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Altered gonadal expression of TGF-β superfamily signaling factors in environmental contaminant-exposed juvenile alligators☆

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ABSTRACT

Environmental contaminant exposure can influence gonadal steroid signaling milieus; however, little research has investigated the vulnerability of non-steroidal signaling pathways in the gonads. Here we use American alligators (*Alligator mississippiensis*) hatched from field-collected eggs to analyze gonadal mRNA transcript levels of the activin–inhibin–follistatin gene expression network and growth differentiation factor 9. The eggs were collected from Lake Woodruff National Wildlife Refuge, a site with minimal anthropogenic influence, and Lake Apopka, a highly contaminated lake adjacent to a former EPA Superfund site. The hatchling alligators were raised for 13 months under controlled conditions, thus limiting differences to embryonic origins. Our data reveal sexually dimorphic mRNA expression in 13-month-old alligator gonads similar to patterns established in vertebrates with genetic sex determination. In addition, we observed a relationship between lake of origin and mRNA expression of activin/inhibin subunits α and βB, follistatin, and growth differentiation factor 9. Our study suggests that embryonic exposure to environmental contaminants can affect future non-steroidal signaling patterns in the gonads of a long-lived species.

1. Introduction

Pollutants can influence steroid hormone concentrations and steroid-dependent tissues in a wide range of vertebrates, and can have particularly profound effects on sex-related endpoints in non-mammalian species due to greater innate reproductive plasticity [1]. Previous research from our laboratory has examined the expression of various steroidogenic factors [2] and steroid receptors [3], as well as circulating steroid concentrations and steroid-dependent tissues [4–6] in juvenile alligators from Lake Woodruff National Wildlife Refuge, FL, USA, an area of minimal anthropogenic influence, and highly polluted Lake Apopka, FL, USA [7–9] (or for review see [10]). These data show that juvenile alligator gonads are physiologically active, and that Lake Apopka alligators display altered endocrine signaling and diminished sexual dimorphism compared to Lake Woodruff animals. Although the physiological relevance of each endpoint with regard to fitness is unclear, the reported differences are pertinent when considered within the context of persistent low egg hatch rates and post hatching survival reported among Lake Apopka alligators [2,11].

The focus of past studies has been primarily on endpoints related to steroid signaling, in part, because many contaminants are known to interact directly with steroid receptors. In comparison, very little research has investigated the vulnerability of non-steroidal signaling mechanisms to contaminant-induced alterations even though they also play a critical role in gonadal development, maturation, and gametogenesis. For instance, the subunits of the transforming growth factor-β (TGFβ) superfamily are involved in paracrine signaling vital for male and female reproductive fitness [12–14]. Dimeric combinations of activin/inhibin signaling subunits (*Inhβ*A, *Inhβ*B, and *Inhα*) produce activin (β–β dimers) or inhibin (α–β dimers) ligands that regulate a broad spectrum of fertility-related biological activities including gonadal steroidogenesis and germ cell maturation [15–18]. Activin signaling activity is modulated, in part, through expression of the activin binding glycoprotein follistatin (*Fst*) [19–21]. Another member of the TGFβ superfamily,

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growth differentiation factor 9 (*Gdf9*), is vital for early ovarian follicle development and contributes to testicular germ cell health [22–25].

Ultimately, it is the combination of—and crosstalk between—steroid and non-steroidal signaling in the gonad that is essential for proper regulation of gonad development and function. Thus, non-steroidal signaling is potentially susceptible to perturbations either directly through contaminant–receptor interactions or indirectly through changes in endogenous steroid hormone milieus. The current study begins to addresses the following two questions. First, are non-steroidal signaling pathways sexually dimorphic in juvenile alligators, a species that exhibits temperature-dependent sex determination? Second, how does embryonic exposure to environmental contaminants affect non-steroidal signaling in alligators, a long-lived, sentinel species of environmental health? To address these questions, we investigated the effects of sex and embryonic environment on gonadal mRNA expressions in 13-month-old American alligators. These animals were hatched from eggs collected from Lake Woodruff and Lake Apopka shortly after oviposition and then raised in a controlled environment, thus limiting environmental differences to embryonic origins. We compared the expression of a group of gonadal factors (Table 1), including activin and inhibin subunits, *Fst*, *Gdf9*, forkhead box L2 (*Foxl2*), and FSH receptor (*Fshr*) that play roles in gonadal differentiation and gametogenesis. *Foxl2* expression is critical for ovary development, health, and fertility [19,26,27]; *Fshr* binds the pituitary-secreted gonadotropin FSH and induces secondary messenger signaling cascades that modulate gonadal gene expression. Activins have been demonstrated to upregulate gonadal *Fshr* expression levels [28].

