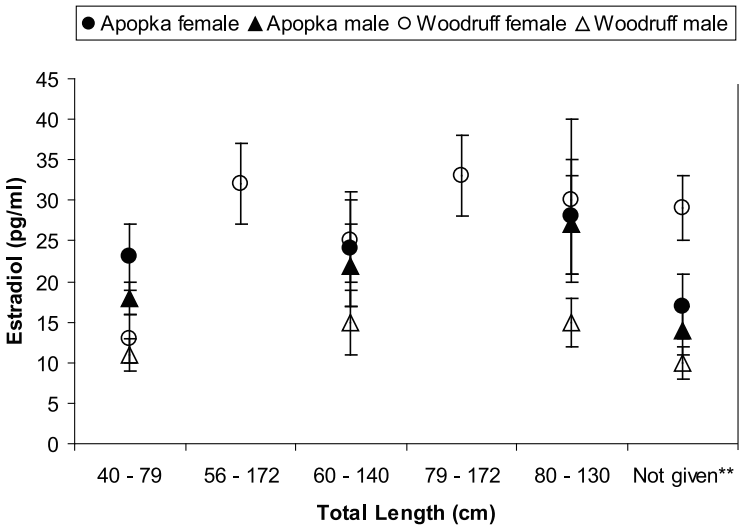
and ERβ.[77,78] Indeed, these two forms of ER were previously found in all vertebrate groups and we have likewise shown that the alligator has two nuclear ERs, ESR1 (ERα) and ESR2 (ERβ). These were cloned and sequenced and shown to have a very high sequence similarity to the estrogen receptors of birds.[79] These receptors have distinct ligand and DNA binding regions that are highly conserved with other species of crocodilians and birds.[79–81]

3. Contaminants and Steroids in Alligators

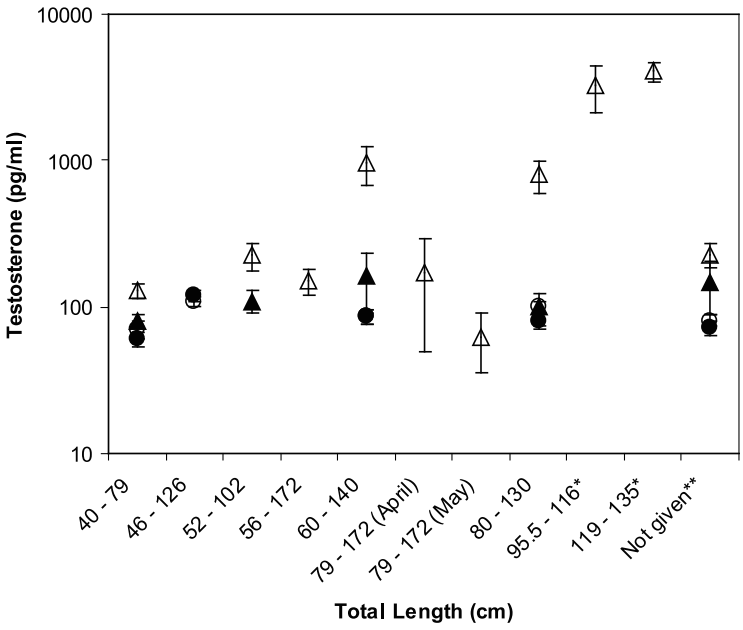
In 1994, we reported that 9-month-old alligators from Lake Apopka, FL, obtained as eggs and grown in the laboratory, showed altered plasma concentrations of sex steroids when compared with animals from reference populations.[82] Females showed elevated estradiol-17β (E_2) concentration, whereas males had reduced plasma testosterone (T) concentration. We also reported that juvenile alligators from Lakes Apopka, Griffin and Okeechobee, FL, all polluted with a complex mixture of agricultural pesticides, their metabolites, nutrients, sewage, and chemicals from storm water runoff, had altered plasma sex steroids relative to alligators from reference sites.[83,84] Primarily, males had reduced plasma T concentration but could also have elevated plasma E_2 concentration, whereas females showed reduced plasma E_2 concentration.

