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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

2.3. Statistical analysis

JMP for windows v7.0.1 (SAS Institute, Cary, NC) was used for statistical analyses. Chi-square tests were used to compare hatching success and post-hatching mortality rates. Gene expression ratios were arcsin transformed to achieve homogeneity of variance as needed. Unpaired Student's *t*-tests compared means from females produced at 30 °C to males produced at 33.5 °C. Two-way ANOVA compared the effects of sex and lake of origin among alligators incubated at 32 °C, and least square means were analyzed using Tukey–Kramer post-hoc comparisons. Significance for all tests was $P < 0.05$. The eggs from Lake Woodruff used in the two study designs did not originate from the same clutches; therefore, statistical comparisons between incubation temperatures within a sex (e.g., high vs. intermediate temperature males) could not be made.

3. Results

3.1. Embryonic and post-hatching mortality

Greater than 90% of all eggs hatched and hatching success did not vary by incubation temperature or lake. Three males from the Lake Woodruff 33.5 °C cohort died during the 13-month study period, but there was no difference in post-hatching mortality between Lake Woodruff 33.5 °C males and 30 °C females ($P = 0.24$). Post-hatching mortality from 32 °C incubated eggs has previously been published [2]. Only 66% of the animals hatched from Lake Apopka eggs survived to 13-month-old, as compared to 96% of animals hatched from eggs obtained from Lake Woodruff ($P < 0.0001$). Mortality was greatest among Lake Apopka females, which exhibited a 40% survival rate. At necropsy, 33 females and 19 males represented Lake Woodruff animals, whereas 8 females and 19 males represented Lake Apopka animals.

3.2. Gonadal mRNA expression

Gonads of Lake Woodruff animals incubated at 30 °C (ovary producing) or 33.5 °C (testis producing) displayed sexually dimorphic mRNA expression levels (Fig. 1). Ovaries expressed greater mRNA levels of *Fst*, *Gdf9*, and *Foxl2* (Fig. 1D–F) than testes, whereas testes expressed greater mRNA levels of *Inhβ*B, *Inhα*, and *Fshr* (Fig. 1A, C and G) compared to ovaries. Expression of *Inhβ*A approached a significant difference (Fig. 1B; $P = 0.06$). Among the activin/inhibin subunits, expression of *Inhβ*B mRNA, regardless of sex, was an

