Sexual Development

Sex Dev 2010;4:62–72
DOI: 10.1159/000277934

Received: May 5, 2009
Accepted: May 13, 2009
Published online: January 22, 2010

Gonadal mRNA Expression Levels of TGFβ Superfamily Signaling Factors Correspond with Post-Hatching Morphological Development in American Alligators

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Key Words
Activin · Alligator · Development · Follistatin · FOXL2 · GDF9 · Inhibin · Ovary · Testis

Abstract
Paracrine factor signaling regulates many aspects of vertebrate gonadal development. We investigated key ovarian and testicular morphological markers of the American alligator *(Alligator mississippiensis)* during the first 5 months post-hatching and correlated gonadal development with mRNA expression levels of a suite of regulatory factors. In both sexes, we observed significant morphology changes, including ovarian follicle assembly and meiotic progression of testicular germ cells. Concomitant with these changes were sexually dimorphic and ontogenetically variable mRNA expressions. In ovaries, FOXL2, aromatase, and follistatin mRNA expression was greater than in testes at all ages. At one week after hatching, we observed ovarian medullary remodeling in association with elevated activin/inhibin βA subunit, follistatin, and aromatase mRNA expressions. Three and 5 months following hatching and concomitant with follicle assembly, ovaries showed increased mRNA expression levels of GDF9 and the mitotic factor PCNA. In testes, the activin/inhibin α and βB subunit transcript levels were greater than in ovaries at all ages. Elevated testicular expression of GDF9 mRNA levels at 5 months after hatching aligned with increased spermatogenic activity. We propose that the mRNA expression levels and concomitant morphological changes observed here affect the establishment of alligator reproductive health and later fertility.

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Timing of vertebrate gametogenesis shows striking differences. Most female germ cells from amniotes enter meiosis during embryonic or post-natal development, whereas male meiosis begins with puberty. Endocrine and paracrine factors regulate these transitions. Here we investigate key morphological markers of American alligator *(Alligator mississippiensis)* ovarian and testicular development during the first 5 months post-hatching and correlate somatic and germ cell development with mRNA expression levels of a suite of gonadal regulatory factors. This comparison establishes sexually dimorphic expressions of many of these gonadal regulatory factors and explores ontogenetic changes in mRNA expression levels both within and between sexes during this period of substantial morphological development.

Ovarian follicle assembly is crucial to adult fertility. Among vertebrates, there are considerable differences in the timing of initial follicular somatic-germ cell interac-

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tions. As oogonia enter into meiotic prophase I, cytoplasmic bridges between germ cell syncytia breakdown [Pepling et al., 1999; Matova and Cooley, 2001; Pepling, 2006]. Subsequently, follicle assembly occurs after oocytes enter into diplotene arrest. Mammalian follicle assembly occurs either in utero (e.g. human, cow, pig), immediately after birth (e.g. rat, mouse), or during an extended postnatal immature period (e.g. rabbit, dog). Non-mammalian vertebrates show similar variability. Birds assemble follicles during the post-hatching period [Callebaut, 1968a, b]. Some reptiles form follicles as embryos [Forbes, 1956]; for example follicle assembly occurs prior to hatching in many species of turtles [Pieau and Dorizzi, 2004]. In the American alligator, we have demonstrated that follicle assembly occurs over a relatively extended post-hatching period [Moore et al., 2008]. Follicles with complete follicular cell layers are not observed until three months after hatching. Here, we expand these morphological observations to 5 months post-hatching and investigate concomitant mRNA expression changes of factors involved in gonadal development, germ cell maturation, and ovarian follicle assembly.

Within seminiferous tubules, male germ cells mature in three main phases: mitotic proliferation of spermatogonia, meiotic maturation of spermatocytes, and production of mature spermatozoa through spermiogenesis. During maturation, germ cells move from the juxta-epithelial, basal compartment towards the central, luminal compartment. Few researchers have investigated alligator testicular development [Gribbins et al., 2006]; though it is clear wild alligators have a prolonged and delayed maturation, followed by an extended pubescent period [Lance, 2003]. Alligators are seasonal breeders and, in the wild, male alligators with snout-vent lengths (SVL) >100 cm produce sperm [Joanen and McNease, 1980]. Therefore, estimated sexual maturity in wild alligators is 10 years of age or greater. However, testosterone cyclicity has been observed in wild juvenile alligators with SVL >38 cm [Rooney et al., 2004]. We have hypothesized that seasonal reproductive cyclicity – based on seasonal changes in plasma sex steroid hormone concentrations – begins years before the achievement of 'sexual maturity'. Male alligator puberty most likely is a neuroendocrine event, like that described in mammals and birds. In many reptiles, hypophysectomy leads to testicular degeneration [van Tienhoven, 1983], but exogenous gonadotropins restore spermatogenesis [Licht and Pearson, 1969]. Furthermore, gonadotropin or androgen treatments of immature male alligators induces testicular development [Forbes, 1937; Ramaswam and Jacob, 1965] and dramatic increases in plasma sex steroid hormone concentrations [Edwards et al., 2004]. We have observed that ovarian development accelerates in animals held under optimum, laboratory conditions [Moore et al., in press]. Under these conditions, the timing of ovarian follicle formation more closely matches development observed in juvenile chickens [Moore et al., 2008]. Here, we demonstrate that under laboratory conditions, alligator testes exhibit profound somatic and germ cell maturation that correlates with changes in mRNA levels of factors associated with testicular maturation.

