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Altered Sex Hormone Concentrations and Gonadal mRNA Expression Levels of Activin Signaling Factors in Hatchling Alligators From a Contaminated Florida Lake

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Abstract

Activins and estrogens participate in regulating the breakdown of ovarian germ cell nests and follicle assembly in mammals. In 1994, our group reported elevated frequencies of abnormal, multioocytic ovarian follicles in 6 month old, environmental contaminant-exposed female alligators after gonadotropin challenge. Here, we investigated if maternal contribution of endocrine disrupting contaminants to the egg subsequently alters estrogen/inhibin/activin signaling in hatchling female offspring, putatively predisposing an increased frequency of multioocytic follicle formation. We quantified basal and exogenous gonadotropin-stimulated concentrations of circulating plasma steroid hormones and ovarian activin signaling factor mRNA abundance in hatchling alligators from the same contaminated (Lake Apopka) and reference (Lake Woodruff) Florida lakes, as examined in 1994. Basal circulating plasma estradiol and testosterone concentrations were greater in alligators from the contaminated environment, whereas activin/inhibin βA subunit and follistatin mRNA abundances were lower than values measured in ovaries from reference lake animals. Challenged, contaminant-exposed animals showed a more robust increase in plasma estradiol concentration following an acute follicle stimulating hormone (FSH) challenge compared with reference site alligators. Aromatase and follistatin mRNA levels increased in response to an extended FSH challenge in the reference site animals, but not in the contaminant-exposed animals. In hatchling alligators, ovarian follicles have not yet formed; therefore, these endocrine differences are likely to affect subsequent ovarian development, including ovarian follicle assembly.

Morphological malformations are often caused by underlying genetic, endocrine, or physiological abnormalities. Multioocytic follicles (MOFs, alternatively called polyovular follicles) are two or more oocytes surrounded by a common follicular envelope of granulosa cells. These malformed follicles are hypothesized to result from oogonial clusters that do not

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properly dissociate and remodel during normal ovarian follicle assembly (Iguchi and Takasugi, '86; Iguchi et al., '86; Kipp et al., 2007a; Mayo et al., 2007). Normally, this morphology is rare. However, increased frequencies of MOFs can be generated in animals by experimental or pharmaceutical prenatal and neonatal exposure to estrogens, such as the natural endogenous estrogen, estradiol-17β (E_2) (Nakamura et al., 2008), pharmaceutical estrogens, such as diethylstilbestrol (DES) (Kim et al., 2009), or phytoestrogens (Jefferson et al., 2006). Additionally, transgenic modifications that lead to over expression of ovarian signaling factors, such as the inhibin α subunit (Inha), also can produce this pathology in rodents (McMullen et al., 2001). Therefore, MOFs can have multiple etiologies, but these factors could converge on a common signaling network regulating follicle assembly (Mayo et al., 2007).

Until a decade ago, research largely viewed increased frequencies of MOFs as a mammalian pathology resulting from improper estrogenic exposure. However, in 1994, our group reported MOFs at a very high frequency in female alligators exposed during embryonic development to environmental contaminants. Lake Apopka, Florida, is contaminated with various pesticides and anthropogenic nutrients (Heinz et al., '91; Guillette et al., '99; Rauschenberger et al., 2007), resulting in reproductive impairments to resident alligators (Woodward et al., '93; Fujisaki et al., 2007; Milnes and Guillette, 2008). Female hatchling alligators from Lake Apopka, after being administered a luteinizing hormone challenge, displayed MOFs (often 3–4 oocytes per follicle) and elevated plasma E_2 concentrations, compared with a low (0–3% of the follicles in an ovary) frequency observed in reference females of a similar age within the same study (Guillette et al., '94b).

Studies of estrogenic exposures using another crocodilian species (*Caiman latirostris*) support our findings of altered gonadal morphology and endocrine physiology. Bisphenol A (BPA) is an industrial chemical shown to have estrogenic and antiandrogenic properties (Richter et al., 2007). Across vertebrates, BPA has the potential to induce a wide range of impacts on gonadal functions (Crain et al., 2007). In ovo exposure of developing caiman to E_2 or BPA resulted in male to female sex reversal (Stoker et al., 2003). Therefore, as earlier observed in alligators (Crain et al., '97), embryonic crocodilian gonads are responsive to exogenous steroidal signaling through both endogenous ligands and endocrine disrupting contaminant exposures. Further, laboratory-raised female caiman exposed in ovo to E_2 or BPA, during the beginning of sex differentiation, displayed elevated circulating E_2 concentrations and higher proportions of advanced follicles as hatchlings. In animals incubated at female-producing temperatures and sex-reversed females incubated at male-producing temperatures, E_2 treatment increased MOF frequencies in juvenile (3- and 12-months post-hatching) caiman (Stoker et al., 2008). Furthermore, BPA exposure increased MOF frequency in sex-reversed females compared with control sex-reversed animals. These findings support a hypothesis that in ovo exposure to endocrine disrupting contaminants can result in long-lasting impacts on reproductive endpoints.