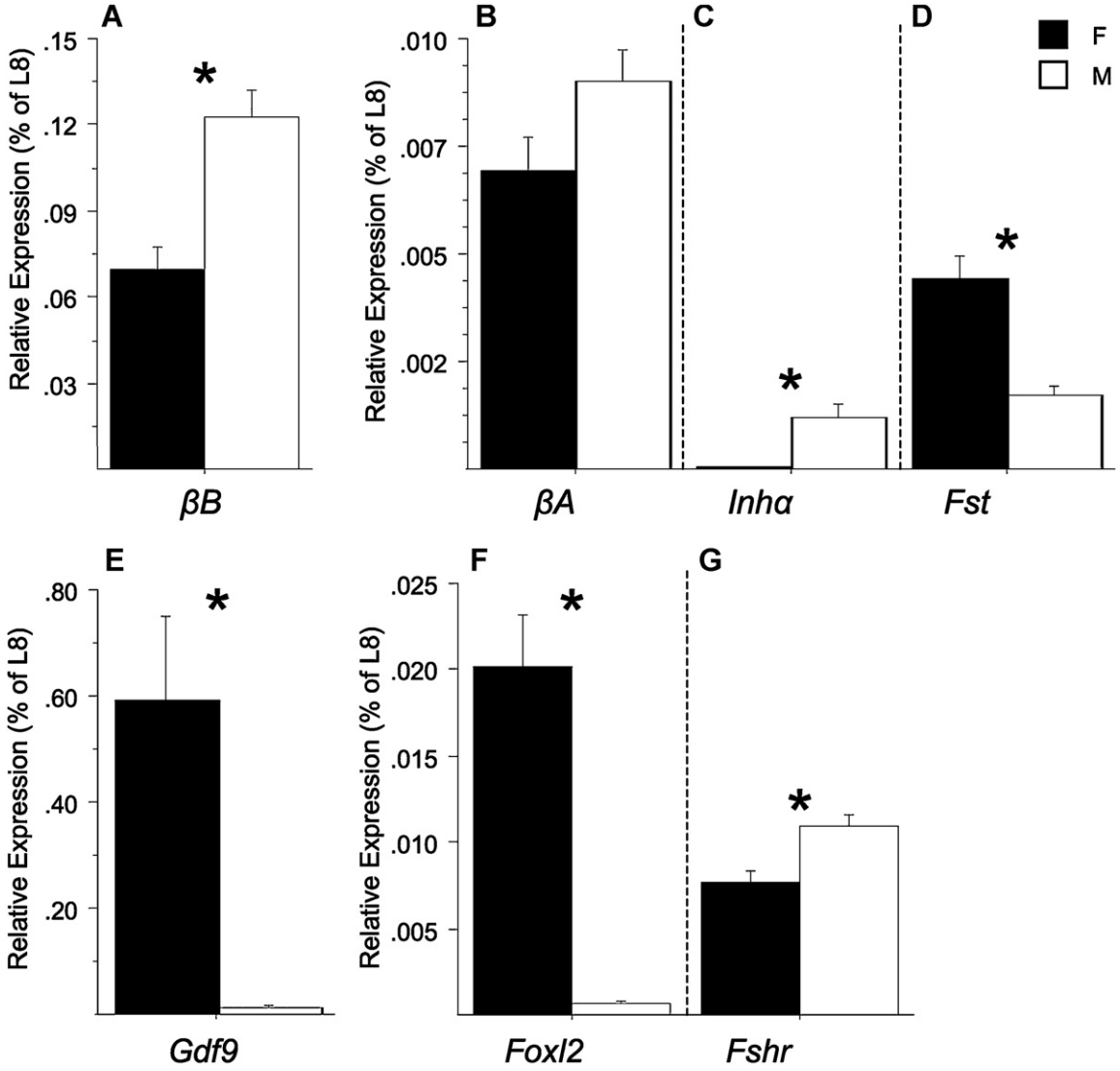

Fig. 1. Gonadal gene expression levels in 13-month-old alligators from 30 °C and 33.5 °C incubations. Mean (±SEM) gonadal mRNA expression of *βB*, *βA*, *Inhα*, *Fst*, *Gdf9*, *Foxl2*, and *Fshr*. Black bars = females/30 °C incubation, white bars = males/33.5 °C incubation. Asterisks denote significant difference in expression between sexes at $P < 0.05$ by unpaired *t*-test.