Ovarian and testicular development requires somatic and germ cell interactions to regulate processes such as proliferation, differentiation, and maturation. Paracrine and autocrine signaling modulates many of these processes. Transforming growth factor-β (TGFβ) superfamily ligand subunits dimerize to produce signaling factors, such as activins and inhibin, that are vital to reproductive maturation and function [de Kretser et al., 2004; Drummond, 2005; Pangas et al., 2007; Barakat et al., 2008]. The production of activins, inhibins, and follistatin mediates activin signaling. 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]. Homo- or heterodimerization of β subunits, βA (INHBA) or βB (INHBB), form activin A (βA-βA), activin B (βB-βB), or activin AB (βA-βB). Ovarian activin signaling regulates follicle formation and initial follicle pool size [Bristol-Gould et al., 2006]. In testes, activin A is a paracrine factor regulating Sertoli cell number in the developing testes [Buzzard et al., 2003]. Loss of activin signaling in mice decreases numbers of spermatogonia and sperm production [Kumar et al., 2001].

Synthesis of inhibins or follistatin antagonizes activin signaling. Inhibins, activin receptor binding and activation antagonists, are heterodimers of a β subunit and an α subunit (INHA) forming either inhibin A (βA-*α*) or inhibin B (βB-*α*). Ovarian inhibin expression is minimal prior to puberty [Raivio and Dunkel, 2002]; however, it is vital to testicular formation and maintenance [Loveland et al., 2007]. Follistatin (FST) is a TGFβ ligand antagonist that binds and neutralizes activins. It is a somatic-cell-produced factor expressed in greater concentrations in embryonic mouse ovaries than testes [Menke and Page, 2002], though FST plays a role in both ovarian and testicular development [de Kretser et al., 2004; Yao et al., 2004; Yao, 2005].

In addition to activin/inhibin signaling components, we examined the mRNA expression levels of additional

factors associated with gonadal differentiation and/or maturation. Growth differentiation factor 9 (GDF9) is a germ cell-secreted TGFβ superfamily ligand that regulates ovarian folliculogenesis through directing granulosa cell proliferation and differentiation [Dong et al., 1996; Carabatsos et al., 1998; Johnson et al., 2005]. Proliferating cell nuclear antigen (PCNA) is a sliding ring clamp that interacts with DNA polymerase during DNA replication and expression is maximal around the S phase of the cell cycle [Krishna et al., 1994]. Ovarian formation is a directed, active process [Ottolenghi et al., 2005, 2007; Yao, 2005] and gonadal expression of the transcription factor forkhead box L2 *(FOXL2)* is a sexually dimorphic, early molecular marker of ovary-specific sex differentiation [Loffler et al., 2003; Uhlenhaut and Treier, 2006; Rhen et al., 2007]. Expression of *FOXL2* is vital for primordial follicle pool formation [Schmidt et al., 2004], granulosa cell differentiation [Schmidt et al., 2004], and activates aromatase *(CYP19A1)* gene transcription [Hudson et al., 2005; Pannetier et al., 2006].

Here, we exploit the relatively slow development of the alligator ovary to study changes in gene expression levels concomitant with morphological development. Additionally, we compare and contrast concomitant testicular morphological development and gene expression. This study establishes sexual dimorphic gene expression patterns and ontogenetic changes in morphology that begin at hatching and provides a baseline for comparative studies as well as future integrative studies of the function of the alligator gonad.

Materials and Methods

We obtained American alligator (*Alligator mississippiensis*, Daudin, 1801) eggs from nests in the Lake Woodruff National Wildlife Refuge (Permit #WX01310) prior to the period of temperature-dependent sex determination [Ferguson and Joanen, 1983] in June 2006. At the University of Florida, eggs (n = 105) from seven clutches were candled to assess viability and divided between two incubation temperatures: 30°C that produces females (n = 55) and 33°C that produces males (n = 50). Within each incubation temperature group, we systematically arranged eggs in trays of damp moss. During incubation, we rotated trays to minimize possible clutch or incubator position biases. The total hatching success rate was 84%: 82% of animals from 30°C (n = 45) and 86% of animals from 33°C (n = 43).

Animal procedures conformed to an IACUC approved protocol. Following hatching, animals were housed within a temperature-controlled animal room in tanks (~20 neonates/0.7 m^3) and experienced a 16:8 photoperiod with heat lamps for basking. Ambient room temperatures ranged from 27 to 31°C. Alligators hatch with a relatively large residual yolk mass; thus, ad libitum hatchling feeding started 10 days after hatching with a small pellet size, commercial crocodilian diet (Mazuri, #5MG1).

