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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.

1. Introduction

Developmental conditions during embryonic and neonatal life influence physiology, including gene and protein expression patterns, leading to altered long-term health (Hochberg et al., 2011). 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 (Zama and Uzumcu, 2010).

In the American alligator (*Alligator mississippiensis*), the ovaries undergo profound morphological development during the first five months after hatching (Moore et al., 2010b). 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 (Moore et al., 2010a). Compared to testes, ovaries express higher mRNA concentrations of follistatin

Abbreviations: CYP11A1, cytochrome P450, family 11, subfamily A, polypeptide 1 (side chain cleavage); CYP17A1, cytochrome P450, family 17, subfamily A, polypeptide 1 (17-alpha-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 alpha; ESR2, estrogen receptor 2 or beta; E2, estradiol-17β; 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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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 (Bristol-Gould et al., 2006; Pangas et al., 2007). In contrast, estrogens suppress activin subunit mRNA expression and subsequent signaling (Kipp et al., 2007), 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 (Raivio and Dunkel, 2002). However, follistatin expression is a vital antagonist of oogonial nest breakdown, germ cell survival, and follicle formation (Kimura et al., 2011; Yao, 2005). 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 (Kipp et al., 2007).

In a previous study, we quantified circulating sex steroids and ovarian gene expression in hatchling American alligators from two lakes in Florida (Moore et al., 2010c). 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 17β (E2) and testosterone as well as diminished basal ovarian expression of *Inhba* and follistatin mRNA (Moore et al., 2010c). 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 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 (*A. 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 (Ferguson and Joanen, 1983). 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 m^3), and subjected to a 16 h:8 h light:dark photoperiod with heat lamps for basking and daily water changes. Ambient room temperatures ranged from 27 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 (Edwards et al., 2004; Lance and Vliet, 1987; Moore et al., 2010c).

Necropsies commenced at 12:00 h on appointed days. Final sample sizes are presented in Fig. 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 1500*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 (Hamlin et al., 2010). 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 (Milnes et al., 2008). Quantitative real-time PCR (Q-PCR) has been used to measure mRNA expression in American alligator tissues (Gunderson et al., 2006; Kohno et al., 2008; Moore et al., 2010a,c,d). 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 20 μl 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