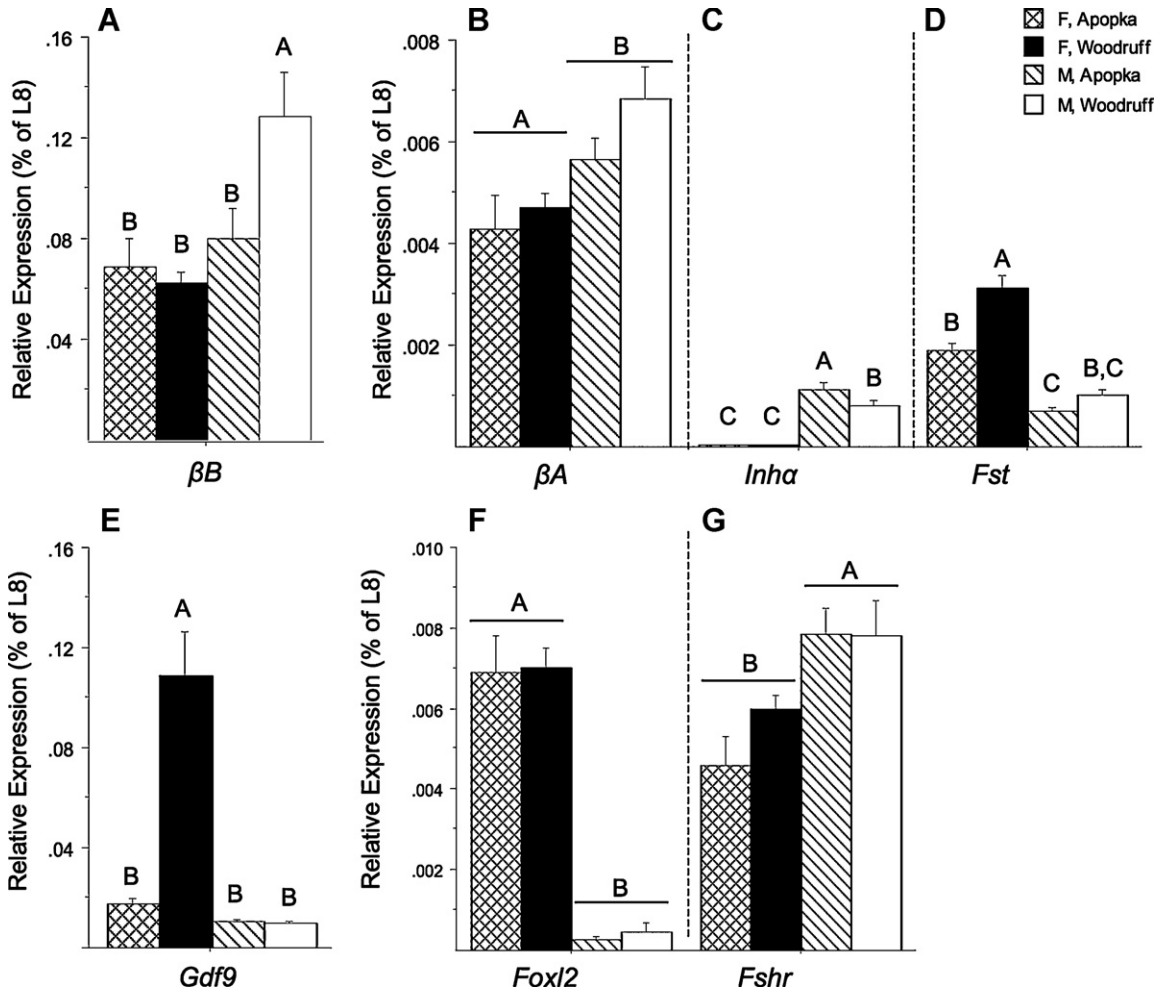

Fig. 2. Gonadal gene expression in 13-month-old Lake Woodruff and Apopka alligators from 32 °C incubations. Mean (±SEM) gonadal mRNA expression of *βB*, *βA*, *Inhα*, *Fst*, *Gdf9*, *Foxl2*, and *Fshr*. Crosshatched bars = Lake Apopka females, black bars = Lake Woodruff females, diagonal lined bars = Lake Apopka males, white bars = Lake Woodruff males. Superscripts denote differences between groups by two-way ANOVA significant at $P < 0.05$. Capital A or B over horizontal bars denotes sex effect. Capital letters over individual error bars denotes lake by sex effect.

order of magnitude greater than *InhβA* expression levels, which was slightly greater than *Inhα*.

Gonadal mRNA expression levels of alligators incubated at 32 °C differed according to sex and the interaction of sex and lake (Fig. 2). Expression of *InhβB* in Lake Woodruff males was greater than that of Lake Apopka males and females from either lake (Fig. 2A). Males from both lakes expressed higher mRNA levels of *InhβA* (Fig. 2B) and *Fshr* (Fig. 2G) compared to females from the same lakes; whereas females from both lakes showed greater expression of *Foxl2* (Fig. 2F) compared to males. Relative expression of *Inhα* mRNA was greatest in Lake Apopka males (Fig. 2C). Transcripts for *Fst* and *Gdf9* were most abundant in Lake Woodruff females (Fig. 2D and E), with nearly five-fold greater expression of *Gdf9* in Lake Woodruff ovaries compared to those of Lake Apopka or testes from either lake. Relative patterns of mRNA expression of activin/inhibin subunits were similar to those observed in 30 °C/33.5 °C gonads, with *InhβB* expression an order of magnitude greater than *InhβA*, and *InhβA* mRNA expression greater than *Inhα* expression.

4. Discussion

4.1. Sexual dimorphism

We observed evidence of sexual dimorphism in all but one of the genes examined in alligators from Lake Woodruff. The *InhβB* and *Inhα* subunits and *Fshr* mRNAs were more highly expressed in testes compared to ovaries. Although the function of these signaling factors have not been examined specifically in alligators, each has been shown to have a role in early sexual development of other vertebrates. For instance, activin signaling plays a paracrine role in regulating Sertoli cell number in the developing testis [34], while ovarian activins regulate follicle formation and initial follicle pool size [16]. In humans, ovarian inhibin expression is minimal prior to puberty [35], but is vital for testicular formation and maintenance [36]. Comparatively less is known concerning early expression of *Fshr*; however, FSH has been shown to regulate multiple members of the TGFβ superfamily [37–40].