Tissues were collected from subsets of alligators after a lethal dose of sodium pentobarbital at: one week (30°C n = 12, 33°C n = 10), 1 month (30°C n = 11, 33°C n = 9), 3 months (30°C n = 10, 33°C n = 8), and 5 months post-hatching (30°C n = 11, 33°C n = 10). At necropsy, one gonad from alternating sides was removed and stored in RNAlater (Ambion) at –20°C until RNA extraction. The contralateral gonad and underlying tissues were Bouin's fixed for 24 h, underwent standard paraffin histology, were sectioned parasagittally at 6 μm, and stained with periodic acid (PAS)-alcian blue (pH 2.5) with hematoxylin counterstaining. Histological sections were examined using an Olympus BH-2 light microscope and photographed with a Pixelink PL-B623CU 3.0 megapixel digital camera.

Microscopic examination showed all gonads from alligators incubated at 30°C were ovaries whereas 68% of gonads from animals incubated at 33°C were testes. Of the females produced from the 33°C incubation, 92% came from three of the seven egg clutches collected for the experiment. We present results only from gonads of 30°C females and 33°C males in this manuscript (n at 30°C/33°C: 1 week 12/8, 1 month 11/6, 3 months 10/8, and 5 months 11/7). Body masses (BM) were recorded prior to necropsy (average age of groups at measurement in days: 1 week = 4.5 days, 1 month = 28.1 days, 3 months = 88.5 days, and 5 months = 145.6 days).

While examining ovarian histology, we observed morphological changes in both cortex and medulla. We characterized states of oogenesis and folliculogenesis at each time point in our study. Chromatin and cytoplasmic morphologies determined various oocyte stages of prophase I. Additionally, oocyte stages were identified according to a system that incorporates both meiotic characteristics and extent of folliculogenesis [Uribe and Guillette, 2000]. Stage-1 (S1) oocytes have a mean diameter of approximately 10 μm, exhibit fibrillar chromatin, and are in loose association with follicular cells. Stage-2 (S2) oocytes have a mean diameter of approximately 25 μm, an incomplete follicular layer of somatic cells, and diplotene nuclei that often display chiasma, lampbrush chromosomes, and several nucleoli. Stage-3 (S3) oocytes, also diplotene, are distinctly larger in diameter (120–200 μm), often display pronounced Balbiani bodies and lampbrush chromosomes, and possess a complete follicular layer of granulosa as well as a developing layer of surrounding thecal cells.

We observed morphological changes in testicular histology of the seminiferous cords and interstitial spaces. With insight from the recent characterization of adult alligator germ cell maturation [Gribbins et al., 2006], cellular morphology and location within tubules characterized germ cell maturation.

RNA isolation and reverse transcription procedures for alligator gonadal tissues have been previously reported from our laboratory [Milnes et al., 2008]. Quantitative real-time PCR (Q-PCR) has been used to measure mRNA expression in the American alligator [Katsu et al., 2004; Gunderson et al., 2006; Kohno et al., 2008]. Table 1 reports primer sequence information, annealing temperatures, and accession numbers. Q-PCR was performed using the MyiQ single color detection system (BioRad) following manufacturer's protocol using iQ SYBR Green Supermix (Bio-Rad) in triplicate reaction volumes of 15 μl with 0.6 μl of RT product and specific primer pairs. We calculated expression levels of mRNA using gene specific, absolute standard curves, which con-

Table 1. Q-PCR Primer data

Gene	Forward primer (5′–3′) Reverse primer (5′–3′)	Annealing temperature, °C	Product size bp	Accession No.
INHBA	ACCCACAGGTTACCGTGCTAA GCCAGAGGTGCCCGCTATA	63.8	67	DQ101152
INHBB	GGGTCAGCTTCCTCCTTTCAC CGGTGCCCGGGTTCA	64.7	70	DQ010153
INHA	ACAATCCACTTGTCCCAGCC CAACTGCCACCGCGC	70.0	68	DQ010151
FST	CGAGTGTGCCCTCCTCAAA TGCCCTGATACTGGACTTCAAGT	66.5	65	DQ010156
FOXL2	ATCAGCAAGTTCCCCTTCTAC GCCTTTCTCGAAAATGTCCTC	65.0	171	EU848473
CYP19A1	CAGCCAGTTGTGGACTTGATCA TTGTCCCCTTTTTCACAGGATAG	63.8	79	AY029233
GDF9	TCAGTTTCCTCCTCTTCTCCAATT ACACACTTGGCTAGAAGGATCATTC	63.0	78	DQ015675
PCNA	AGCAGAAGACAATGCAGACAC CTAAGCCATATTGGAGATGCA	62.0	199	FJ824113

tain 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 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 $p < 0.05$. We analyzed data by two-way ANOVA and employed least square means Tukey-Kramer post-hoc comparisons, when appropriate, comparing body measurements and relative mRNA expressions by sex, age, and sex by age. To investigate possible interaction between mRNA expressions, we performed selected linear regressions within sex and age groups.

