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Gene – environment interactions: the potential role of contaminants in somatic growth and the development of the reproductive system of the American alligator

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Abstract

Developing organisms interpret and integrate environmental signals to produce adaptive phenotypes that are prospectively suited for probable demands in later life. This plasticity can be disrupted when embryos are impacted by exogenous contaminants, such as environmental pollutants, producing potentially deleterious and long-lasting mismatches between phenotype and the future environment. We investigated the ability for *in ovo* environmental contaminant exposure to alter the growth trajectory and ovarian function of alligators at five months after hatching. Alligators collected as eggs from polluted Lake Apopka, FL, hatched with smaller body masses but grew faster during the first five months after hatching, as compared to reference-site alligators. Further, ovaries from Lake Apopka alligators displayed lower basal expression levels of inhibin beta A mRNA as well as decreased responsiveness of aromatase and follistatin mRNA expression levels to treatment with follicle stimulating hormone. We posit that these differences predispose these animals to increased risks of disease and reproductive dysfunction at adulthood.

Keywords

Alligator; Ovary; Hormone; Aromatase; Activin; Follistatin; Growth

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1. Introduction

Developmental conditions during embryonic and neonatal life influence physiology, including gene and protein expression patterns, leading to altered long-term health [19]. Developmental plasticity allows the production of multiple phenotypes from a given genotype to better match the dictates and requirements of a variable environment. Often these alterations are beneficial in preparing an organism for differing surroundings. However, improper cues in the form of exposure to hormonally-active, endocrine-disrupting environmental pollutants can deleteriously influence development, resulting in a phenotype that generates a lower fitness. In females, these exposures can result in reproductive disorders, not just during the exposure period, but also in the long term due to epigenetic alterations to ovarian regulatory systems via stable modification to cell-specific transcription profiles [50].

In the American alligator (*Alligator mississippiensis*), the ovaries undergo profound morphological development during the first five months after hatching [31]. Ovarian follicles form with well defined basement membranes and distinct granulosa and theca cells. However, follicle formation and oogenesis may continue after this period, as evidenced by occurrences of loosely associated oocytes and somatic cells lacking a basement membrane and by oogonia displaying mitotic chromatin at five months after hatching. Therefore, unlike in mice, which demonstrate a five-day window of follicle formation following birth, folliculogenesis in alligators is extended and allows a greater opportunity to investigate ovarian development over long time scales. We have identified sexually dimorphic gene expressions associated with this process in neonatal alligators [30]. Compared to testes, ovaries express higher mRNA concentrations of follistatin and aromatase (*Cyp19A1*) and lower mRNA levels of inhibin alpha and inhibin beta B subunits. These dimorphic gonadal gene expressions align with observations in the gonads of other vertebrates.

Inhibin alpha (*Inha*) and beta (*Inhba* and *Inhbb*) subunits associate in various combinations to yield activins and inhibin proteins, with dimers of the beta subunits forming activins and heterodimers of the alpha and beta subunits forming inhibin proteins. Activins stimulate ovarian follicle formation and growth and are vital for female fertility [2, 39]. In contrast, estrogens suppress activin subunit mRNA expression and subsequent signaling [23], thereby maintaining oogonial nests. Therefore, follicle formation is regulated, in part, by the antagonism between an activin-mediated promotion of germ cell proliferation and follicle formation and an estrogen-mediated silencing of activin function.

Activin signaling also is antagonized both by inhibin proteins and by follistatin, the activin-neutralizing binding protein. Inhibin proteins act as activin receptor antagonists at the cell membrane, thus blocking activin signaling function. Ovarian expression of the alpha subunit of inhibin (and thus inhibin proteins) is minimal prior to puberty [40]. However, follistatin expression is a vital antagonist of oogonial nest breakdown, germ cell survival, and follicle formation [22, 49]. Although the number of developing follicles increases when ovarian follistatin expression is reduced, this condition also leads to accelerated depletion of follicles and diminished fertility. Further, follistatin decreases the expression of estrogen receptor beta (*Esr2*), in contrast to activins, which increase the expression of estrogen receptors alpha (*Esr1*) and beta in mammalian models [23].

In a previous study, we quantified circulating sex steroids and ovarian gene expression in hatchling American alligators from two lakes in Florida [32]. Lake Apopka, Florida, is characterized by elevated pesticide and agricultural nutrient pollution compared to a more pristine reference site, Lake Woodruff. Alligators hatched from eggs collected at contaminated Lake Apopka exhibit elevated plasma concentrations of both estradiol 17 β

(E2) and testosterone as well as diminished basal ovarian expression of *Inhba* and follistatin mRNA [32]. Further, females from the reference site, Lake Woodruff, responded to five days of treatment with follicle stimulating hormone (FSH) by increasing ovarian mRNA expression of follistatin and aromatase (*Cyp19A1*) whereas females from contaminated Lake Apopka females did not. Thus, *in ovo* exposure to environmental contaminants modulates post-hatching gonadal function, at least in the short term. The goal of the present study was to determine if maternal effects and/or the early nest environment can entrain gonadal function over longer time scales. From the same group of eggs collected from Lake Apopka and Lake Woodruff for our previous study, we hatched a second cohort of animals and raised them together under laboratory-controlled conditions for five months. We assessed growth rates and, at five months, administered FSH as in our previous study. Here, we present the growth dynamics, circulating sex steroid concentrations, and ovarian mRNA expression levels of steroidogenic enzymes, receptors, and activin signaling factors from those animals.