At hatching, alligator ovaries do not possess follicles. Follicle assembly occurs slowly over many months post-hatching (Forbes, '40; Moore et al., 2010b, 2008), and the mechanisms regulating this process are being investigated. In mice, follicle assembly occurs over the first 3 days postnatal and some of the signaling mechanisms that regulate follicle assembly are

becoming clear. Transforming growth factor β (TGF-β) superfamily signaling plays a vital role in follicle assembly and establishment of the ovarian follicle pool (Drummond, 2005; Trombly et al., 2009). Activins are transforming growth factor ligands that act as ovarian paracrine signals and regulate a variety of endpoints, including growth and cellular differentiation (Pangas et al., 2007; Onagbesan et al., 2009). Activin signaling plays a role in regulating follicle assembly in neonatal mice. Augmentation of activin levels in neonatal mice increases germ and granulosa cell proliferation and primordial follicle numbers in juvenile animals (Bristol-Gould et al., 2006).

TGF-β superfamily ligands form from large precursor proteins that are processed and assembled into mature dimers. Activin ligands are homo or heterodimers of two β subunits (Inhba and Inhbb). Activin ligands act as agonists, work through membrane-bound activin receptor complexes, stimulate Smad-mediated secondary messenger cascades, and ultimately modulate gene expression (Ethier and Findlay, 2001). Production of inhibins or follistatin (Fst) antagonizes activin signaling. Inhibin ligands, activin receptor binding and activation antagonists, are heterodimers of a β subunit and an α subunit (Inha), forming either inhibin A (Inhba+Inha) or inhibin B (Inhbb+Inha). Follistatin is a TGF-β ligand antagonist that binds and neutralizes activins (Nakamura et al., '92; Welt et al., 2002). We have demonstrated the expression of *Inhba*, *Inhbb*, *Fst*, and *Inha* mRNA in alligator gonads during the first 5 months post-hatching (Moore et al., 2010a). Furthermore, expression levels were sexually dimorphic, with testes expressing elevated levels of *Inha* and *Inhbb* mRNA and ovaries expressing elevated Fst mRNA levels.

During germ cell nest breakdown and subsequent follicle assembly, an activin-dominated signaling milieu could be critical (Mayo et al., 2007; Trombly et al., 2009). Activins participate in signaling crosstalk with steroid hormones. Estrogens suppress activin gene expression (Kipp et al., 2007a) while, in turn, activins induce the expression of estrogen receptors (Kipp et al., 2007b).

In rodents, it is hypothesized that high levels of maternal steroids impede follicle assembly and after birth, steroid levels fall and potentiate ovarian follicle assembly (Kezele and Skinner, 2003; Pepling, 2006; Chen et al., 2007), possibly via TGF-β superfamily signaling pathways (Mayo et al., 2007). Follicle stimulating hormone (FSH) may also play a role in regulating follicle assembly. When E_2 levels fall in the postnatal mouse, serum FSH levels rise from birth to day 7. These changes encompass the period of primordial follicle formation. In vitro organ culture of mouse ovaries in the presence of low E_2 (fetal level) allows FSH to upregulate the expression levels of FSH receptor (*Fshr*), activin βA subunit, and oocyte-specific transcription factors associated with primordial follicle formation (Lei et al., 2010). Furthermore, FSH facilitates the breakdown of germ cell nests and primordial follicle formation at both high (maternal) and low (fetal) E_2 organ culture conditions. These results support a hypothesis that the regulation of ovarian follicle formation involves an integration of estrogen, activin, and FSH signaling.

In contrast to eutherian embryonic development, embryonic alligators do not maintain a direct maternal endocrine connection during embryonic development. However, alligator eggs are invested with a substantial, maternally derived yolk that supplies nutrient

throughout in ovo and post-hatching development. Environmental contaminants, including pesticides, are passed from mother to yolk (Rauschenberger et al., 2007). Furthermore, Lake Apopka contaminants at concentrations observed in alligator eggs interact with alligator estrogen receptors in vitro (Vonier et al., '96) and produce male to female sex reversal after in ovo exposure of turtle (*Trachemys scripta elegans*) embryos (Willingham and Crews, '99). Thus, Lake Apopka is contaminated with a mix of chemicals that potentially impact multiple physiological systems; however, these results demonstrate that many Lake Apopka contaminants display estrogenic activity.

We have observed that alligators hatched from eggs removed from wild nests before sex determination and incubated under identical conditions with eggs from reference populations exhibited an increased frequency of MOFs. Therefore, factors that lead to an increased frequency of MOFs are not exogenous factors experienced during development in the nest, such as temperature or humidity, but more likely pass maternally to the embryo via the egg. We propose that in female alligators from Lake Apopka, the maternal contribution of contaminants to the egg yolk subsequently alters inhibin/activin signaling in female offspring putatively through alteration of estrogenic signaling and, therefore, predisposes increased frequency of MOF formation.

Here, we employ the paradigm of ovarian follicle assembly regulation through interactions between estrogen, activin, and FSH signaling. We examine both basal and FSH-stimulated levels of circulating E_2 and testosterone (T) and gonadal mRNA expression levels of *Inha*, *Inhba*, *Fst*, aromatase (*Cyp19a1*), and *Fshr*. Comparatively, ovaries of both embryonic and hatchling chicken are responsive to exogenous FSH, both in vitro (Pedernera et al., '99) and in vivo (Gonzalez-Moran, '98; Mendez-Herrera et al., '98; Sanchez-Bringas et al., 2006), resulting in elevated circulating estradiol and ovarian cell proliferation. Furthermore, in cultured chicken granulosa, activin signaling is necessary to maintain morphological differentiation (Schmierer et al., 2003), whereas FSH increases expression of *Inha*, *Fst*, and *Inhba* mRNA (Davis et al., 2001; Safi et al., 2003). Differences in the levels of these factors between contaminant exposed and reference animals could differentially increase in a gonadotropin-challenged ovary, as occurred just before necropsy in the cohort described in our earlier work. We hypothesize that early exposure to abnormal estrogenic signaling alters both basal and stimulated levels of circulating steroid hormones and transcript levels of activin signaling factors.