Results

Growth and Gonadal Morphology

Under our laboratory animal room conditions (16:8 photoperiod and ambient room temperatures ranging from 27 to 31 °C), alligator growth was robust over the period of this study (age-mean male BM (g) ± SEM, female BM ± SEM): 1 week – 56.9 ± 4.4, 56.7 ± 4.5; 1 month – 80 ± 19, 103 ± 12; 3 months – 412 ± 56, 493 ± 58; 5 months – 901 ± 118, 985 ± 81. At 5 months, the average alligator BM was more than 15 times greater than that recorded at 1 week of age. At 1 and 3 months, the female BM was greater than male BM (p = 0.009 for each).

Alligator ovaries are composed of a cortex of germ and somatic cells overlying a medulla of connective tissue and lacunae containing secretory materials (fig. 1A–D). Mesonephric and adrenal tissues juxtapose gonadal tissues (fig. 1A). During the 5 months after hatching, we observed cortex expansion and follicle assembly. At 1 week and 1 month after hatching, the cortex is composed of oogonia nests, S1 oocytes, and somatic pre-follicular cells (fig. 1A, B). The medulla fragments and lacunae expand during this period. Cortex thickness increases at 3 and 5 months after hatching, concomitant with enlarging germ cell nests and follicle assembly around S2 and S3 oocytes (fig. 1C, D). The cytoplasm of S2 and S3 oocytes contains Balbiani bodies. Follicles containing S3 oocytes have a full complement of granulosa cells and, externally, a thin layer of fibroblast-forming thecal layers (fig. 1G, H). At 5 months, S3 oocytes were more numerous than at 3 months and we continued to observe oogonia with mitotic chromatin within germ cell nests (fig. 1H).

From 1 week to 1 month after hatching, alligator testes are characterized by seminiferous cords lined with Sertoli cells and spermatogonia A and are separated by sparse interstitial tissues (fig. 2A, B, E and F). At 3 months after hatching, we observed spermatogonia B above the basement membrane, primary spermatocytes with more central locations within tubules, and an increase in interstitial tissues with clearly observed Leydig cells (fig. 2C, G). At 5 months after hatching, seminiferous tubules con-

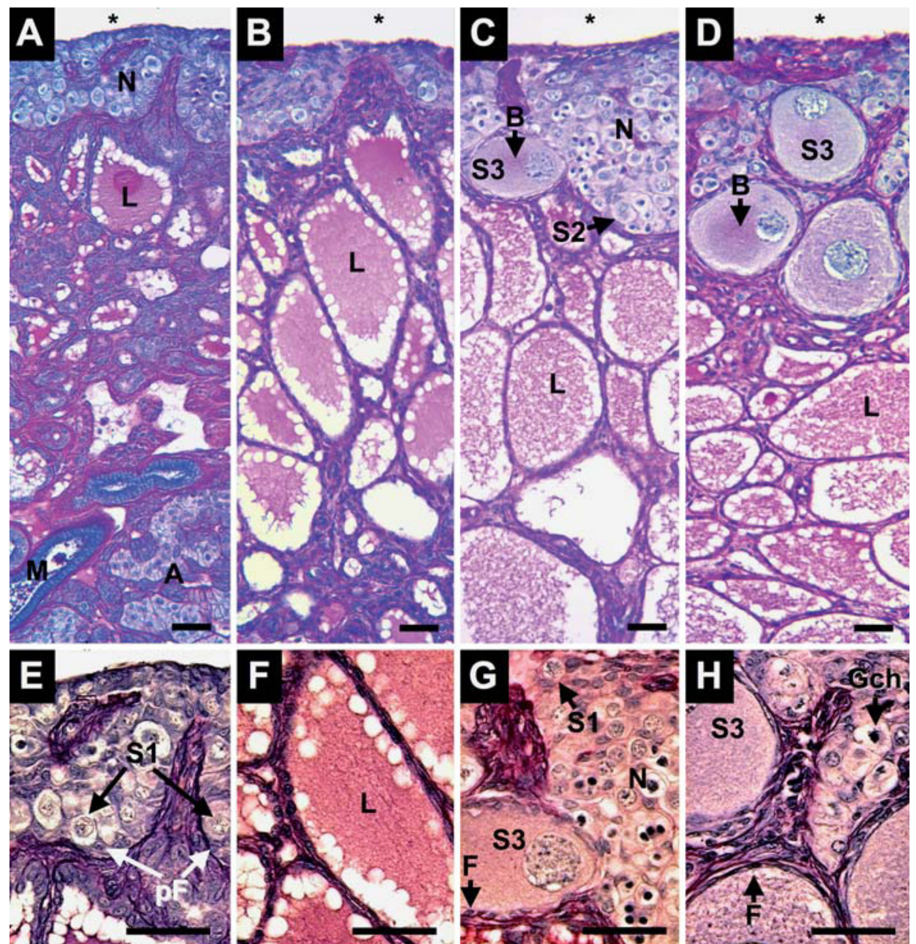

Fig. 1. Ovary of *Alligator mississippiensis* at 1 week (**A**, **E**), 1 month (**B**, **F**), 3 months (**C**, **G**), and 5 months (**D**, **H**) after hatching in parasaggital view (**A–D**) and enlarged detail (**E–H**). At 1 week after hatching the ovarian cortex contains nests of germ cells (N) along the coelomic (*) border. These nests contain Stage-1 oocytes (S1) in close interaction with pre-follicular cells (pF). Underlying the cortex, lacunae (L) form within degenerating medullary cords. The gonad overlies mesonephric (M) and adrenal tissues (A). At 1 month after hatching, the medullary lacunae (L) have expanded. At 3 and 5 months after hatching, the cortex contains Stage-2 (S2) and -3 oocytes (S3), displaying Balbiani bodies (B). S3 oocytes are contained in follicles with a complete complement of squamous to cuboidal follicular cells (F). Germ cell nests continue to present oogonia with mitotic chromosomes (Gch) at 5 months after hatching. Scale bars = 50 μm.

tained centrally located round spermatids along with spermatogonia and spermatocytes (fig. 2D, H). Interstitial tissues and Leydig cell clusters continued to enlarge.