2. Materials and Methods

2.1 Egg collection and Experimental Procedures

Clutches of American alligator (*Alligator mississippiensis*, Daudin, 1801) eggs were collected from nests at Lake Woodruff National Wildlife Refuge (n = 6 nests) and Lake Apopka (n = 5 nests), Florida on June 27th and 28th, respectively, 2005 (Permit #WX01310) prior to the period of temperature-dependent sex determination [9]. Eggs were candled to assess viability and each given a unique mark with soft pencil. A subset of viable eggs from these clutches was 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 protocols approved by the University of Florida's Institutional Care and Use Committee. At hatching, animals were web tagged with numbered Monel tags, co-housed in a temperature-controlled animal room in tanks (~20 neonates / 0.7 m³), and subjected to a 16h:8h light:dark photoperiod with heat lamps for basking and daily water changes. Ambient room temperatures ranged from 27°C to 31°C. Hatching occurred from August 31st through September 15th. Hatchlings were systematically assigned based on hatch order to treatment groups for an FSH challenge study administered at approximately five months after hatching (average age = 141 days old; range = 131–153 days old). During the five-month growth period, body mass, total length, and snout-vent length (SVL) were recorded for each animal approximately every month (hatching, 10/25, 11/27, 12/19, 1/19/2006, and at necropsy). Animals that lost tags during this period were excluded from the study. Six Lake Apopka hatchlings and one Lake Woodruff hatchling died within a day of hatching. Four additional Lake Apopka animals died during the growth period compared to none from Lake Woodruff. Data from dead animals were not analyzed.

Animals in the FSH challenge study received a daily intramuscular injection of either 0.8% sterile saline vehicle (isotonic to alligator blood) or an FSH dose (50 ng/gram body mass) to the base of the tail. Injection volumes were ~90 µl and were administered between 11:00 and 12:00 h. Animals either received one injection with necropsy on the following day (2-day animals) or one injection per day for four consecutive days with necropsy on the following day (5-day animals). Because reptile FSH preparations are not commercially available, we treated with ovine FSH (Sigma-Aldrich #F8174). Previous experimentation has shown robust hormonal and/or gonadal responsiveness to ovine FSH treatment in alligators [8, 25, 32].

Necropsies commenced at 12:00 h on appointed days. Final sample sizes are presented in Figure 2A. Immediately prior to euthanasia, 1 ml of blood was collected from the supravertebral blood vessel, followed by administration of a lethal intravenous dose (0.06 mg/g body mass) of sodium pentobarbital (Sigma). Blood was collected in a heparinized Vacutainer (BD Diagnostics) and kept on ice until centrifugation at 1,500 g for 20 min at 4°C. Plasma was stored at -80°C for subsequent radioimmunoassay (RIA). Plasma E2 and testosterone concentrations were analyzed with a 96-well FlashPlate PLUS system (Perkin Elmer, Shelton, CT) as previously described [17]. At necropsy, one ovary from each animal was fixed in RNAlater (Ambion) and stored at -80°C for subsequent RNA extraction. Standard paraffin histology of the contralateral ovary confirmed sex.

Our standard total RNA isolation and reverse transcription procedures have been previously reported in detail [27]. Quantitative real-time PCR (Q-PCR) has been used to measure mRNA expression in American alligator tissues [16, 24, 30, 32, 33]. Table 1 reports Q-PCR primer sequence information, annealing temperatures, and accession numbers. The MyiQ single color detection system (BioRad, Hercules, CA) was used for Q-PCR following the manufacturer's protocol. We used iQ SYBR Green Supermix (BioRad) in triplicate reaction volumes of 15 µl with 0.6 µl of RT product (from a 20ul RT reaction containing 160 ng of total RNA by iScript cDNA synthesis kit, BioRad) and specific primer pairs. Q-PCR expression levels were calculated using gene-specific, absolute standard curves, which contain the target cDNA at known concentrations. The use of absolute standard curves allows statistical comparisons of mRNA expression levels of different genes within and among samples. Sample means were normalized using ribosomal protein 18S mRNA expression.

