THE ROLE OF TGFβ SIGNALING IN OVARIAN DEVELOPMENT AND FOLLICLE ASSEMBLY IN THE AMERICAN ALLIGATOR

By

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To my parents, Richard and Maureen, who taught me to
clean-up after myself and leave places better than you found them

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THE ROLE OF TGFβ SIGNALING IN OVARIAN DEVELOPMENT AND FOLLICLE ASSEMBLY IN THE AMERICAN ALLIGATOR

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Formation of ovarian follicles, germ cells surrounded by a cohort of supporting somatic cells, is critical of later female reproductive health. As a follicle matures, it fosters the production of a viable egg that, after ovulation, is available for fertilization. Follicles can form improperly. When two or more germ cells become enclosed a single follicle this malformation is a multioocytic follicle (MOF). In laboratory experiments, estrogenic treatments of female rodents before follicle assembly increase MOF frequencies. Further, this estrogenic treatment appears to interfere with appropriate activin signaling. Interactions between estrogen and activin signaling modulate follicle assembly processes. Yearling alligators from Lake Apopka, a site extensively contaminated with endocrine disrupting compounds, show elevated MOF frequencies. Here, we investigate ovarian follicle assembly dynamics and activin signaling in hatchling alligators to understand possible causation and characterize mechanism underlying these malformations of alligator ovaries.

Results demonstrated that alligator follicle assembly occurs over many months following hatching. Complete follicles were not observed under laboratory conditions until three months after hatching. This prolonged developmental process putatively results in a larger window of time in which follicle assembly could be disrupted. Gonadal expression levels of some activin

signaling factor mRNA are sexually dimorphic. In 13-month old contaminant exposed alligators, we observed diminished or lost sexual dimorphisms in both male and female alligator gonads. Additionally, these sexually dimorphic expressions of these factors are also present in hatchling animal gonads, both before and during the period of follicle assembly in females. Further, contaminant exposure altered mRNA expression levels of some of these factors. In some gene expressions, mRNA expression levels differences between contaminant exposed and reference site animals became more pronounced when ovaries were gonadotrophin challenged. Ovaries in contaminant-exposed animals showed both greater and lesser expression levels of factors vital for female reproductive health when compared to ovaries from reference site alligators. Therefore, these reproductive differences could affect later hypothalamus-pituitary-gonadal signaling dynamics after puberty. Taken together, these studies demonstrate that exposure to contaminants found in Lake Apopka, even before hatching, can have lasting effects on gonadal endocrine activity and may comprise the causation of Lake Apopka alligator ovarian MOFs.

CHAPTER 1
MULTIOOCYTIC FOLLICLES, ENVIRONMENTAL CONTAMINANTS, AND AMERICAN ALLIGATORS

Causes of Infertility

Current estimates of infertility suggest that more than 2 million U.S. couples are affected (one in 12 couples are unable to conceive after one year of unprotected intercourse). Although various reasons have been hypothesized for the growing rate of this phenomenon, such as life style alterations (late age first pregnancies; STDs, other complications such as fibroids and endometriosis), environmental factors could also be a major factor. The National Institute for Occupational Safety and Health (NIOSH) reports that "Physical and biological agents in the workplace that may affect fertility and pregnancy outcomes are practically unstudied. The inadequacy of current knowledge coupled with the ever-growing variety of workplace exposures poses a potentially serious public health problem" (Drozdowsky and Whittaker, 1999). Although numerous occupational exposures have been demonstrated to impair fertility [e.g., heavy metals (lead), some pesticides, and solvents (Sharara et al., 1998), the overall contribution of environmental exposures to infertility is unknown, but a growing scientific database suggests that exposure to various environmental factors, both *in utero* and neonatally, could dramatically affect fertility as an adult (Hotchkiss et al., 2008).