Gene Expression

Expression levels of *RPL8* were not different among sexes, ages, or sex by age comparisons. *INHBA* (fig. 3A, B; $p = 0.003$), *IHBBB* (fig. 3C, D; $p = 0.004$), *INHA* (fig. 3E, F; $p < 0.001$), *FST* (fig. 3G, H; $p < 0.001$), *GDF9* (fig. 3I, J; $p = 0.42$), *PCNA* (fig. 3K, L; $p = 0.039$), and *CYP19A1* (fig. 3O, P; $p = 0.008$) mRNA expression levels showed significant sex by age interactions. We observed sexually dimorphic expression patterns that were uniform across all ages. Female *FOXL2* mRNA expression levels were greater than male expression levels, which were at the limit of detection (fig. 3M, N; $p > 0.001$). On the other hand, testicular expression levels of *INHBB* and *INHA* mRNA were greater than ovarian levels (fig. 3C–F) and female *INHA* expression levels were at the limit of detection (fig. 3E). Testicular levels of these transcripts trended to decrease as age increased (fig. 3D, F). Conversely, ovarian *FST* and *CYP19A1* mRNA expression levels were greater than that observed in testicular tissue (fig. 3G, H and 3O, P, respectively). Testicular *INHBB* levels were over an order of magnitude greater than *INHBA* levels (fig. 3B, D). Ovarian *INHBA*, *INHBB*, *FST*, and *CYP19A1* mRNA expression levels shared a similar expression pattern: decreased expression levels between 1 week and 1 month, a trend to increase at 3 months, and subsequently decreased levels at 5 months (fig. 3A, C, G, and O). Ovarian *GDF9* and *PCNA* mRNA expression levels increased with age between 1 and 3 months (fig. 3I, K). Testicular *GDF9* mRNA expression was greater in months 3 and 5 than at 1 week and 1 month (fig. 3J) and *PCNA* mRNA expression levels showed an increasing trend between 3 and 5 months after hatching (fig. 3L).

Linear regression of ovarian *FOXL2* by *CYP19A1* mRNA expression by age groups showed significant lin-