2.2 Statistical Analysis

JMP for windows version 8.0 (SAS Institute, Cary, NC) was used to perform all statistical analyses. Morphometric data were log transformed and gene expression ratios were arcsin transformed to achieve homogeneous variances. For morphometric data, repeated measures ANOVA were used to compare body mass, SVL, and total length growth trajectories. Further, specific growth rates for body mass, SVL, and total length (specific growth rate = $100 * (\ln[\text{size}_t] - \ln[\text{size}_0]) / t$, where t = time in days) and body condition indices (condition index = $\text{body mass} / \text{SVL}^3$) were calculated for each animal and compared using repeated measure ANOVA and Tukey-Kramer post hoc tests, when appropriate.

Allometric analysis was used to evaluate the relationships between morphometric parameters. Measurements of body mass and tail length (the dependent variables) and SVL (the independent variable) at each of the five time points indicated above for each animal were log-transformed and fitted to the allometric equation $y = ax^b$. Values for b (the slope) and $\log(a)$ (the intercept) were determined for each alligator for the five-month growth period by both simple linear regression (SLR) and reduced major axis regression (RMA). Confidence intervals (95% and 99%) of regression slopes and intercepts for animals from each source lake were determined based on a Student's t distribution. Confidence intervals that did not include the values of b predicted by isometry ($b = 3$ for the relationship between body mass and SVL; $b = 1$ for the relationship between tail length and SVL) indicated allometry with respect to SVL. Regression coefficients ($\log(a)$ and b) were also compared by Student's t -tests to assess differences in allometric parameters between source lakes.

Gene expression levels of vehicle treatment groups were evaluated using matched pairs t -tests to establish baseline dimorphic expression patterns. Steroid hormone concentrations and mRNA expression levels of control and FSH-treated animals in the two-day and five-day FSH challenge studies were compared using two-way ANOVA with treatment and lake

of origin as the two factors. Pairwise comparisons were then evaluated using least square means Tukey-Kramer post-tests. Significance was set at $P < 0.05$.

3. Results

3.1 Growth Morphometrics

The body mass of hatchlings from contaminated Lake Apopka was less than that of hatchlings from Lake Woodruff (Fig. 1A left; $p < 0.001$); however, snout vent length (SVL) and total length did not differ by lake of origin (SVL: Apopka 11.9 ± 0.1 cm, Woodruff 11.8 ± 0.1 cm; total length: Apopka 25.0 ± 0.2 cm, Woodruff 24.7 ± 0.1 cm). During the five-month growth period, the average body mass of Lake Apopka hatchlings changed from 8% less than Lake Woodruff animals at hatching to 8% greater at the final periodic measuring point (Fig. 1A right, $p = 0.03$) and at necropsy ($p = 0.05$; Lake Apopka body mass: 335 ± 11 g, Lake Woodruff body mass: 308 ± 8 g). However, SVL and total length did not significantly differ by lake of origin during the growth period or at necropsy (SVL: Lake Apopka 23.0 ± 1.8 cm, Lake Woodruff 23.5 ± 1.4 cm; total length: Lake Apopka 47.6 ± 3.9 cm, Lake Woodruff 46.4 ± 3.1 cm).

Lake Woodruff animals exhibited a faster body mass specific growth rate from hatch to first measurement at about 1 month of age (Fig. 1B: $p = 0.01$), whereas Lake Apopka animals grew at a faster rate during the second and third monthly measurement intervals ($p < 0.001$ and $= 0.01$, respectively). This pattern of growth also is observed using specific growth rates for SVL and total length, with faster growth in Lake Woodruff animals in the first interval and faster growth in Lake Apopka animals in the second and third intervals (data not shown).

The 99% confidence intervals for all observed slope coefficients for simple linear regression and reduced major axis regression analyses of morphometric parameters did not include the values expected for isometry ($b = 3$ for the relationship between body mass and SVL; $b = 1$ for the relationship between tail length and SVL). These results indicate that body mass and tail length of alligators from both source lakes increased allometrically with respect to SVL (Table 2). Slope coefficients and intercepts for the relationship between body mass and SVL (but not for the relationship tail length and SVL) differed significantly between source lakes. The larger slope coefficients for the relationship between body mass and SVL in Lake Apopka alligators compared to Lake Woodruff alligators implies that the latter had a leaner growth trajectory than the former. This conclusion is further supported by changes in condition indices during the growth period. At hatching, Lake Woodruff animals had a 9% greater condition index ($p > 0.001$) than Lake Apopka animals (data not shown), but body condition did not differ by lake of origin at the final growth measurement or at necropsy.

3.2 Serum Sex Steroid Hormone Concentrations

The two-day and five-day FSH challenge did not alter circulating testosterone concentrations (Fig. 2A); however an interaction between lake and treatment was observed. Lake Apopka controls were greater than Lake Woodruff controls and intermediate testosterone concentrations were measured in FSH-treated animals from both lakes ($p = 0.004$). Baseline testosterone concentrations were also different by lake according to a t-test ($p = 0.025$). Plasma E2 concentrations increased in response to FSH treatment relative to controls (Fig. 2B) in both the two-day (27% increase, $p = 0.002$) and the five-day (260% increase, $p < 0.001$) FSH treatment groups.