MATERIALS AND METHODS

We collected American alligator (*Alligator mississippiensis*, Daudin, 1801) eggs from nests at Lake Woodruff National Wildlife Refuge and Lake Apopka on June 27 and 28, 2005, respectively (Permit #WX01310), before the period of temperature-dependent sex determination (Ferguson and Joanen, '83). Eggs were candled to assess viability at the University of Florida. Two of the eight clutches collected from Lake Apopka were entirely nonviable, whereas all Lake Woodruff clutches ($n = 15$) contained viable eggs. We used seven Lake Woodruff and six Lake Apopka clutches of eggs for this study (remaining clutches were assigned to other experiments). A subset of viable eggs from these clutches were systematically intermixed, placed into trays of damp sphagnum moss, and incubated at

a female-producing temperature of 30°C. Daily rotation of trays minimized regional temperature effects within incubators.

Animal procedures conformed to an IACUC-approved protocol. Hatching animals were web tagged with numbered Monel tags and measured for body masses (BM), snout–vent lengths (SVL), and total lengths (TL). Alligators were co-housed in a temperature-controlled animal room in tanks (~20 neonates/0.7 m^3) and experienced a 16:8 photoperiod with heat lamps for basking and daily water changes. Ambient room temperatures ranged from 27 to 31°C. We supplied no food during the experimental period because alligators subsist off the internalized yolk sac during this time (first 2 weeks post-hatching).

Hatching order systematically assigned animals to one of four experimental groups: necropsy at 1, 2, or 5 days after hatching or to a grow-out experiment not addressed in this article. Those animals examined either 2 or 5 days after hatching were part of similar FSH challenge studies of differing durations. Because reptile FSH preparations are not commercially available, we treated the animals with ovine FSH (Sigma-Aldrich #F8174-1VL). Earlier experimentation has shown robust hormonal and/or ovarian responsiveness to treatment in alligators (Lance and Vliet, '87; Edwards et al., 2004) and other reptiles (Jones et al., '75; Jones and Swain, 2000), and FSH directly modulates activin signaling in a variety of species (Knight, '96; Kumar et al., '97; Davis et al., 2001). Challenge study animals received either a sham needle insertion or IM injections of 0.8% sterile saline vehicle (isotonic to alligator blood), low dose (10 ng/g BM FSH) or high dose (50 ng/g BM FSH) to the base of the tail on a daily basis. We administered all treatments in an injection volume of 90 μL between 11:00 and 12:00 hr. Animals examined 2 days after hatching received one treatment on the day after hatching, whereas those examined 5 days after hatching received one treatment per day for the four consecutive post-hatching days.

Necropsies commenced at 12:00 hr on appointed days. Immediately before euthanasia, 1 mL of blood was collected from the supravertebral blood vessel, followed by a lethal dose (0.5 mg/g BM) of sodium pentobarbital (Sigma). Blood collected in a heparinized Vacutainer (BD Diagnostics, Franklin Lakes, NJ) was kept on ice until centrifugation at 1,500 g for 20 min at 4°C. Drawn-off plasma was stored at −80°C until radioimmunoassay. Plasma E2 and T concentrations were analyzed with a 96-well FlashPlate PLUS system (Perkin Elmer, Shelton, CT) earlier validated for *A. mississippiensis*. One ovary (from alternating sides) was dissected away and frozen in liquid nitrogen and stored at −80°C until RNA extraction. At hatching, the relatively translucent alligator ovary overlies and stands in contrast to the more vascularized mesonephric kidney and the more medially located, white colored adrenal tissues. Standard paraffin histology of the contralateral gonad confirmed sex.

Our standard RNA isolation and reverse transcription (RT) procedures have been earlier reported in detail (Milnes et al., 2008). Quantitative real-time PCR (Q-PCR) has been used to measure mRNA expression in American alligator tissues (Katsu et al., 2004; Gunderson et al., 2006; Kohno et al., 2008). Table 1 shows primer sequence information, annealing temperatures, and accession numbers. The MyiQ single color detection system (BioRad, Hercules, CA) performed Q-PCR following manufacturer's protocol using iQ SYBR Green Supermix (BioRad) in triplicate reaction volumes of 15 μL, with 0.6 μL of RT product and

specific primer pairs. Reactions were performed with relative standard curves of serially diluted cDNA. Sample means were normalized using ribosomal protein L8 (*Rpl8*) expression (Kohno et al., 2008; Milnes et al., 2008).