2. Materials and methods

2.1. Experimental design and animal care

All fieldwork was conducted under Florida Fish and Wildlife Conservation Commission (FWC) and the US Fish and Wildlife Service permits (#WX01310). Laboratory work involving alligators was performed under Institutional Animal Care and Use Committee guidelines at the University of Florida. Egg collection, handling, and incubation methods have been previously published in detail [2,29]. Complete clutches of eggs were collected soon after oviposition from Lake Apopka and Lake Woodruff National Wildlife Refuge, FL, USA. To confirm the embryonic development stage according to criteria set forth by Ferguson [30] at least one egg per clutch was opened. All eggs were incubated at 32 °C until assigned to their respective incubation cohort at stage 19, which proceeds the thermosensitive period of sex determination.

Viable eggs, as determined by candling, were allocated into either one of two study designs. The first study used only eggs from Lake Woodruff, in which 13 were incubated at an all-female producing temperature, 30 °C, and 17 were incubated at an all-male producing temperature, 33.5 °C [31]. Groups were assembled from nine different clutches with a maximum of three eggs from any clutch at either incubation temperature. The sample size imbalance is an artifact of using these animals in several developmental studies, one of which required additional males. The second study design consisted of 60 eggs from Lake Apopka and 60 eggs from Lake Woodruff, all incubated at 32 °C, a temperature that produces males and females. These groups were assembled using ten eggs from each of six clutches collected from each study site, as previously published [2]. Upon hatching, alligators were web-tagged and co-housed in tanks within a greenhouse enclosure under natural lighting for 13 months at the University of Florida. Animals were fed commercial alligator chow (Burris Mill and Feed, Franklinton, LA) ad libitum, health was checked daily, and water changes were performed every other day.

2.2. Tissue collection, RNA isolation, and quantitative real-time PCR

At the time of necropsy, approximately 13 months after hatching, mean body mass was 301.2 g and mean snout-vent length was 23.2 cm. Sex was determined by visual inspections of gonad morphology and the presence or absence of oviducts. Gonads were then removed, flash frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. RNA isolation and reverse transcription procedures have previously been published in detail [2]. Quantitative real-time PCR (Q-PCR) has been used to measure mRNA expression of each gene of interest in alligators [3,32,33], and primer sequence information, annealing temperatures, and accession numbers are reported in Table 1. Q-PCR was performed in the MyiQ single color detection system (BioRad, Hercules, CA) following the manufacturer's protocol using iQ SYBR Green Supermix (BioRad) in triplicate reaction volumes of 15 μl with 2 μl of RT product and specific primer pairs. Expression levels of mRNA 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. All sample means were normalized using ribosomal protein L8 (*Rpl8*) expression [2,33].

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 L8 (*Rpl8*)	GGTGTGGCTATGAATCCTGT ACGACGAGCAGCAATAAGAC	60.0	64	ES316580
Inhibin α (*Inhα*)	ACAATCCACTTGTCCCAGCC CAACTGCCACCGCGC	70.0	68	DQ010151
Activin βA (*InhβA*)	ACCCACAGGTTACCGTGCTAA GCCAGAGGTGCCCGCTATA	63.8	67	DQ101152
Activin βB (*InhβB*)	GGGTCAGCTTCCTCCTTTCAC CGGTGCCCGGGTTCA	64.7	70	DQ010153
Follistatin (*Fst*)	CGAGTGTGCCCTCCTCAAA TGCCCTGATACTGGACTTCAAGT	66.5	65	DQ010156
Forkhead box L2 (*Foxl2*)	ATCAGCAAGTTCCCCTTCTAC GCCTTTCTCGAAAATGTCCTC	65.0	171	EU848473
Growth differentiation factor 9 (*Gdf9*)	TCAGTTTCCTCCTCTTCTCCAATT ACACACTTGGCTAGAAGGATCATTC	63.0	78	DQ015675
Follicle-stimulating hormone receptor (*Fshr*)	GAAATTACCAAACGAGGTTTTTCAA GGGCAGGAAACTGATTCTTGTC	60.0	81	DQ010157