3.3 Ovarian Genes Expression Levels

3.3.1 Steroidogenic Enzymes—Both the two-day and five-day FSH treatments increased ovarian expression levels of *Cyp11A1* (Fig. 3A: two-day $p < 0.001$, five-day $p = 0.005$) and *Cyp17A1* (Fig. 3B: two-day $p = 0.046$, five-day: $p = 0.003$) regardless of lake of origin. The two-day FSH treatment did not alter *Cyp19A1* levels, whereas a lake-by-treatment effect of the five-day challenge was observed, with FSH-treated animals from Lake Woodruff exhibiting expression levels greater than levels observed from similarly treated Lake Apopka animals and controls from both lakes (Fig. 3C: $p = 0.041$).

3.3.2 Hormone Receptors—The two-day and five-day FSH challenges increased ovarian mRNA expression levels of FSH receptor (Fig. 4A: 2-day $p = 0.001$; 5-day $p < 0.001$). Androgen receptor expression levels were increased by the five-day FSH challenge (Fig. 4B: $p = 0.003$) regardless of lake of origin. Control animals for the two-day FSH treatment exhibited differing expression levels based on lake of origin for both FSH receptor and androgen receptor ($p = 0.001$ and $= 0.029$, respectively). FSH treatment increased ovarian *Esr1* expression in both the two-day and five-day FSH treatment groups (Fig. 4C: $p = 0.035$ and $= 0.037$, respectively) but did not increase *Esr2* expression levels (Fig. 4D), which were significantly less than *Esr1*.

3.3.3 Activin/Inhibin Signaling Factors—FSH treatment did not alter ovarian *Inhba* mRNA levels following either the two-day or the five-day FSH treatment (Fig. 5A). However, neonates from Lake Woodruff in the two-day control group exhibited expression levels that were greater than those observed in animals from Lake Apopka ($p = 0.020$). Further, animals treated with FSH for five days demonstrated a lake of origin effect (ANOVA), with greater expression in ovarian tissues taken from Lake Woodruff females compared to tissue from Apopka females ($p = 0.029$). Both two-day and five-day FSH treatments robustly increased *Inha* expression levels (Fig. 5B: $p = 0.005$ and < 0.001 , respectively) in neonates from both lakes of origin. Ovarian follistatin expression (Fig. 5C) increased in two-day FSH-treated animals from both lakes ($p = 0.001$). However, animals treated with FSH for five days showed a lake-by-treatment effect, with a significant elevation of follistatin in Lake Woodruff animals over similar levels in FSH-treated Lake Apopka ovaries and controls from both lakes ($p = 0.017$). *Inhbb* expression levels were approximately an order of magnitude greater than *Inhba*, though neither challenge increased expression levels (Fig. 5D).

4. Discussion

Alligators in this study were collected as eggs from two source lakes, including one with high levels of environmental contaminants, and were subsequently raised for five months after hatching in a controlled laboratory environment. Thus, source lakes influenced animals in this study only via maternal contributions to the eggs and via the very early nest environment. In a previous study, we demonstrated that this influence was sufficient to disrupt neonatal physiology in eggs collected from the contaminated site. In the present study, we investigated if this *in ovo* exposure to environmental contaminants could impact performance over longer time scales, putatively through gene-environment interactions resulting in long-term alterations in developmental programming.

Alligators from contaminated Lake Apopka have consistently demonstrated decreased reproductive success [27, 32] and altered reproductive function [29] associated with persistently high body burdens of persistent organochlorine pesticides, including DDT and its metabolites [10]. In alligators, organochlorine pesticide contaminants pass from mother to the yolk of the eggs and result in poor clutch viability [41]. These contaminants interfere

with reproductive function through effects on gene expression and hormone signaling. For example, the DDT metabolite p,p'-DDE modulates aromatase gene expression [1], possesses anti-androgenic activity through competitive binding of the androgen receptor without activation of androgen receptor-dependent pathways [3, 12, 13], and is associated with reproductive and endocrine alterations in alligators [14, 28].

4.1 Growth Trajectories

Exposure to hormonally active environment contaminants during vulnerable developmental periods may alter the programming of metabolic set points through gene-environment interactions. In the present study, we observed that hatchlings from contaminated Lake Apopka were on average 8% smaller in body mass and were leaner (e.g., they had lower condition indices) when compared to Lake Woodruff hatchlings. However, we also observed that Lake Apopka animals grew more rapidly after hatching and were thus 8% larger than Lake Woodruff animals by five months of age. Specifically, we observed faster specific growth rates for body mass, snout-vent length, and total length in the Lake Apopka animals during the second and third growth measurement intervals compared to Lake Woodruff animals. Further, the allometry of growth differed for Lake Apopka and Lake Woodruff alligators, with alligators from the contaminated lake gaining body mass relative to body length at a faster rate than those from the reference lake during the five-month study. These results were obtained under laboratory conditions with a controlled diet. Thus, the observed effects are associated with pre-hatching environmental effects.