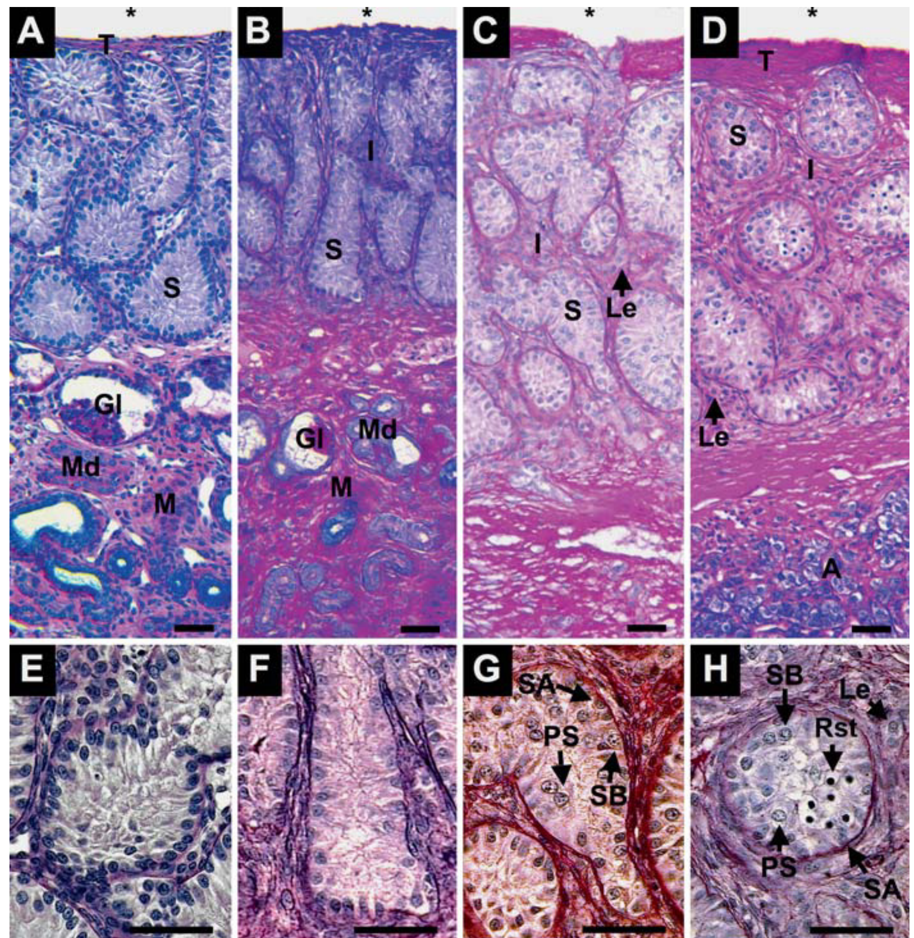

Fig. 2. Testis of *Alligator mississippiensis* at 1 week (**A**, **E**), 1 month (**B**, **F**), 3 months (**C**, **G**), and 5 months (**D**, **H**) after hatching in parasagittal view (**A–D**) and enlarged detail (**E–H**). The coelomic (*) border is defined by a tunica (T) that thickens with age. The gonads contain well-defined seminiferous tubules (S) lined with spermatogonia A (SA) and overlie mesonephros tissue (M) containing glomeruli (Gl) and mesonephric ducts (Md) and adrenal tissues (A). At 3 and 5 months after hatching, interstitial tissues (I) between tubules expand and present Leydig cells (Le). At 3 months after hatching, seminiferous tubules present spermatogonia A and B (SA, SB) and primary spermatocytes (PS). At 5 months after hatching, tubules contain germ cells including round spermatids (Rst). Scale bars = 50 μm.

ear relationships at 1 week ($p = 0.005$, $R^2 = 0.56$) and 3 months ($p = 0.001$, $R^2 = 0.71$), however not at 1 month ($p = 0.40$, $R^2 = 0.02$) and 5 months ($p = 0.18$, $R^2 = 0.19$).

Discussion

During the first 5 months after hatching, we observed a considerable increase in body mass, significant changes in gonadal morphology, development of male and female germ cells, and sex-specific and ontogenetically variable mRNA expression patterns. As observed in previous studies, newly hatched alligator gonads are not quiescent [Guillette et al., 1994; Milnes et al., 2008; Moore et al., 2008]. Analogous to the importance of appropriate mammalian follicle assembly [Bristol-Gould et al., 2006] or post-natal Sertoli cell proliferation [Buzzard et al., 2003], we propose that the mRNA expression levels and concomitant morphological changes observed here affect the establishment of alligator reproductive health and later fertility.

Appropriate activin signaling is vital for both ovarian and testicular maturation [Loveland et al., 2007; Pangas et al., 2007]. In this study, we observed sexually dimorphic expressions of *INHBB*, *INHA*, and *FST* mRNA in alligator hatchling gonads. In mammals and birds, ovarian *INHA* mRNA expression prior to puberty is minimal [Raivio and Dunkel, 2002; Billiar et al., 2003; da Silva et al., 2004; Onagbesan et al., 2004]; however, embryonic and immature expression is vital to testicular formation and maintenance [Drummond et al., 2004; Loveland et al., 2007]. The absence of *INHA* in ovaries allows an activin-signaling milieu critical for germ cell survival and proliferation [da Silva et al., 2004; Bristol-Gould et al., 2006]. In male humans and rodents, *INHA* mRNA is primarily expressed in Sertoli cells and a post-natal inhibin B peak is hypothesized to result from Sertoli cell proliferation [Meachem et al., 2001; Buzzard et al., 2004; Bara-