Studies of wildlife populations exposed to various environmental contaminants have documented significant declines in fecundity (Guillette and Gunderson, 2001; Jobling and Tyler, 2003; Tyler et al., 1998). Importantly, in these populations the production of fewer offspring is related to varying mechanisms, from altered sperm or egg production to early embryonic mortality. In humans, major debate and a significant research effort has focused on the role of environmental factors and changes in male semen quality and fertility over the last decade (Swan et al., 2003; Swan et al., 1997). A central theme of the work on semen quality is the hypothesis

that embryonic, fetal and neonatal exposure to various xenobiotic factors could be the basis for adult disease, including subfertility or infertility (Edwards et al., 2006a; Skakkebaek et al., 2001).

Although semen quality is under intense study, less studied are the possible environmental causes of ovarian disease, specifically follicular failure. Recently, a group of physicians and researchers who are experts in women's reproductive health convened and took up the mantle of coalescing and synthesizing the diversity of research regarding environmentally-induced female reproductive disorders into a unified, consolidated platform addressing the commonalities between the etiologies of these disorders and the large-scale steps to needed to understand these complex interactions (Crain et al., 2008). One tenant of this platform, in addition to other recent research, is that normal ovarian follicle assembly can be altered by both genetic and environmental factors, possibly compromising later reproductive success (Crain et al., 2008; Hunt and Hassold, 2008). Changes in the quality or quantity of oocytes is more difficult to measure than changes in semen quality due to the inaccessibility of female gametes and the timing of female gametogenesis in many species. What is apparent from current research is that there are critical developmental windows in which ovarian development and subsequent heath can be profoundly impacted by environmental factors.

Multioocytic follicles (MOFs, alternatively called polyovular follicles) are characterized by two or more oocytes surrounded by a common follicular envelope of granulosa cells. They are hypothesized to result from oogonial clusters that do not remodel into single oocytes during normal follicle assembly (Iguchi and Takasugi, 1986; Iguchi et al., 1986). MOFs have been reported at low, but variable frequencies in rodents, dogs, cats, rabbits, rhesus monkey, women, chicken and various reptiles (Amer and Shahin, 1975; Suzuki et al., 2002) (Fig. 1-1). While a

low frequency of MOFs is presumed normal in an ovary, a high frequency is pathological. The success rates of *in vitro* fertilization of ova from MOFs of neonatally diethylstilbestrol (DES) treated mice are reduced in comparison to ova from uniovular follicles from both control or neonatally DES treated mice: 23% compared to 60 and 52%, respectively (Iguchi, Fukazawa et al. 1990). Furthermore, increased embryonic loss has been observed in mice. At least one study has suggested a relationship between biovularity (MOFs) and the formation of ovarian teratomas, as 26 of 31 young women (mean age 27) with mature teratomas had MOFs whereas only 1 of 30 control females lacking teratomas had MOFs (Muretto et al., 2001). Oocytes in MOFs also can be multinucleated. MOFs have been generated in rodents and humans by experimental or medicinal prenatal and neonatal exposure to estrogens, such as the natural endogenous estrogen, estradiol-17β (E_2), pharmaceutical estrogens like DES or phytoestrogens (Iguchi, 1992). Overexpression, by transgenic modification, of the inhibin α subunit has also produced this pathology in rodents (McMullen et al., 2001). The peptide hormone inhibin plays a major endocrine role in regulating the hypothamo-pituitary-gonad (HPG) axis as well as paracrine regulatory roles in vertebrate gonads. Therefore, to gain a better understanding of the origin of adult ovarian dysfunction, a comprehensive understanding of the ontogeny of normal ovarian follicle formation and underlying mechanisms is required.