Our results parallel those of various experimental and epidemiological studies that have demonstrated disrupted body weight homeostasis [18] and growth abnormalities in response to developmental exposure to endocrine-active compounds, including natural and synthetic estrogens. For example, female neonatal mice treated with the synthetic estrogen diethylstilbestrol (DES) during the first five days after birth exhibited decreased growth during the treatment period. However, these females subsequently grew faster than controls, resulting in greater body mass and larger adipose stores in adult mice after 75 days [35]. In humans, gestational exposure to DDE is associated with low birth weight, faster body mass growth in the first five months [26], and increased body mass index in adulthood [21]. These alterations have been attributed to estrogenic effects of DDE via androgen inhibition and/or weak estrogenic interactions.

In our study, alligators from a contaminated lake hatched at a smaller size but grew more quickly in the neonatal period than those from a reference lake, mirroring the results of DES and DDE exposure studies in humans and mice, respectively. Alligators hatch with a residual yolk that they metabolize during the first weeks of life, after which they begin feeding. Our study tracked growth throughout this shift. Although Lake Apopka animals demonstrated decreased growth and smaller body condition indices during the period of terminal yolk consumption, they exhibited faster increases in both body mass and putative body stores during subsequent ad libitum feeding. We hypothesize that the transition from maternally-derived, and potentially pollutant-contaminated, yolk consumption to feeding marks an abnormal metabolic change in these animals and could be associated with epigenetic programming via chronic developmental contaminant exposure.

4.2 Reproductive Hormones and Gene Expression

Alligators from both the contaminated and the reference lake showed endocrine responsiveness to FSH treatment through increased circulating plasma sex steroid concentrations and ovarian mRNA expression patterns. Serum estradiol concentrations in alligators from both lakes increased in response to FSH, with the magnitude of the response corresponding to the duration of FSH treatment. These increases coincided with increased

ovarian mRNA expression of steroidogenic enzyme genes. Expression of cholesterol side-chain cleavage (*Cyp11A1*) and 17 α -hydroxylase (*Cyp17A1*) increased in response to both durations of FSH treatment, whereas aromatase (*Cyp19A1*) expression increased only after five days of FSH treatment and only in alligators from Lake Woodruff. These results imply modification of a specific step of sex steroid hormone metabolism; we did not observe expression level difference at all points of steroidogenic enzyme expression, only with *Cyp19A1* expression. This pattern mirrors the expression levels observed in previous experimentation using FSH-treated hatchling alligators [32]. Therefore, the Lake Apopka ovarian phenotype seems to be more discrete and specific than a global alteration of the entire steroidogenic pathway. Further, we did not observe differences in estradiol levels resulting from these differences in CYP19A1 mRNA expression levels. Circulating hormone levels are a function of hormone synthesis, transport, and degradation/excretion. Steroidogenic enzyme expression is only one factor that influences circulating hormone levels. Within the scope of this study, we cannot reconcile the difference between variable CYP19A1 mRNA expression levels and circulating estradiol concentrations and propose that further integrated study of sex steroid hormone metabolism in alligators is warranted.

The expression of FSH receptor mRNA also increased in response to both durations of FSH treatment, thus providing evidence of positive feedback in response to ligand binding. Expression of both androgen receptor and *Esr1*, but not *Esr2*, mRNA also increased after FSH treatment in animals from both lakes. FSH treatment did not increase *Inhba* or *Inhbb* mRNA expression levels. However, FSH treatment did result in increased *Inha* and follistatin mRNA expression after both two days and five days of FSH treatment. Both of these proteins act as activin signaling inhibitors, either as activin receptor antagonists or as activin-neutralizing binding proteins. These results demonstrate that the gonads of alligators from both source lakes are competent to respond to FSH stimulation. Further, they provide evidence that feedback systems, which impact ovarian endocrine signaling and downstream gene expression, are functional in neonatal alligators.

Despite this functionality, we observed evidence of persistent reproductive differences in five-month old female alligators from contaminated Lake Apopka, as compared to reference site alligators. Gene expression differences observed in this study show similarities to those observed in similar investigations of Lake Apopka hatchlings [32]. In both studies, Lake Apopka alligators displayed a lack of ovarian follistatin and *Cyp19A1* mRNA expression responsiveness after a five-day FSH challenge, as compared to the positive responsiveness of Lake Woodruff animals. Follistatin and *Cyp19A1* play vital roles in ovarian development, regulation, and overall health. Altered ovarian expression of these genes is associated with female reproductive pathologies, most notably polycystic ovary syndrome. In hatchling Lake Woodruff alligators, ovarian follistatin and *Cyp19A1* mRNA expression levels are markedly greater than in testis, implying a greater functional role for these proteins in ovaries [30]. Given the essential reproductive regulatory role of these transcripts, the lack of responsiveness to FSH in Lake Apopka females suggests a lasting and critical reproductive alteration resulting from *in ovo* exposure to environmental contaminants.