JMP for windows version 7.0.2 (SAS Institute, Cary, NC) performed all statistical analyses. Morphometric data were log transformed and gene expression ratios were arcsin transformed to achieve homogeneous variances, as needed. Significance was set at $P<0.05$. Unpaired Student's *t*-tests compared BM, SVL, and TL measurement by lake of origin for all hatching alligators from the collected clutches and the subset of alligators systematically assigned to the experiments detailed in this article. Circulating steroid or gonadal mRNA expression levels were not different between sham and vehicle-treated animals (lowest observed *P*-value by independent *t*-tests; *Fst* mRNA $P = 0.17$). We combined these groups into a single control treatment group for further statistical analysis.

Two-way ANOVA followed by least square means Tukey–Kramer post-tests, when appropriate, compared steroid hormone concentrations and mRNA expression levels between control groups of differing ages (Fig. 1, ANOVA factors: age, lake of origin, or interaction) and between control and FSH treatment levels for animals in the 2 and 5 day old FSH challenge studies (Figs. 2, 3, respectively; ANOVA factors: treatment, lake of origin, or interaction). Statistical analysis between control groups of differing ages and FSH challenge experiments shared control animals of respective ages and lake of origin. We further investigated significant observed treatment effects revealed by two-way ANOVA with subsequent one-way ANOVA by individual lake of origin and Dunnett's post-tests, if appropriate, to quantify lake of origin differences in FSH responsiveness compared with controls within each age group.

RESULTS

Upon collection, egg viability for the 13 clutches used for this study was 81% (237/291) from Lake Woodruff and 68% (181/267) from Lake Apopka. Of the subset of these eggs co-incubated, 86% (range by individual clutches: 67–100%) from Lake Woodruff ($n = 125/145$) and 59% (range by individual clutches: 18–92%) from Lake Apopka ($n = 99/167$) hatched. Of these, 54 Lake Woodruff and 37 Lake Apopka alligators were allocated to the hatchling experimental groups (Figs. 1–3 present sample sizes). At hatching, body morphometrics differed by lake or origin (Table 2). Average Lake Woodruff alligator body mass was greater than those measured in the Lake Apopka hatchling alligators between all hatchlings and the subset systematically assigned to the hatchling experimental groups.

Both gross anatomy at necropsy and gonadal histology observations confirmed that all hatchling animals were female. The ovarian cortex of all females contained oogonial clusters and meiotic germ cells showing varying degrees of physical interaction with somatic cells. However, ovaries from these neonates, whether 1, 2 or 5 days old from either lake, did not possess complete follicles. Ovarian expression levels of *Rpl8* mRNA were not different between control groups or between FSH challenge study groups ($P = 0.78$, 0.92, and 0.41, respectively).

Comparing control group females from both lakes at 1, 2, or 5 days after hatching, Lake Apopka alligators had greater plasma concentrations of E_2 (Fig. 1A; $P<0.001$) and T (Fig. 1B; $P<0.001$) compared with Lake Woodruff females. Gonadal *Inha* mRNA expression showed a lake by age effect (Fig. 1C; $P = 0.009$), with Lake Apopka expression levels greater than Lake Woodruff 1 day after hatching, but less than Lake Woodruff expression levels 5 days after hatching. Lake Woodruff alligators expressed greater gonadal mRNA levels of *Inhba* (Fig. 1D; $P = 0.017$) and *Fst* (Fig. 1E; $P = 0.026$) compared with those from Lake Apopka. Expression of *Cyp19a1* (Fig. 1F; $P = 0.33$ or *Fshr* mRNA, data not shown; $P = 0.25$) did not significantly vary by age or lake of origin.

Within 2 days of birth, FSH-challenged animals, plasma E_2 and T concentrations were greater in Lake Apopka alligators compared with Lake Woodruff (Fig. 2A, B; $P<0.001$ for each). Additionally, FSH treatment resulted in a significant elevation of plasma E_2 concentration irrespective of lake (Fig. 2A; $P = 0.008$). Analysis of treatment effects by individual lake of origin showed that the circulating E_2 concentrations for Lake Apopka animals responded to both low and high FSH doses ($P = 0.027$ and 0.015, respectively), whereas Lake Woodruff animals responded only to high dose FSH ($P = 0.49$ and 0.007, respectively). Gonadal expression of *Inha* mRNA showed a lake by treatment effect (Fig. 2C; $P = 0.002$), with Lake Apopka alligators showing a greater expression level response to the high FSH dose than Lake Woodruff alligators.

Further refinement of statistical analysis using Dunnett's post-test analysis of treatment effects by individual lake of origin showed that, both Lake Woodruff and Lake Apopka, 2 day old animals responded to both low and high dosages with elevated *Inha* levels (Woodruff: low $P = 0.006$, high $P<0.001$; Apopka: low $P = 0.003$, high $P<0.001$). Significant differences in *Inhba*, *Fst*, *Cyp19a1*, and *Fshr* mRNA expression levels were not observed (Fig. 2D–F; data not shown).