Multioocytic Follicles and Alligators

Until a decade ago, MOFs were largely viewed as a pathology observed in mammals exposed to pharmaceutical estrogens. However, in 1994 our group reported that MOFs occurred at a very high frequency in female alligators exposed during embryonic development to environmental contaminants (Fig. 1-1). Lake Apopka is contaminated with various pesticides and anthropogenic nutrients (Guillette et al., 1999a), resulting in reproductive impairment of

alligators (Fujisaki et al., 2007; Milnes and Guillette, 2008). Female alligator hatchlings from Lake Apopka when given an luteinizing hormone (LH) challenge displayed MOFs (often 3-4 oocytes per follicle) and elevated plasma E_2 concentrations compared to a low (0 - 3% of the follicles in an ovary) frequency in reference females of a similar age within the same study (Guillette et al., 1994b). Embryos and neonates from clutches of eggs collected from Lake Apopka exhibit elevated levels (≥ 50%) of embryonic mortality that occurs either early in gastrulation or just after hatching (Masson, 1995; Rotstein et al., 2002). Mortality occurred in rodents at the same embryonic stage when oocytes came from MOFs (Iguchi et al., 1990). Embryonic mortality in alligators has been correlated with organochloride pesticide levels in eggs both in the field and under laboratory conditions (Rauschenberger et al., 2007) and is consistently elevated in wild populations exposed to mixtures of pesticides and nutrients (Heinz et al., 1991b; Woodward et al., 1993). Although first reported in 1994, elevated embryonic mortality and MOFs continue to be reported from females from this contaminated population. Neonates that are obtained from eggs removed from the wild nests prior to sex determination and incubated under identical conditions with eggs from reference populations exhibited MOFs. Therefore, factors that lead to an increased frequency of MOFs are not exogenous factors experienced during development in the nest such as temperature or humidity, but are most likely passed maternally to the embryo via the egg. We hypothesize that in female alligators from Lake Apopka, the maternal contribution of endocrine disrupting contaminants with estrogenic activity to the egg yolk subsequently alters inhibin/activin signaling in the female offspring that predisposes MOF development. Importantly, the frequency of MOFs in the ovary may increase dramatically if the female is experimentally challenged with a gonadotropin treatment, as occurred just prior to necropsy in the cohort described in our previous work. This further

supports the hypothesis that early exposure to an abnormal estrogenic signal alters normal ovarian responsiveness within the HPG axis as reference females within the same challenge did not display MOFs.

Studies of estrogenic exposures using another crocodilian (*Caiman latirostris*) support our findings of altered gonadal morphology and physiology. Bisphenol A (BPA) is an industrial chemical shown to have estrogenic and anti-androgenic properties (Richter et al., 2007). *In ovo* exposure of developing caiman gonads to E_2 or BPA resulted in male to female sex reversal (Stoker et al., 2003). Therefore, as previously observed in alligators (Crain et al., 1997), embryonic crocodilian gonads are responsive to exogenous steroidal signaling through both endogenous ligands and contaminant exposures. Furthermore, laboratory raised female caiman exposed *in ovo* to E_2 or BPA during the beginning of sex differentiation displayed increased frequencies of MOFs, higher proportions of advanced follicles, and increased circulating E_2 levels as juvenile (3 and 12 months post-hatch) animals (Stoker et al., 2008). This finding supports the hypothesis that prenatal exposures can result in long-lasting impacts to reproductive endpoints.

As with the 'canary in the coal mine', we propose that if MOFs could be induced in wildlife by contaminant exposure with estrogenic activity, similar responses could also be observed in natural populations of mammals, including humans(Crain et al., 2008). This hypothesis is based on the conserved nature of follicle assembly and maturation. Understanding and documenting these conserved mechanisms among vertebrates will strengthen causal and mechanistic relationships, allowing a better understanding of this phenomenon in human populations. Unfortunately, studies to test this hypothesis have not been performed in natural populations of any mammal (e.g. rodent), including humans, to date.