Figure 5 shows the summary of the mean plasma estradiol and testosterone concentrations from several comparative studies of juvenile and subadult alligators sampled from Lakes Apopka and Woodruff in April and May, the months that coincide with adult mating season.[74,83,85–88] Across these studies, mean estradiol concentration was highest among Woodruff females, followed by Apopka females and then Apopka males (Fig. 5A). Woodruff males had the lowest plasma estradiol concentrations. This trend is true of all sampled size classes except the smallest group, in which Apopka males and females exhibited the highest mean plasma estradiol concentrations. Potentially, these data indicate an earlier onset of puberty among Apopka females. Mean plasma testosterone concentrations were also size-dependent, particularly among Woodruff males. We have consistently observed a decrease in sexually dimorphic testosterone concentrations among Apopka juveniles, compared with those captured from Lake Woodruff (Fig. 5B)

Given our previous observations of altered steroid concentrations in animals that were nearly a year old and in wild juveniles, we examined neonates to determine if a clear pattern was established early in life. Neonatal alligators, less than 1 month of age, from Lake Apopka were compared with those of a reference population (Lake Woodruff).[89] By examining neonatal animals, this study limited contaminant exposure to that from maternal contribution to the egg. Circulating testosterone concentration and aromatase activity were examined as well as phallus size among males and oviduct epithelial cell height (ECH) among females (androgen- and estrogen-dependent tissues, respectively). Neonatal male alligators from Lake Apopka exhibited higher plasma testosterone concentrations than those from Lake Woodruff,[89] contrary to previously published studies of 9-month-old animals and wild juveniles. Phallus tip length and cuff diameter, however, were smaller in males from Lake Apopka as reported in other studies for older animals.[89] No differences were noted in gonadal aromatase activity in neonatal females from Lake Apopka when they were compared with Lake Woodruff females and no differences were noted in oviduct ECH.[89] Interestingly, if gene expression profiles are examined for the enzymes associated with steroidogenesis (*e.g.*, aromatase, P450scc, 3β-HSD) or for StAR, one can detect a loss of the normal sexually dimorphic pattern in contaminant-exposed animals. That is, the testis of neonatal males from Lake Woodruff expresses higher levels of P450scc, 3β-HSD and StAR but a lower level of aromatase when compared with neonatal ovarian tissue. In contrast, this dimorphism is lost in gonadal tis-



(A)



(B)

Fig. 5. Summary of plasma hormone data published by our laboratory between 1996 and 2004.[74,83,85–88] Graphs show (A) mean estradiol 17β and (B) mean testosterone concentrations ±1 SE for juvenile alligators as reported in the original publications. Male and female alligators of varying lengths were captured in April or May from Lake Apopka (contaminated) or Lake Woodruff (reference) in central Florida. All animals were captured and sampled in the field, except those marked with an asterisk (*), indicating that they were taken as eggs from their lake of origin, hatched in the laboratory, and raised in an outdoor zoo environment. **Reference 87 indicates that animals were juveniles, but does not specify lengths.

sue of neonatal alligators from Lake Apopka (Milnes, Katsu, Guillette and Iguchi, unpublished data). Thus, although plasma T concentration does not differ between neonates, our data suggest that gene expression is altered for various factors that are essential for steroidogenesis.

Can we identify what factors in the eggs – contaminants for example – contribute to these alterations? We and others previously reported that alligators from Lake Apopka have elevated parts per billion (ppb) to parts per million (ppm) concentrations of *p,p'*-DDE and other organochlorine pesticide metabolites in their eggs[90] and blood.[91] Additionally, it has been reported that elevated levels of toxaphene have been found in the tissues of fish and alligators from Lake Apopka.[92] Toxaphene and *p,p'*-DDE, as with many other organochlorine pesticides or metabolites, have been shown to act as endocrine disrupting contaminants. Toxaphene has estrogenic action[93] whereas the action of *p,p'*-DDE is more complex, showing strong anti-androgenic actions in mammalian systems[94] and estrogenic actions in some reptile and fish systems.[95,96] For example, dosing the eggs of alligator, a species that exhibits temperature-dependent sex determination, with several dichlorodiphenyltrichloroethane (DDT) metabolites, trans-Nonachlor, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), or atrazine has produced alterations in sex determination, secondary sex characteristics, gonadal anatomy, and plasma steroid concentrations that are, in part, similar to those reported in wild populations.[97–99]

Embryonic exposure to *p,p'*-DDE in alligators generated a female biased sex ratio among hatchlings exposed to *p,p'*-DDE at 100 ppb wet egg mass at an intermediate incubation temperature but no effect on sex determination was observed for *p,p'*-DDE at higher temperatures that produced all males.[96] *p,p'*-DDE failed to affect plasma T, gonadal aromatase activity, oviductal ECH, or phallus morphology at the concentrations used.[96] These data show that the gonadal response to a contaminant could be due to the interaction of dose and temperature as was observed in this study. In contrast to the results for *p,p'*-DDE, exposure of developing alligator embryos to varying concentrations of toxaphene, a broad-spectrum pesticide found in relatively high concentration in Lake Apopka alligator egg yolk, indicates that toxaphene had little or no effect on sexual differentiation but male plasma T concentrations were higher in animals treated with 0.01 and 10 μg toxaphene/kg (based on mean egg mass) than in control males.[92] Interestingly, the elevated plasma T concentration concentration in neonates exposed to toxaphene is similar to that reported in neonates that come from eggs collected from Lake Apopka, suggesting that this specific alteration could be due to maternally deposited toxaphene in the egg. In contrast to the observed differences in plasma concentrations, *in vitro* testicular T production was not different when toxaphene-exposed and control neonates were compared, suggesting that the difference in plasma T could be due to differences in hypothalamic-pituitary stimulation of the gonad or hepatic steroid degradation.[92]