Within 5 days of birth, FSH-challenged study animals, plasma E_2 and T concentrations were greater in Lake Apopka alligators compared with Lake Woodruff (Fig. 3A, B; $P<0.005$ and 0.001, respectively). Additionally, FSH treatment resulted in a significant elevation of plasma E_2 concentration (Fig. 3A; $P<0.001$). Analysis of treatment effects by individual lake of origin showed circulating E_2 concentrations of both Lake Apopka and Lake Woodruff animals responded only following high doses of FSH ($P<0.001$ and 0.005, respectively). Both lake of origin and treatment effects were observed in mRNA expression levels of *Inha* (Fig. 3C; lake of origin $P<0.03$, treatment $P<0.001$), *Fst* (Fig. 3E; lake of origin $P = 0.003$, treatment $P = 0.03$), *Cyp19a1* (Fig. 3F; lake of origin $P = 0.001$, treatment $P = 0.004$), and *Fshr* (data not shown; lake of origin $P = 0.006$, treatment $P = 0.002$). Lake of origin effects showed that Lake Woodruff animals expressed greater levels of gonadal *Inha*, *Fst*, *Cyp19a1*, and *Fshr* mRNA than Lake Apopka animals.

Further refinement of statistical analysis using Dunnett's post-test analysis of treatment effects by individual lake of origin showed that both Lake Woodruff and Apopka 5 day old animals responded to both low and high dosages of FSH with elevated *Inha* levels (Woodruff: low $P = 0.033$, high $P<0.001$; Apopka: low $P = 0.044$, high $P<0.001$). In contrast, only Lake Woodruff animals responded to the FSH challenges with elevated

mRNA levels of: *Fst* (Woodruff: low $P = 0.09$, high $P = 0.022$; Apopka: low $P = 0.63$, high $P = 0.39$), *Cyp19a1* (Woodruff: low $P = 0.033$, high $P<0.001$; Apopka: low $P = 0.42$, high $P = 0.42$), and *Fshr* (Woodruff: low $P = 0.47$, high $P = 0.006$; Apopka: low $P = 0.42$, high $P = 0.17$).

DISCUSSION

Recent research in rodents has demonstrated that an interaction between activin and estrogen signaling participates in the breakdown of germ cell nests and the assembly of ovarian follicles. Here, we investigated the basal and gonadotropin stimulated levels of circulating hormones and gonadal mRNA expression of activin signaling factors in hatchling alligators from a contaminated and a reference environment. We observed evidence of elevated circulating steroid hormones (E_2 and T) in alligators from the contaminated environment and, conversely, greater ovarian mRNA expression levels of activin signaling factors (*Inhba* and *Fst*) in alligators from the reference environment. Additionally, *Inha* mRNA expression levels in ovaries from Lake Apopka females changed substantially during the post-hatching period from greater than that measured in ovaries from Lake Woodruff females 1 day after hatching to lesser than those observed at 5 days after hatching. Hatchling alligator ovaries have germ cell nests that will develop into follicles over a period of several months; therefore, these endocrine differences are liable to affect subsequent ovarian development.

We observed these endocrine differences in animals that exhibited differing egg viability rates at the nest (Woodruff: 81%, Apopka: 68%), post-incubation hatch rates (Woodruff: 86%, Apopka: 59%), and average hatching BM (Woodruff: 62.0±0.7 g, Apopka: 55.2±1.2 g). Differences in hatching success continues observations of decreased egg viability and hatching success in Lake Apopka eggs observed for almost three decades (Fujisaki et al., 2007; Milnes and Guillette, 2008).

Although the role of FSH in ovarian nest breakdown and follicle assembly is unknown in alligators, we observed clear differences in response to a gonadotrophin challenge. Although alligators from both lakes showed responses to a single high dose FSH injection, only Lake Apopka animals showed a significant E_2 increase to the low dose treatment, and ovarian *Inha* mRNA expression levels in Lake Apopka animals responded more robustly than ovaries from Lake Woodruff alligators to the high dose treatment. In 5 day old challenged alligators, we observed alterations in circulating E_2 concentrations and ovarian *Inha*, *Fst*, *Cyp19a1*, and *Fshr* mRNA expression levels. Animals from both lakes responded with elevated plasma E_2 concentrations to high dose treatments and increased *Inha* mRNA expression to both treatment levels. However, only Lake Woodruff animals responded to FSH with elevated *Fst*, *Cyp19a1*, and *Fshr* mRNA expressions. Therefore, *Fst* mRNA expression in Lake Woodruff animals is both greater at basal levels and is more inducible by FSH challenge than in Lake Apopka alligators. In mice, Wnt4 signaling suppresses male gonadal tissue differentiation during ovary development and regulates *Fst* expression (Yao et al., 2004). Because *Fst* expression also is critical for female germ cell survival, this Wnt4-Fst signaling cascade is both anti-testis and pro-ovary. Therefore, lower basal and stimulated *Fst* expression levels could be a robust sign of impaired reproductive health of Lake Apopka alligators.

Aromatase (translated from *Cyp19a1* mRNA) is an enzyme that converts androgens to estrogens. Although we observed elevated circulating E_2 concentrations in untreated Lake Apopka alligators and in FSH-stimulated alligators from both lakes, *Cyp19a1* mRNA expression levels were not different between non-stimulated alligators from either lakes and the FSH challenge elevated expression levels only in 5 day old Lake Woodruff alligators. Circulating steroid hormone concentrations in an organism are the result of an integration of synthesis, plasma storage, hepatic biotransformation and clearance. Changes to these parameters can alter measured concentrations of any steroid. We observed elevated basal T concentrations in Lake Apopka animals, therefore, supplying an elevated level of available substrate for aromatization. Additionally, our laboratory group demonstrated that contaminant exposures can change expression levels of enzymes involved in hepatic steroid degradation (Gunderson et al., 2001). Finally, measured mRNA levels do not always predict rates of protein translation, length of mRNA stability, or length of enzymatic activity of the transcribed product. Therefore, numerous physiological processes can dissociate *Cyp19a1* mRNA levels from circulating E_2 concentrations.