At five-months-old, the alligator ovary has produced numerous follicles; however, continuing meiosis and follicle formation is also observed [31]. The location of *Cyp19A1* and *Fst* mRNA expression has not been demonstrated in alligator ovaries of this age. Using comparative deduction, we have proposed that alligator ovary development is comparable to chickens in follicle formation morphology and associated mRNA expression localization [32]. In chicken ovaries two weeks after hatching, steroidogenic cells migrate from the underlying medullary cords toward the germ cell-containing cortex and incorporate into the thecal layers of developing follicles [34]. Whereas, in five-week old chicken ovaries, aromatase is only detected in the thecal cells of developing follicles, granulosa cells do not

express aromatase [11, 38]. Activin signaling factors are exclusively expressed by avian granulosa cells of the pre-hierarchical follicles [37]. Morphologically, the ovaries examined in this study are between those of the two-month old and five-month-old chicken ovary. We propose that in the developing five-month-old alligator ovary that activin signaling factors, including follistatin, are found primarily in the follicular cells of the cortex. Further, aromatase activity is associated with both follicular cells of the cortex and also, to some extent, in migrating steroidogenic cell of the medullary cords. Localization of aromatase and follistatin mRNA expression in alligator ovaries will inform on potential co-localization and signaling crosstalk interactions.

Promoter regions for both follistatin [45] and *Cyp19A1* [4] can be epigenetically modified via DNA methylation, resulting in altered gene expression. Further, DNA hypomethylation is observed in DDT-exposed laboratory rodents [44] and is correlated with DDT and DDT metabolite levels measured in human populations [42]. Lake Apopka animals are exposed to a complex mix of organochlorine pesticides including DDT and its metabolites, toxaphene, chlordane, and dieldrin [10]. We hypothesize that the persistent differences in ovarian responsiveness of follistatin and *Cyp19A1* genes between the contaminant-exposed Lake Apopka and reference-site Lake Woodruff yearling alligators may lie in epigenetic misprogramming. In other words, the environment may induce changes in gene programming during early development, with subsequent impacts on phenotype and disease susceptibility.

We observed additional ovarian differences between alligators from the two source lakes. Ovaries from Lake Woodruff alligators express higher basal levels of *Inhba* mRNA expression as well as a greater FSH-induced increase in *Inhba* mRNA expression compared to ovaries from Lake Apopka alligators. These results are similar to previous observations of greater *Inhba* mRNA expression levels in hatchling alligators from the reference site [32]. Thus, evidence of a difference in ovarian *Inhba* mRNA expression levels between Lake Apopka and Woodruff alligators has been observed at two different time points, one during early, post-hatching ovarian development and the second during the period of active ovarian folliculogenesis at five months of age. The *Inhba* subunit homodimerizes to form activin A, which promotes germ cell nest breakdown and follicle formation. Epigenetic modifications to the *Inhba* promoter region have been suggested to increase expression levels of activin in esophageal cancers and to increase proliferation [43]. A putative chronic activin A deficiency in ovaries from Lake Apopka alligators, as predicted from lower *Inhba* mRNA expression levels, associated with a decreased ability to upregulate follistatin and *Cyp19A1* expression levels, would result in significant alterations in ovarian development and physiology.

5. Conclusions

Alligators are important sentinels of ecosystem health in the wetlands of the southeastern United States. Alligator studies offer potential insight into environmentally-induced defects reported in other wildlife and human populations exposed to a wide array of endocrine-disruptive contaminants. Our data presented here and in several previous studies demonstrate that neonatal and young juvenile alligators from contaminated Lake Apopka exhibit altered gonadal development, plasma hormone concentrations, and growth. In other words, we are observing responses to contaminant exposure at multiple levels of biological organization, from gene expression to whole-organism physiology. Further, we observe these effects consistently in offspring from multiple clutches, which represent many different maternal and paternal genomes. A study of the population genetics of alligators in the southeastern USA has suggested that animals from Lake Apopka are not genetically unique and that gene flow occurs throughout the region [7]. In short, it is unlikely that the effects we describe at Lake Apopka result from genetic differences among populations.

Rather, differences in reproductive function among alligators from different source lakes appear to result from environmental influences. Because our neonates are hatched in controlled laboratory conditions from eggs collected within a week of laying, these animals are only exposed to their lakes of origin via maternal contributions to the eggs and via the very early nest environment. Our data clearly suggest that these influences, in the form of nutrients or contaminants deposited in the yolk and/or albumen, shape the developing embryo and modulate ovarian development. Importantly, we have documented the effects of contaminant exposure on reproduction function for more than a decade. Given the reproductive cycle of the female alligator, with most individuals laying nests only every other year [15, 48], our observations of altered reproductive function in Lake Apopka alligators indicates that these effects are widespread and affect most, if not all, of the females in the contaminated lake. Further, given that these alterations are seen year after year, it is unlikely that they are due to changes in diet alone that might occur with changes in food abundance and availability that occur with regional drought or flooding. We hypothesize that the effects we observe are due to an interaction between the embryonic genome and environmentally-derived endocrine active contaminants (from the maternal diet) that are deposited in the egg. These gene-by-environment interactions lead to lifelong alterations in the phenotype, including but not limited to the reproductive system.