In contrast to the activin and inhibin subunits, *Fst*, *Gdf9*, and *Foxl2* were more highly expressed in ovaries as compared to testes. Follistatin, an antagonist of TGFβ ligands including activins, is expressed in greater concentrations in embryonic mouse ovaries than testes [41], but plays a role in the development of both [17,19,20,42]. Growth differentiation factor 9 regulates folliculogenesis, and in chickens *Gdf9* mRNA expression is greater in less mature follicles [23,43,44]. Though expressed at higher levels in ovaries compared to testes, *Gdf9* mRNA expression has been localized in human testes and rodent spermatocytes and spermatids [45] and has been shown to regulate Sertoli cell function [25]. Gonadal expression of the transcription factor forkhead box L2 (*Foxl2*) is an early molecular marker of ovary-specific sex differentiation [26,46]. It is vital for primor-

dial follicle pool formation [47], granulosa cell differentiation [47], and regulation of aromatase (*Cyp19a1*) expression [48,49]. Expression of *Foxl2* mRNA has been detected in chicken [50] and duck [51] ovaries at orders of magnitude greater than in testes, similar to the dimorphism we observed in juvenile alligators. As previously stated, direct statistical comparisons of mRNA expression could not be made between females from eggs incubated at 30 °C and 32 °C or between males produced at 32 °C and 33.5 °C. It is worth noting, however, that the patterns of sexual dimorphism were consistent among the Lake Woodruff alligators regardless of incubation temperature, and that these patterns were similar to those established in species with genetic sex determination.

4.2. Embryonic effects

The alligators used for this study were collected as eggs within 1–2 weeks post ovipositioning and reared under carefully controlled conditions so that we could isolate the effects of embryonic origins from those resulting from post-hatching environmental variables. By incubating eggs at 32 °C, a temperature that produces both sexes, we were able to investigate sexual dimorphism of non-steroidal signaling factors without the confounding effects of incubation temperature differences. Sexual dimorphic expression of *Inhβ*B was observed in Lake Woodruff alligators, whereas there were no differences between males and females from Lake Apopka. The subunit, *Inhβ*B was the most highly expressed TGFβ mRNA in males in this study, and testes from Lake Woodruff alligators had greater mRNA concentrations of *Inhβ*B compared to testes from Lake Apopka alligators. A similar pattern of mRNA expression was reported for steroidogenic factor 1 (*Nr5a1*) and steroidogenic acute regulatory protein (*Star*) in these same animals [2]. In contrast to *Inhβ*B, testes obtained from Lake Apopka males expressed *Inhα* at a greater level than testes obtained from Lake Woodruff males. Because *Inhβ*B and *Inhα* are subunits that make up one-half of 4 different potential ligands, it is impossible to fully assess the implications of these differences with regard to proper sexual development, but our results warrant future studies of activin and inhibin concentrations in relation to embryonic environment.

Gonadal expression of *Gdf9* was sexually dimorphic in alligators from Lake Woodruff, but not from Lake Apopka. This was the most highly expressed TGFβ mRNA in females in this study, and ovaries from Lake Apopka alligators had *Gdf9* mRNA concentrations ~5 times lower than those from Lake Woodruff. Ovarian *Fst* mRNA expression was also lower in Lake Apopka alligators compared to Lake Woodruff. Interestingly, *Gdf9* deficient mouse ovaries express decreased *Fst*, compared to controls [52], and mice lacking *Gdf9* have increased frequencies of multi-oocytic follicles (MOFs) [53]. Previously, our laboratory has shown that yearling Lake Apopka alligator's ovaries exhibit increased frequencies of MOF [54], suggesting decreased *Gdf9* as a potential etiology for this condition.

5. Conclusions

Our data reveal sexually dimorphic mRNA expression patterns in the gonads of juvenile alligators from Lake Woodruff (reference site) that were similar to patterns established in other vertebrates for TGFβ-related signaling. The loss of sexually dimorphic mRNA expression in alligators from Lake Apopka (contaminant-exposed animals) is consistent with previously reported losses of sexual dimorphism in Lake Apopka alligators, such as phallus length [55,56], steroidogenic enzymes [2,57], and circulating steroid levels [6,54,58]. Our research suggests a relationship between contaminant exposure and altered TGFβ signaling in a sentinel, wetlands species. We hypothesize that environmental alteration in gonadal TGFβ superfamily signaling could be more prevalent than reflected in the current literature and presents an important, new research frontier.

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