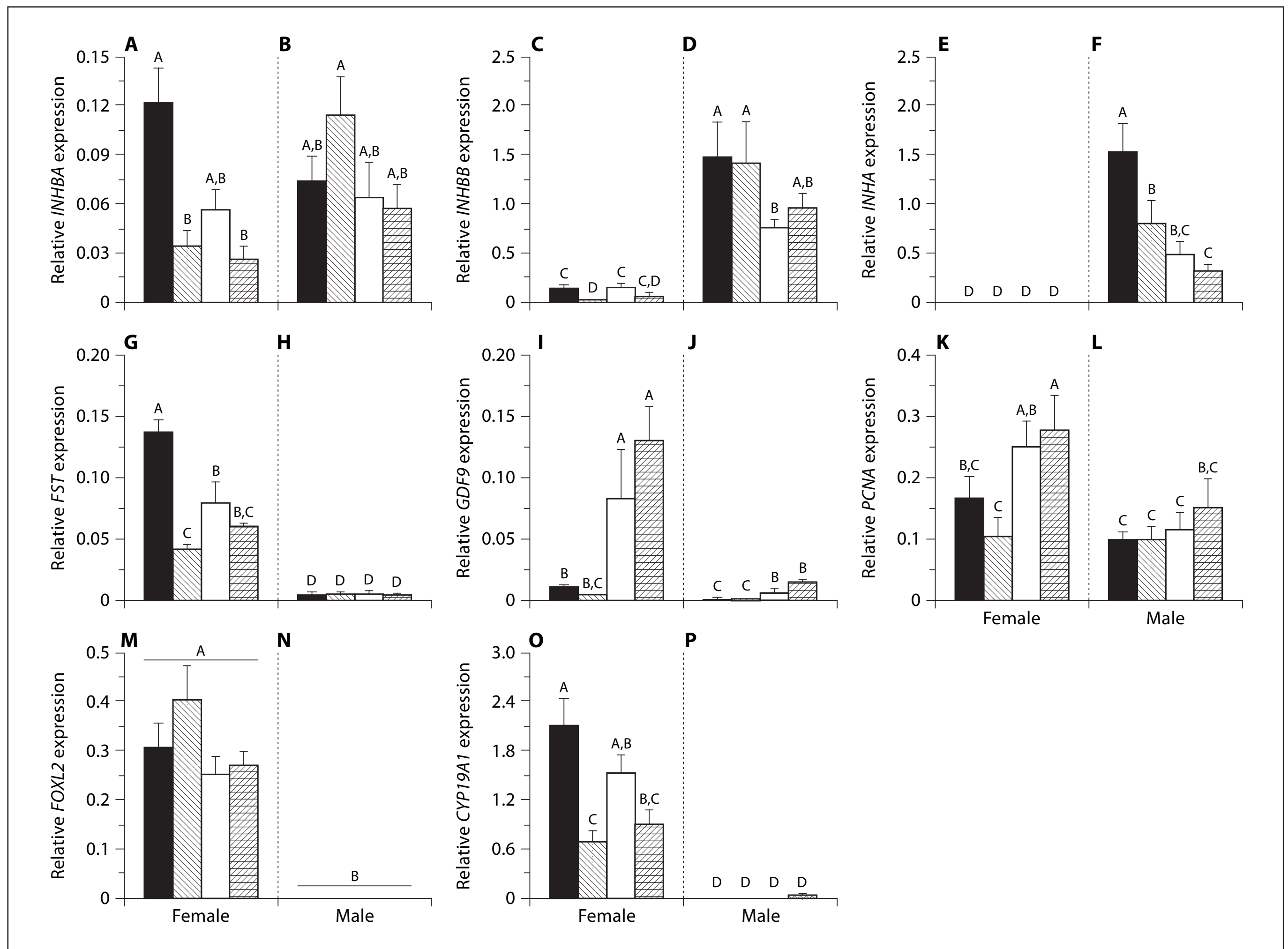

Fig. 3. Mean (± SEM) gonadal mRNA expression of *INHBA* (**A**, **B**), *INHBB* (**C**, **D**), *INHA* (**E**, **F**), *FST* (**G**, **H**), *GDF9* (**I**, **J**), *PCNA* (**K**, **L**), *FOXL2* (**M**, **N**), *CYP19A1* (**O**, **P**) in female (**A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O**) and male (**B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P**) alligators. Days after hatching: black bars = 7, diagonal lined bars = 30, white bars = 90, crosshatched bars = 150. All sample means were normalized using ribosomal protein L8 *(RPL8)* expression. Different letters above the bars of each graph of mRNA transcript levels indicate statistical significance at $p < 0.05$ across both ages and sexes.

kat et al., 2008]. Sertoli cells proliferate for seven weeks after hatching in chicken [Bozkurt et al., 2007]. Our observations of post-hatching elevated testicular *INHA* and *INHBB* mRNA levels are in line with these observations. We propose that decreasing testicular *INHA* and *INHBB* mRNA levels over time could represent either a cessation of Sertoli cell proliferation or a decrease in concentration of Sertoli cell-derived mRNA in cDNA samples due to increased mRNA from increasing numbers of germ and interstitial cells.

In the developing mouse testes, *INHBB* mRNA expression is greater than in ovaries [Yao et al., 2006]. Elevated *INHBB* levels modulate the formation of testis-specific vascularity, putatively through production of activin B or inhibin B. This is in agreement with sexually dimorphic *INHBB* mRNA levels observed in post-hatching alligators. Further, the activin antagonist *Fst* is expressed at greater levels in embryonic mouse ovary than testis [Menke and Page, 2002], promotes the survival of meiotic germ cells in the cortex, and antagonizing testis-specific, *INHBB*-mediated vascular formation [Yao et al., 2004]. Conversely, *FST* mRNA overexpression leads to Leydig cell hyperplasia, an arrest of spermatogenesis, and seminiferous tubular degeneration [Guo et al., 1998]. El-

evated testicular *INHA* and *INHBB* and elevated ovarian *FST* mRNA levels are in line with mammalian gene expression patterns that result in appropriate sex development and maintenance.

We observed a robust increase in *GDF9* mRNA expression in 3- and 5-month-old ovaries. This increase coincided with the assembly of complete follicles around S3 oocytes. This is in agreement with the reported role of GDF9 in primary and primordial follicles which is to promote granulosa and possibly thecal cell proliferation and differentiation [Trombly et al., 2009]. Though expressed in greater amounts in ovaries, *GDF9* mRNA expression has been localized in human testes and rodent spermatocytes and spermatids [Fitzpatrick et al., 1998] and regulates Sertoli cell function [Nicholls et al., in press]. In agreement with these findings, we observed increased *GDF9* mRNA expression concomitant with initiation of spermatogenesis at 3 and 5 months after hatching. Further, rat testicular *GDF9* expression was greatest in round spermatids [Nicholls et al., 2009]. We observed the greatest mean expression of *GDF9* mRNA in alligator testes at 5 months, the time round spermatids were first observed.