We previously examined the hepatic biotransformation of steroids and reported that juvenile alligators from lakes Apopka and Okeechobee show altered biotransformation of androgens when compared with animals from reference populations.[67] One enzyme important in steroid biotransformation is the hepatic enzyme CYP3A that plays a broad role in biotransforming both exogenous compounds and endogenous hormones such as testosterone and estradiol. Organochlorine compounds are known to induce CYP3A expression in other vertebrates; thus, we have examined whether CYP3A induction by organochlorine contaminants could increase the biotransformation and clearance of sex steroids by CYP3A and provide a plausible mechanism for the reported alterations in endogenous sex steroid concentrations in juvenile alligators. Toxaphene exposure significantly induced CYP3A77 gene expression (3.5-fold) in juvenile alligators within 24 h of exposure but plasma T concentrations did not change significantly following treatment.[100] Interestingly, we did not observe differences in hepatic CYP3A77 gene expression when juveniles

from lakes Apopka and Woodruff were compared.[100] These experimental studies are beginning to provide some basis for the causal relationships between embryonic pesticide exposure and reproductive abnormalities that have been lacking in descriptive field studies of wild populations.

3.1 *Nitrate: a new area of study*

We have recently published a retrospective regression analysis of the association between nitrate contamination in seven Florida lakes and altered steroid hormones measured in resident alligators.[101] Sourced primarily from atmospheric deposition, municipal sewage, and agricultural runoff (manure and fertilizer), nitrogen has been called the second global pollutant behind carbon dioxide.[102] The regression analysis showed that plasma T concentrations in juvenile male and female alligators were negatively correlated with mean total nitrogen concentration in lake water. Plasma E_2 concentrations, on the other hand, were positively correlated with total lake water nitrogen concentration for males, and lake water nitrate-nitrogen concentration for females.[101] Although this analysis is by no means definitive, the data do suggest that some components of nitrogen pollution have the potential to affect the regulation of plasma sex steroid concentration. In a study of mosquito fish captured from eight Florida springs contaminated with a range of nitrate concentrations (1–5 mg/L NO_3-N), we observed a significant negative relationship between nitrate concentration and both embryo dry weight and percentage of mature females that were reproductively active.[103] These results suggest that nitrate can interfere with reproductive function, including steroidogenesis in wildlife at ecological relevant concentrations.

On the basis of an extensive literature review and our own observations, we have extended a hypothesis originally proposed by Panesar and Chan.[104] That is, we have proposed that nitrate can alter steroidogenesis *in vivo* by (1) its conversion to nitrite and nitric oxide (NO); NO has been shown to inhibit StAR, 3β-HSD, and P450scc, (2) altering cellular chloride ion concentration, which has been shown to affect steroidogenesis, and/or (3) binding to the heme molecule of P450 steroidogenic enzymes and altering their action.[101] Extensive future research is needed on this globally distributed endocrine disrupting contaminant.

4. Conclusions

Steroid hormones play a central role in the reproductive biology of all vertebrate species and a disruption in steroid signaling by environmental contaminants, via various mechanisms, is a major concern. Although the hypothalamo-pituitary regulation of gonadal steroidogenesis is ubiquitous among vertebrates, accumulating data indicates that paracrine regulation at the level of the gonad is also an essential component. Furthermore, we have documented here that variation exists in the regulation of various steroidogenic cells with development and sexual maturation. Moreover, ovarian steroidogenesis in birds and mammals has distinctive elements, in that granulosa and thecal cells in these groups perform different functions in the steroidogenic pathway, depending on follicular stage. All these factors contribute to a concern that endocrine disrupting contaminants can alter steroidogenesis as the gonad develops, or later in the gametogenic and steroidogenic processes. Also, our review underscores that an environmental impact (or lack of impact) on steroidogenesis in an animal of a given age may not be directly translated to animals of differing ages or animals of other species due to variations in the patterns of steroid production or ontogenetic factors. Given its central role in the reproductive biology of all vertebrate species, future studies must examine steroidogenesis in more detail, specifically the ontogenic and mechanistic effects of endocrine disrupting contaminants.

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