Conserved Pathways to Ovarian Follicle Formation

Recent research has made clear that ovary formation is a directed, active process rather than passive, default development (Mayo et al., 2007; Ottolenghi et al., 2005; Ottolenghi et al., 2007b; Yao, 2005b). Integrated gene expression cascades result in ovary-specific morphology and interruptions of these cascades results in malformation (Crain et al., 2008; Pepling, 2006) or even female-to-male sex reversal (Ottolenghi et al., 2007a). Many of these ovary-specific factors (i.e. Foxl2, Wnt4, Gdf9, and follistatin) are conserved across vertebrates and perform similar gonadal functions (Hudson et al., 2005; Vizziano-Cantonnet et al., 2008), though often dictated by different sex determination mechanisms (Modi and Crews, 2005b; Yao and Capel, 2005) and substantial differences in expression timing and dosage may occur (Yao and Capel, 2005). Adult vertebrate ovaries show many similarities in structure and function, though some clear differences in ovary differentiation exist between vertebrates. Turtle and mouse bipotential gonads display differing primitive sex cord structures and initial locations of germ cells, possibly due to differing migration routs (Yao et al., 2004a). Comparing steroidogenic cell development between embryonic chicken to mouse ovaries, it has been observed that these cells differentiate and migrate from the nephrogenous mesenchyme in both species, however this differentiation and migration is much earlier in chickens (Sekido and Lovell-Badge, 2007). Therefore, while many conserved processes of ovarian development and maturation are often directed by similar molecular mechanisms across vertebrates and result in similar morphological maturation of the ovaries, exceptions and variations do exist. Compared to the regulation of folliculogenesis, the molecular mechanisms controlling primordial follicle formation are still poorly understood (da Silva et al., 2004; Maheshwari and Fowler, 2008; Mayo et al., 2007).

An ovarian follicle, the functional unit of the female gonad, is composed of a germ cell, the oocyte, surrounded by somatic cells, an inner layer of granulosa cells and an outer layer of thecal

cells. The surrounding somatic cells both produce and respond to hormones, participate in regulation of follicular development, nurture oocytes maturation, and ultimately impact the fertility of the ovary. Oocyte formation and follicular assembly is highly conserved among vertebrates (Matova and Cooley, 2001; Pepling, 2006; Pepling et al., 1999) and is bidirectionally coordinated between somatic and germ cells, as observed with the involvement of cell-cell Notch signaling (Trombly et al., 2009b). During early mitotic proliferation, oogonia in the embryonic ovary multiply with incomplete cytokenesis forming cytoplasmically-interconnected clusters called germ line cysts or nests (Gondos and Zamboni, 1969; Pepling, 2006). These interconnected nests are well established in mammals and birds (Ukeshima and Fujimoto, 1991) and are the sites where oogonia enter meiosis, becoming oocytes. Within defined, developmentally conserved steps there is a precipitous germ cell loss through apoptosis after which the remaining oogonia are enveloped by pre-granulosa cells (McNatty et al., 2000). During normal follicle assembly, pre-granulosa cells initially surround oocyte nests (Weakley, 1967). These nests still possess intercellular bridges between oocytes. Oocytes progress through prophase I of meiosis until arresting at diplotene stage. Finally, monolayers of granulosa cells envelop single oocytes as intercellular bridges break, forming primordial, uniovular follicles (Mazatid et al., 2005; Pepling and Spradling, 2001). A majority of oocytes undergo apoptosis rather than form follicles and this programmed cell death could be required for follicle assembly (Pepling, 2006). During further maturation, steroidogenic thecal cells later encircle the follicle. Thus, an elevated frequency of ovarian MOFs following follicle formation marks a failure of cell-cell interactions and the breakdown of oocyte nests during this critical developmental window.

Activins and Inhibins

Activins and inhibins, extracellular polypeptide ligands, are members of the structurally related transforming growth factor-β (TGFβ) superfamily, which also includes the bone morphogenic proteins (BMPs), Mullerian inhibiting substance (MIS; anti-Mullerian hormone, AMH) and growth differentiation factor 9 (GDF-9). These secreted proteins can function via endocrine or paracrine signaling. During embryonic development, TGF superfamily members exhibit a broad spectrum of biological activities in regulating cell proliferation, differentiation, and apoptosis (da Silva et al., 2004). Members of this family are assembled from large precursor proteins that are processed and assembled into mature dimers. Activins are dimers of two β subunits, βA or βB, assembled into three possible activin dimers: activin A (βA + βA), activin B (βB + βB), or activin AB (βA + βB). Inhibins are heterodimers of one β subunits and a unique α subunit to produce two isoforms: inhibin A (α + βA) or inhibin B (α + βB). Within the normal female reproductive axis, inhibins act at the pituitary and the ovary. The inhibin α subunit and mature inhibin dimers are produced by the ovarian granulosa cells (Weng et al., 2006), whereas the β subunits and activins are produced by somatic and germ cells of the ovary (Pangas and Woodruff, 2000).