Using reported means, standard errors, and samples sizes from our 1994 study, we calculated that the LH challenge significantly increased circulating concentrations of E_2 in Lake Apopka alligators ($P = 0.047$), but not in Lake Woodruff animals ($P = 0.74$). Comparing this earlier work to data presented here, basal circulating E_2 concentrations were greater in Lake Apopka than in Lake Woodruff alligators in both studies. Furthermore, Lake Apopka animals showed greater E_2 responsiveness following treatment with either LH (Guillette et al., '94a) or FSH (this study). In ovo exposure of caiman embryos to exogenous E_2 or BPA resulted in increased circulating E_2 concentrations in 10 day old hatchlings, although this difference was no longer observed at 3–12 months post-hatching (Stoker et al., 2008). Taken together, these three studies strengthen a hypothesis that embryonic exposure to endocrine active substances can affect the endocrine milieu of hatching crocodilians, and likely other vertebrate species as well.

Although numerous studies have demonstrated increased MOF frequencies in estrogenically treated rodents (Iguchi and Takasugi, '86; Iguchi et al., '90; Nakamura et al., 2008; Kim et al., 2009;), only recently have studies reported the effects of similar treatments on activin signaling pathways. Treatments with E_2 or DES of neonatal mice resulted in increased MOF frequencies and altered activin signaling 19 d postnatally (Kipp et al., 2007a). Alterations of activin signaling were observed as decreased *Inha*, *Inhba*, and *Inhbb* mRNA expressions and decreased Inhba and activin A ovarian protein levels. Treatments did not alter *Fst* mRNA levels. We observed consistently decreased *Inhba* and a rapid decrease in *Inha* mRNA expression levels in contaminant-exposed alligators over the observed period. *Fst* mRNA levels decreased in contaminant-exposed alligators, compared with no alteration observed in estrogenically treated mice. In contrast to the increased maturation of follicles observed in caiman treated in ovo with E_2 (Stoker et al., 2008), neonatal E_2 and DES treatment of mice resulted in fewer small antral follicles at day 19, as compared with controls. Therefore, although these exposure experiments share some similarity in molecular and physiological effects, these studies are difficult to compare given the different status of the follicle cohorts,

before follicle formation in the alligator and caiman or at the time of follicle formation in mice.

In the mouse ovary, estrogen and activin signaling proteins colocalize in granulosa cells. Although the location of synthesis of these factors is unknown in the pre-follicular crocodilian ovary, we can compare spatial expression patterns in other vertebrates with morphologically similar ovaries (Moore et al., 2008) to formulate a hypothesis. Steroidogenic cells differentiate and migrate from the nephrogenous mesenchyme in chicken and mouse ovaries (Sekido and Lovell-Badge, 2007). In embryonic, pre-follicular chicken left ovaries, aromatase protein localizes to the medullary cords and not to the overlying cortex (Govoroun et al., 2004). Similarly, embryonic turtle ovaries express aromatase around the regressing medullary cords and at the cortex/medulla boundary after sex differentiation (Ramsey et al., 2007). In chicken ovaries 2 weeks after hatching, medullary steroidogenic cells migrate toward the cortex and incorporate into the thecal layers of developing follicles (Narbaitz and DeRobertis, '68; Pedernera et al., '88). Whereas in 5 week old chicken ovaries, aromatase is only detected in the thecal cells of developing follicles, granulosa cells do not express aromatase (Oreal et al., 2002; Govoroun et al., 2004). Furthermore, avian granulosa cells of the prehierarchical follicles exclusively express most activin signaling factors (Onagbesan et al., 2009). In light of the ovarian cortex possessing follicular cells that express activin, inhibin, and follistatin and an ovarian medulla containing the majority of steroidogenic cells before migration and differentiation into theca around small follicles, we hypothesize that in the pre-follicular alligator ovary, activin and estrogen signaling segregate spatially between cortex and medulla, respectively.

Embryonic chicken ovarian medullary cells bind FSH (Woods et al., '91) and this gonadotropin induces steroidogenesis (Gomez et al., 2001). At hatching, chickens treated with FSH in ovo respond with increased plasma E_2 concentrations, thickening of the ovarian cortex and medullary cords (Gonzalez-Moran and Mancilla, '98), increased number of oogonia, and impeded meiosis observed as diminished numbers of oocytes (Gonzalez-Moran, '98). Furthermore, these researchers hypothesized that an FSH challenge does not directly augment germ or somatic cell numbers, direct somatic differentiation, or change entry into meiosis in the ovarian cortex, but rather produces a transient effect owing to altered medullary steroidogenesis. We expand this hypothesis to state that an FSH challenge alters cortical activin signaling by way of elevating plasma and tissue E_2 concentrations that interact with activin signaling dynamics. Furthermore, our data presented in this study indicate that exposure to environmental contaminants can alter this response and interaction.