If *GDF9* mRNA expression promotes somatic cell proliferation, we hypothesized that we could detect an associated change in mitotic activity. As a molecular marker of mitotic activity, we quantified levels of gonadal *PCNA* mRNA expression. Increased PCNA protein in granulosa cells marks the earliest stages of follicle growth [Oktay et al., 1995]. In testes, PCNA protein marks the proliferation of Sertoli cells and spermatogonia [Schlatt and Weinbauer, 1994]. When compared to levels at 1 month, we observed increased ovarian *PCNA* mRNA expression at 3 and 5 months after hatching. During these later developmental periods, the ovarian cortex expanded with the presence of enlarged follicles. Follicular, thecal, and germ cell proliferation could contribute to elevated ovarian *PCNA* mRNA levels. In alligator testes, we observed a trend toward greater *PCNA* mRNA levels, though this increase was not statistically significant.

Among vertebrates, *FOXL2* expression is vital for ovarian development [Ottolenghi et al., 2007] and has high amino acid sequence conservation in the forkhead domain [Oshima et al., 2008]. In *FOXL2* knockouts, meiotic prophase oocytes form, but *FST* mRNA expression is decreased and primordial follicles do not assemble [Uda et al., 2004; Ottolenghi et al., 2005]. As expected, we observed highly dimorphic *FOXL2* mRNA in juvenile alligator gonads. In pre-follicular chicken ovaries, *FOXL2* and aromatase expression co-localize in the medulla [Govoroun et al., 2004] and positively correlate with expression levels during embryonic ovarian development in turtles [Rhen et al., 2007] and chicken [Govoroun et al., 2004]. FOXL2 transcription factor binds to and regulates the CYP19A1 promoter [Pannetier et al., 2006; Wang et al., 2007]. Further, *CYP19A1* transcript expression could potentiate a positive feedback on *FOXL2* expression via an estradiol-17β mechanism [Hudson et al., 2005].

Similar to *FOXL2* transcript levels, in this study we observed sexually dimorphic juvenile alligator gonadal *CYP19A1* mRNA expression levels. In support of *FOXL2* regulation of *CYP19A1* mRNA expression, we observed positive correlations between expression levels of these factors at 1 week and 3 months after hatching, however not at 1 month or 5 months. We propose that because 1 week after hatching encompasses the greatest period of medullary fragmentation and 3 months marks the first observation of thecal cell layers around S3 oocytes completing follicle assembly that greater estrogenic signaling characterizes these periods. Gonadal aromatase activity is sexually dimorphic in embryonic alligator gonads and ovarian aromatase activity increases from sex determination until hatching [Smith et al., 1995]. In turtles, ovarian aromatase activity falls after hatching, and then rises during the following months [Belaid et al., 2001]. Estrogens impede testicular cord formation and possibly maintain female turtle medullary morphology and gene expression after sex differentiation [Belaid et al., 2001; Pieau and Dorizzi, 2004]. We hypothesize elevated *CYP19A1* mRNA levels at 1 week after hatching, driven by *FOXL2* mRNA expression, plays a roll in alligator medullary restructuring.

In chicken hatchlings, steroidogenic cells migrate from the medulla into the cortex and surround assembling follicles as theca [Narbaitz and Adler, 1966; Narbaitz and DeRobertis, 1968; Gonzalez-Moran et al., 1985; Drummond, 2005]. In small chicken follicles, the single thecal layer synthesize estrogens, not the granulosa [Nitta et al., 1991a, b]. In rats, aromatase transcription increases during primordial follicle assembly [Kezele et al., 2005], whereas decreases in estrogenic signaling are suggested to regulate follicle assembly [Britt et al., 2004; Chen et al., 2007]. We observed follicle formation around S3 oocytes at 3 months after hatching concomitant with increased *CYP19A1* expression compared to 1 month after hatching. This increase in *CYP19A1* expression and putative resulting aromatase activity could be associated with the initiation of limited follicle formation while germ cell nests with mitotic oogonia continue to persist.

Recent research has demonstrated substantial ovarian estrogen-activin signaling cross talk [Kipp et al., 2007a]. That is, TGFβ and steroid signaling interplay regulates follicle assembly, with estrogens impeding germ cell nest breakdown and activins promoting granulosa cell maturation and follicle assembly [Kipp et al., 2007b; Mayo et al., 2007; Trombly et al., 2009]. We observed expression patterns of *INHBA*, *INHBB*, and *FST* mRNA that were similar to *CYP19A1* and propose that this coordinated change in expression levels relates to integrated estrogen-activin signaling that is observed in neonatal and juvenile rodent ovaries.

Acknowledgements

Continuing logistical support of our alligator research by the Florida Fish and Wildlife Conservation Commission made this study possible; specifically we thank Allan Woodward for his continued assistance with fieldwork and permitting. Grants to L.J.G. from the NIH (R21 HD047885-01; R21 ES014053-01) and the Howard Hughes Medical Institute Professors program supported this work.

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