Activins and inhibins were first identified as opposing non-steroidal gonadal hormones regulating follicle-stimulating hormone (FSH) synthesis and secretion from the gonadotrope (Woodruff, 1999). Since their initial discovery, activins and inhibins have also been characterized as paracrine factors within the ovary, modulating follicle growth and steroidogenesis. The actions of activins are regulated, in part, by the activin-binding protein follistatin, that sequesters activin and initiates its degradation (Thompson et al., 2005). Follistatin is produced in granulosa cells and regulates local paracrine and autocrine actions of

activin (Knight, 1996). The majority of circulating activin is bound by follistatin, suggesting that many if not all, of the actions of activins are paracrine or autocrine in nature. This is in contrast to inhibin, which acts as a traditional endocrine hormone between the ovary and the pituitary, in addition to having paracrine effects within the ovary.

The inhibitory action of inhibin on activin has been explained by competitive binding of inhibin to the type II receptor of activin (ActRII), but the low affinity binding of inhibin to the type II receptor does not explain its action when activin and inhibin are present in equal amounts (Martens et al., 1997). However, the TGF-β type III receptor, betaglycan, has been shown to promote a high affinity association between inhibin and ActRII in mammals, thereby providing a mechanism of activin antagonism even at low concentrations of inhibin (Lewis et al., 2000; Makanji et al., 2008). The production of α subunits alone could also limit activin signaling by way of competing for β subunit monomers and therefore limiting activin production. (Antenos et al., 2008).

Inhibins, Activins, Estrogens, Follicle Stimulating Hormone, and Follicle Assembly

Given the induction of MOFs following transgenetically-induced over expression of inhibin α subunit, we have hypothesized that environmental contaminants with estrogenic action disrupt estrogen-inhibin/activin signaling during follicle assembly, leading to the formation of MOFs. During normal follicle assembly, fetal ovaries in humans (da Silva et al., 2004) baboons (Billiar et al., 2003), and chickens (Bruggeman et al., 2002; Onagbesan et al., 2004; Safi et al., 2001) preferentially express activin β subunits rather than the inhibin α subunit. In neonatal mice, circulating inhibin B levels drop during follicle formation, putatively increasing the ovarian activin to inhibin ratio (Weng et al., 2006). Multioocytic follicles are also observed in conditional activin βA-null mouse ovaries, but not in βB-null mouse ovaries. Therefore, this

malformation is more likely linked to βA associated signaling cascades (Trombly et al., 2009a). However, mice lacking both βA and βB activin subunits still produce early stage follicles implying that activins promote, but are not necessary for follicle assembly (Pangas et al., 2007).

Baboon ovaries during late gestation do not show inhibin immunoreactivity in the granulosa cells of primordial follicles, nor is the inhibin receptor, betaglycan, expressed. Inhibin subunit α mRNA is detectable in the ovary, however inhibin protein is not. Paradoxically, when depleted of estrogens through use of an aromatase inhibitor, same stage ovaries display an eight fold inhibin immunoreactivity increase in both pregranulosa cells around germ cell nests and granulosa cells of primordial follicles concomitant with reduced follicle assembly (Billiar et al., 2003). Normal follicle assembly can be rescued with the administration of E_2, even in the presence of an aromatase inhibitor. Altered inhibin α subunit expression has been associated with ovarian pathogenesis through direct autocrine/paracrine mechanisms. Importantly, these data suggest that estrogen regulates the encapsulation of oocytes by follicular cells by regulating the inhibin:activin ratio and that it is not the absolute presence or absence of an estrogenic signal that leads to a pathological expression of inhibin, rather that the correct 'dose' is seen by the ovary as an absence of estrogens as well as the presence of excessive estrogens appears to lead to abnormal inhibin expression and improper follicle assembly (Pepe et al., 2006). Similarly, in neonatal hamster ovaries *in vitro,* elevated E_2 dosages showed increased somatic cell apoptosis, whereas when deprived of E_2 signaling *in vivo,* through the use of the estrogen receptor antagonist ICI 182,720, apoptosis also increased and follicle assembly was reduced (Wang and Roy, 2007). Again, these data demonstrate that exposure to an appropriate E_2 dose is needed for proper ovarian development.