Amniote follicle formation is a function of initial germ cell proliferation followed by precipitous loss concomitant with complex interactions between the oocytes and somatic cells (Pepling and Spradling, 2001). The survival of oocytes depends on follicular assembly and those that fail to be surrounded by granulosa cells forming normal follicles undergo apoptosis (Pru and Tilly, 2001). A majority of the morphological processes described here are conserved among vertebrates (Pepling et al., '99; Matova and Cooley, 2001) and appear to share many of the molecular mechanisms underpinning gonadal maturation. As with the “canary in the coal mine,” we propose that if MOFs can be induced in wildlife by contaminant exposure with estrogenic activity, putatively through alteration in activin

signaling, similar responses could also be observed in natural populations of mammals, including humans (Crain et al., 2008). Understanding and documenting these conserved mechanisms among vertebrates will strengthen causal and mechanistic relationships, allowing a better understanding of this phenomenon across vertebrates and allowing us to reduce or prevent these abnormalities in future populations.

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LITERATURE CITED

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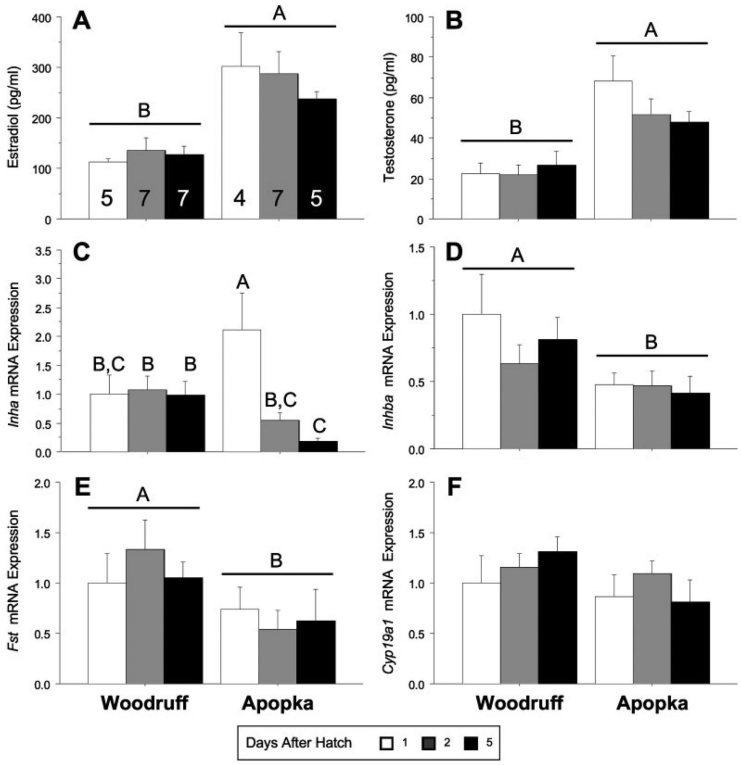

Figure 1.

Basal circulating steroids concentrations and ovarian mRNA expression levels, bars report means±SEM, in hatchling alligators: estradiol and sample sizes (**A**), testosterone (**B**), *Inha* (**C**), *Inhba* (**D**), *Fst* (**E**), and *Cyp19a1* (**F**) in Lake Woodruff and Lake Apopka alligators. Days after hatching: white bars = one, gray bars = two, and black bars = five. All mRNA expression sample means are normalized using ribosomal protein L8 (*Rpl8*) expression and standardized for each endpoint to Lake Woodruff female, day 1 expression = one. Horizontal line above bars indicates statistical significance by lake of origin ($P<0.05$). Different letters above the bars indicate age by lake of origin statistical significances.

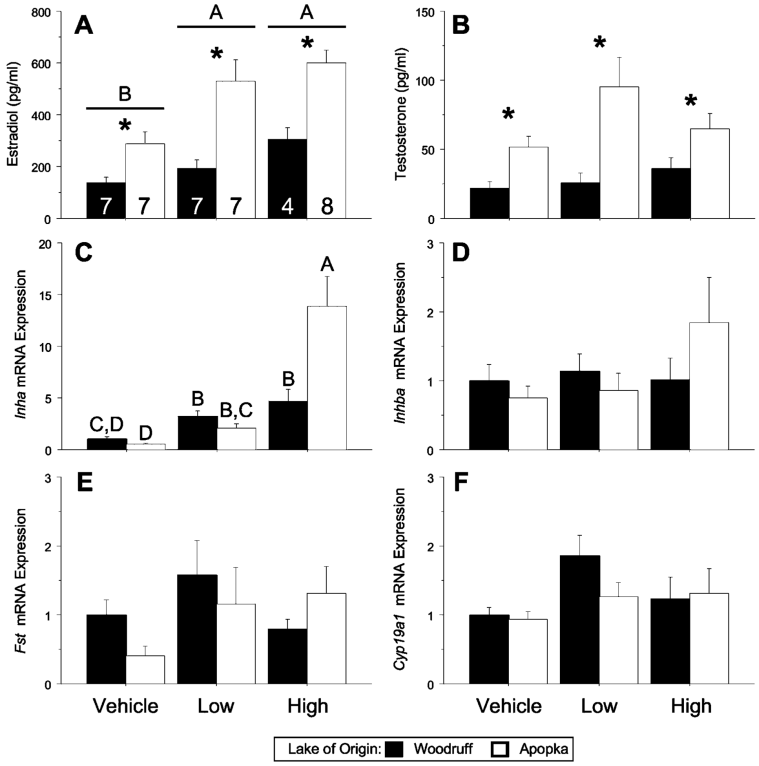

Figure 2.