Our results are in agreement with other studies have shown that estrogenic drugs and chemicals can have unexpected stimulatory effects on the differentiation of adipocytes, postnatal growth, and obesity during critical developmental periods [5, 20, 36]. These findings are associated with studies showing that administration of supplementary estrogen to rodents, even at very low doses, disrupts normal development of the reproductive system [46, 47]. Therefore, our results may be more broadly applicable to larger suite of contaminant-exposed animals.

These observations also suggest that several common reproductive abnormalities in women could have, as their basis, an environmental cause and manifest through gene-by-environment (environmental contaminant) effects. We have noted in our studies that the ovaries of Lake Apopka female alligators respond to FSH treatment in much the same way as human ovaries exhibiting polycystic ovarian disease or premature ovarian failure. Although there is some evidence that these disease states can be due to genetic mutation, a very large number of these cases have no genetic basis. In a review of the literature, it has been suggested that these and other diseases of the female reproductive system could result from exposure to endocrine-disrupting contaminants during the embryonic or neonatal periods [6]. Our data demonstrating similar responses in a sentinel wildlife species suggest that more research is required to understand the potential long-term impacts of contaminant exposure during early life. Further, an understanding of gene-by-environment interactions is critical to our success in establishing the causes of such disease states.

Highlights

- Alligators collected as eggs from polluted Lake Apopka, FL, grew faster during the first five months after hatching, as compared to reference-site alligators.
- Ovaries of these Lake Apopka alligators displayed decreased responsiveness of aromatase and follistatin mRNA expression levels to treatment with follicle stimulating hormone.
- Taken together with lower basal expression levels of ovarian inhibin beta A mRNA, alligators from a polluted lake show impacts of developmental contaminant exposure at multiple levels of biological organization.

Highlights

- Alligators from polluted Lake Apopka, FL grew faster during the first five months after hatching
- 5-month-old alligator ovaries displayed decreased mRNA level responsiveness to FSH treatment.
- Pollution exposed alligators showed impacts at multiple levels of biological organization

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Abbreviations

CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1 (side chain cleavage)
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1 (17- α -hydroxylase)
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1 (aromatase)
DDT	1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane
DES	diethylstilbestrol
ESR1	estrogen receptor 1 or α
ESR2	estrogen receptor 2 or β
E2	estradiol-17 β
INHA	inhibin alpha subunit
INHBA	inhibin beta A subunit
INHBB	inhibin beta B subunit
p,p'-DDE	1,1-dichloro-2,2-bis ethylene
SVL	snout-vent length

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Fig. 1.

A) Body mass (mean $\pm$ SE) of female alligators from contaminated Lake Apopka (closed bars/ solid circles and dashed lines) and Lake Woodruff (open bars/ open circles and solid lines) at hatching (left figure) and throughout the first five months of life (right figure). Asterisks denote significant differences between source lakes within time points as determined by t-test (left) or t-test after repeated measures analysis (right). B) Specific growth rates (mean $\pm$ SE) calculated using body mass for female *Alligator mississippiensis* from Lake Apopka (solid bars, n=54) and Lake Woodruff (open bars, n=45) during four time intervals following hatching. Asterisks denote post-hoc differences between

measurements within a time point (t-test, $p < 0.05$) after repeated measures analysis indicated a significant lake effect.

Fig. 2.

Circulating plasma concentrations (mean $\pm$ SEM) of 17 β -estradiol (A) and testosterone (B) in five-month-old female alligators following treatment with follicle stimulating hormone (50 ng/gram body mass daily) for two or five days. Horizontal lines over bars denote statistical significance by treatment. Different letters above bars indicate significant differences between source lakes within treatment durations.

Fig. 3. Ovarian mRNA expression levels (mean $\pm$ SEM) of steroidogenic enzymes *Cyp11A1* (A), *Cyp17A1* (B), and *Cyp19A1* (C) in five-month-old female alligators following treatment with follicle stimulating hormone (50 ng/gram body mass daily) for two or five days. All mRNA expression sample means are normalized using 18S ribosomal expression levels. Horizontal lines with differing capital letters denote statistical significance by treatment (3a and 3b) or lake of origin by treatment (3c).

Fig. 4.

Ovarian mRNA expression levels (mean $\pm$ SEM) of FSH receptor (A), androgen receptor (B), *Esr1* (C), and *Esr2* (D) in five-month-old female alligators following treatment with follicle stimulating hormone (50 ng/gram body mass daily) for two or five days. All mRNA expression sample means are normalized using 18S ribosomal expression levels. Horizontal lines with capital letters over bars denote statistical significance by treatment. Asterisks denote significant differences in control expression levels between source lakes.