Follicle formation occurs during the first five post-natal days in neonatal mice and estradiol and progesterone have been demonstrated to inhibit primordial follicle assembly and primordial to primary follicle development *in vitro*, putatively through limiting oocyte apoptosis rates and impeding nest breakdown (Kezele and Skinner, 2003). Neonatal E_2 or DES exposure for 5 days after birth generated MOFs in mice (Iguchi et al., 1990; Iguchi and Takasugi, 1986) with exposed mice expressing MOFs at a rate 120 – 340 times greater than controls. Treatment exposure must begin within three days of birth in mice to increase the formation of MOFs, for females exposed to DES treatment starting five days after birth were not different from controls; thus, a critical window exists during the development of the ovary (Iguchi and Takasugi, 1986). The expression of MOFs increased linearly in neonatally DES-treated mice from 10 to 30 neonatal days. Non-metabolizable progestins also impede follicle assembly, indicating that progestin affects are direct and not the result of acting as a substrate to synthesize other steroids (Chen et al., 2007). Further, estrogens and progestins inhibit nest breakdown and follicle assembly in an additive manner. Neonatal treatment with aromatizable androgens increased MOFs, a result not observed with treatment of the non-aromatizable androgen DHT (Iguchi et al., 1988). Additionally, co-treatment with an aromatase inhibitor and testosterone resulted in fewer MOFs than testosterone alone. Therefore, androgens can be aromatized to an estrogen and induce MOFs. Taken together, these studies show that normal steroidogenic signaling, estrogens and progestins, appropriately modulates follicle formation whereas elevated steroid signaling abnormally slows nest breakdown and induces MOFs. It has been hypothesized that the decrease of maternally derived steroids, in post-partum mice, initiates follicle formation in mice and rats (Chen et al., 2007; Kezele and Skinner, 2003; Pepling, 2006). If sex steroids regulate follicle formation in alligator ovaries, they do not have direct maternal endocrine communication during

their in ovo development. Alligator eggs have a megalecithal yolk containing both maternally derived nutrients (Speake and Thompson, 1999), steroids (Elf, 2003) and environmental contaminants (Heinz et al., 1991a) which are taken up by the embryo during development. We hypothesize that the depletion of these stores could influence ovarian dynamics and follicle assembly.

Over the last decade, a growing societal trend has promoted the use of dietary supplements with 'all natural estrogens' of plant origin, the phytoestrogens. Although the beneficial or detrimental aspects of the use of these compounds in perimenopausal women is still under study, use of these compounds by pregnant women and or infants could be of concern based on several recent studies. Neonatal treatment (days 1-5) of mice with the phytoestrogen geinstein resulted in a dose dependant increase in MOFs with females exhibiting up to four oocytes per follicle (Chen et al., 2007; Jefferson et al., 2002). Genistein treatment induced MOF's in ESR1 (ERα) knock-out mice, but not in ESR2 (ERβ) knock-out mice (Jefferson et al., 2002). Furthermore, the effect of DES inducing MOFs has been shown to be mediated through ESR2 (Kirigaya et al., 2004) and associated with an overall suppression of follicle development in mice (Kim et al., 2009b), thus implicating a common ESR2 mediated mechanism to genistein and DES induced MOF formation. However, other researchers have found MOFs induced with both ESR1 and ESR2 ligands (Nakamura et al., 2008). Ovarian ESR2 expression is exclusively expressed in rat and mouse granulosa cells (Lenie and Smitz, 2008; Sar and Welsch, 1999) and folliculogenesis proceeds normally in ESR1 knockout mice up to the preantral stage (Schomberg et al., 1999), but ESR2 knockout mice show decreased fertility (Dupont et al., 2000) and reduced primary follicle numbers (Emmen et al., 2005). Therefore, the mechanism of MOF induction by geinstein appears to be an ESR2-granulosa cell mediated event, but follicle formation is not abolished in