Circulating steroid concentrations and ovarian mRNA expression levels in 2 day old FSH-challenged alligators. Bars report means±SEM: estradiol and sample sizes (**A**), testosterone (**B**), *Inha* (**C**), *Inhba* (**D**), *Fst* (**E**), and *Cyp19a1* (F) in Lake Woodruff (black bars) and Lake Apopka (white bars) alligators. All mRNA expression sample means are normalized using ribosomal protein L8 (*Rpl8*) expression and standardized for each endpoint to Lake Woodruff female, vehicle treated expression = one. Horizontal lines above bars indicate statistical significance by treatment ($P<0.05$). Asterisks above bars indicate significant difference by lake of origin. Different letters above the bars indicate age by lake of origin statistical significances.

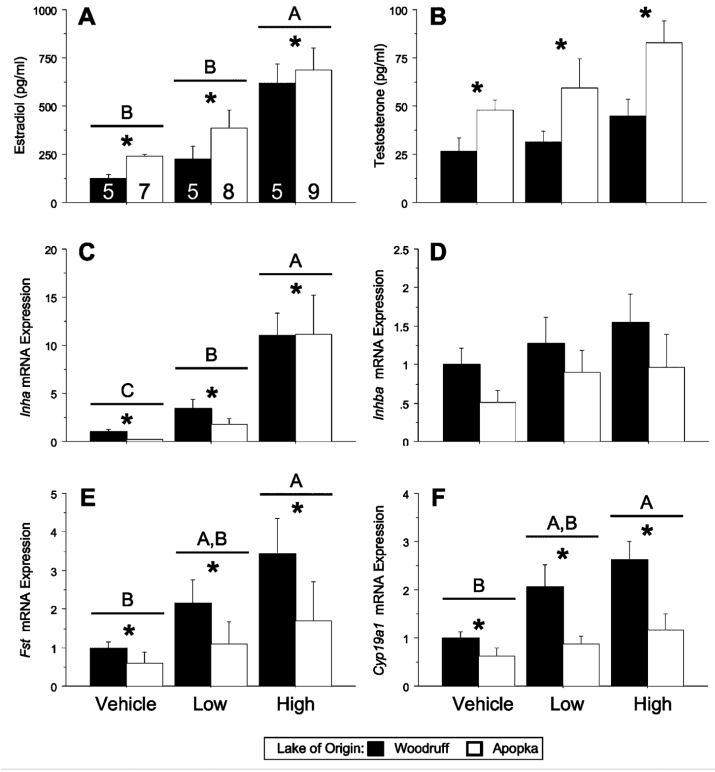

Figure 3.

Circulating steroid concentrations and ovarian mRNA expression levels in 5 day old FSH-challenged alligators. Bars report means±SEM: estradiol and sample sizes (**A**), testosterone (**B**), *Inha* (**C**), *Inhba* (**D**), *Fst* (**E**), and *Cyp19a1* (**F**) in Lake Woodruff (black bars) and Lake Apopka (white bars) alligators. All mRNA expression sample means are normalized using ribosomal protein L8 (*Rpl8*) expression and standardized for each endpoint to Lake Woodruff female, vehicle treated expression = one. Horizontal lines above bars indicate statistical significance by treatment ($P<0.05$). Asterisks above bars indicate significant difference by lake of origin. Different letters above the bars indicate age by lake of origin statistical significances.

Table 1

Quantitative real-time PCR primers for alligator gonadal factors.

Transcript	Forward primer (5′–3′) Reverse primer (5′–3′)	Anneal (°C)	Product (bp)	Accession
Ribosomal protein L8 (*Rp18*)	GGTGTGGCTATGAATCCTGT ACGACGAGCAGCAATAAGAC	60.0	64	Katsu et al., 2004
Inhibin α (*Inha*)	ACAATCCACTTGTCCCAGCC CAACTGCCACCGCGC	70.0	68	DQ010151
Activin βA (*Inhba*)	ACCCACAGGTTACCGTGCTAA GCCAGAGGTGCCCGCTATA	63.8	67	DQ101152
Follistatin (*Fst*)	CGAGTGTGCCCTCCTCAAA TGCCCTGATACTGGACTTCAAGT	66.5	65	DQ010156
Aromatase *(Cyp19a1*)	CAGCCAGTTGTGGACTTGATCA TTGTCCCCTTTTTCACAGGATAG	62.0	79	AY029233
Follicle stimulating hormone receptor (*Fshr*)	GAAATTACCAAACGAGGTTTTTCAA GGGCAGGAAACTGATTCTTGTC	60.0	81	DQ010157

Table 2

Body morphometrics of hatchling alligators.

	Total hatchlings		Experimental hatchlings	
Lake of origin	**Woodruff**	**Apopka**	**Woodruff**	**Apopka**
Body mass (g)	61.9 ± 0.5	56.7 ± 0.6**	62.0 ± 0.7	55.2 ± 1.2**
Snout–vent length (cm)	11.7 ± 0.1	11.8 ± 0.8	11.7 ± 0.6	11.6 ± 0.9
Total length (cm)	24.5 ± 0.1	24.7 ± 0.1	24.5 ± 0.1	24.1 ± 0.2*