Fig. 5. Ovarian mRNA expression levels (mean ± SEM) of *Inhba* (A), *Inha* (B), follistatin (C), and *Inhbb* (D) in five-month-old female alligators following treatment with follicle stimulating hormone (50 ng/g body mass daily) for two or five days. All mRNA expression sample means are normalized using 18S ribosomal expression levels. Horizontal lines with capital letters over bars denote statistical significance by treatment. Brackets with different letters above bars indicate significant differences between source lakes. Asterisks denote significant differences in control expression levels between source lakes.

TABLE 1

Quantitative real-time PCR primers for alligator gonadal factors

Genes	Forward Primer (5' - 3') Reverse Primer (5' - 3')	Anneal (°C)	Product (bp)	Accession
Ribosomal Protein 18S (<i>18S</i>)	GTCCGAAGCGTTACTTTGA TCTGATCGTCTTCGAACCTC	65.5	270	AF173605
Cholesterol Side-Chain Cleavage (<i>Cyp11A1</i>)	TCTGGAGTCAGTGTGCCATGTC TCATGCTCACC GCATCGAT	60.0	101	DQ007995
17-Alpha-Hydroxylase/17,20 Lyase (<i>Cyp17A1</i>)	CCAGAAAAAGTTCACCGAGCAC CGGCTGTTGTTGTTCTCCATG	63.8	79	DQ007997
Aromatase (<i>Cyp19A1</i>)	CAGCCAGTTGTGGACTTGATCA TTGTCCCTTTTTCACAGGATAG	63.8	79	AY029233
Follicle Stimulating Hormone Receptor (<i>Fshr</i>)	GAAATTACAAACGAGGTTTTCAA GGGCAGGAACTGATTCTTGTC	60.0	81	DQ010157
Androgen Receptor (<i>Ar</i>)	TGTGTT CAGGCCATGACAACA GCCCATTT CACCACATGCA	67.5	103	AB186356
Estrogen Receptor α (<i>Esr1</i>)	AAGCTGCCCCTTCAACTTTTA TGGACATCCTCTCCCTGCC	66.5	72	AB115909
Estrogen Receptor β (<i>Esr2</i>)	AAGACCAGGCGCAAAAGCT GCCACATTT CATCATTCCAC	66.0	72	AB115910
Inhibin α (<i>Inha</i>)	CAACTGCCACCGCGC ACAATCCACTTGTTCCAGCC	70.0	68	FJ457911
Activin β A (<i>Inhba</i>)	ACCCACAGGTTACCGTGCTAA GCCAGAGGTGCCCGCTATA	63.8	67	DQ010152
Activin β B (<i>Inhbb</i>)	GGGTCAGCTTCCTCTTCAC CGGTGCCCCGGGTCA	64.7	70	DQ010153
Follistatin (<i>Fst</i>)	CGAGTGTGCCCTCTCAAA TGCCCTGATACTGGACTTCAAGT	66.5	65	DQ010156

Allometric growth parameters for the equation $\log(y) = b \cdot \log(x) + \log(a)$ as determined by least-squared regression (LSR) and reduced major axis regression (RMA), where y is body mass (BM) or tail length and x is snout-vent length. Values of b and $\log(a)$, reported as mean (95% confidence interval), were determined for each alligator during the four month growth interval. Lowercase letters within the table (a and b) represent significantly different means ($p < 0.05$) between source lakes within regression models as determined using unpaired Student's t -tests. The p -values reported in the last column represent the significance for comparisons of observed slopes (b) with the slopes expected for isometric growth ($b = 3$ for $y = \text{BM}$ and $b = 1$ for $y = \text{tail length}$).

Table 2

Lake	n	Regression	y	b	log(a)	r ²	Isometry?
Apopka	45	LSR	BM	2.68^a (2.59, 2.77)	-1.13^a (-1.25, -1.02)	0.98	No: $p < 0.01$
		RMA		2.70^a (2.61, 2.80)	-1.16^a (-1.28, -1.04)		
Woodruff	54	LSR	BM	2.46^b (2.41, 2.51)	-0.85^b (-0.91, -0.79)	0.98	No: $p < 0.01$
		RMA		2.48^b (2.43, 2.52)	-0.87^b (-0.93, -0.82)		
Apopka	45	LSR	Tail	0.95^a (0.92, 0.98)	0.09^a (0.06, 0.13)	0.98	No: $p < 0.01$
		RMA		0.96^a (0.93, 0.98)	0.08^a (0.05, 0.12)		
Woodruff	54	LSR	Tail	0.94^a (0.92, 0.96)	0.10^a (0.07, 0.13)	0.98	No: $p < 0.01$
		RMA		0.95^a (0.93, 0.97)	0.09^a (0.07, 0.12)		