ESR2 knockout mice so ESR2 is not required for follicle formation. While the etiology of MOF formation is becoming clearer, even in light of the complexity of ovarian regulatory mechanisms, many other pathways leading to this morphology may exist and defining a simple pathway may not be likely. For example, the involvement of non-nuclear, membrane bound estrogen receptors in estrogen mediated MOF formation has not been investigated (Revankar et al., 2005). These findings do not preclude activins and inhibins being a crucial part of an E_2 modulated, downstream mechanism as "suggest(ed) that estrogen regulates fetal ovarian folliculogenesis by controlling, in a cell-specific manner, α subunit expression and thus the ratio of activin to inhibin" (Billiar et al., 2003). Putatively, estrogenic activity by way of ESR2 modulates the activin to inhibin ratio and thus, the overall development of the ovarian follicles.

Recent evidence of activin interaction between the establishment of the follicle pool and also steroid signaling pathways has bolstered this hypothesis. When activin was administered to neonatal mice during the period of oogonial nest break down and follicle assembly, primodial follicle number was increased by 30% by way of somatic and oogonia proliferation (Bristol-Gould et al., 2006). Activin induces ESR1 and ESR2 mRNA expression and protein in cultured granulosa cells in a dose dependent manner (Kipp et al., 2007c). This, in turn, enhanced estrogen action in the ovary. Conversely, follistatin was demonstrated to decrease ESR2 mRNA levels. Ovarian estrogen receptor mRNA and protein levels are decreased in a transgenic mouse that over expresses the inhibin α subunit, resulting in decreased activin levels. ESR1 decreased primarily in theca, whereas ESR2 decreased in granulosa. These results lead to the identification of an activin-estrogen signaling regulatory circuit. This was further supported by demonstration that both neonatal treatment of E_2 or DES decreased activin β subunit expression and promoter activity, concomitant with decreased antral follicle production and increased MOF formation

(Kipp et al., 2007b). The identification of this integrated signaling circuit may prove crucial to understanding the basis of MOF formation.

Concomitant with E_2 or DES induced decreased activin signaling and MOF formation, circulating levels of follicular stimulating hormone (FSH) increase along with circulating inhibin levels (Kipp et al., 2007b). While this change in FSH levels is most likely a secondary effect of altered activin/inhibin signaling, a possible role of FSH in modulating follicle assembly is debated. Follicle stimulating hormone induces the production of inhibin α subunit (Tuuri et al., 1996), follistatin (Tuuri et al., 1996), and activin βA *in vitro* (Tuuri et al., 1996; Welt and Schneyer, 2001) and *in vivo (Welt et al., 1999a)* in mature granulosa cells. However, FSH cannot stimulate activin βB production in granulosa cells in rodents due to a lack of a cAMP response element in the activin βB subunit promoter (Dykema and Mayo, 1994; Feng et al., 1989). Activin synergizes with FSH regulating granulosa cell proliferation and differentiation and also stimulates follicle stimulating receptor (Fshr) expression in granulosa cells (Findlay et al., 2001). Both betaglycan (Liu et al., 2003) and follistatin (Michel et al., 1992) expression co-localizes in rodent ovarian cells expressing inhibin α and their expressions are up regulated by FSH and/or cAMP. Examination of granulosa cells from FSH-deficient knockout mice shows decreased expression of aromatase and inhibin/activin subunit mRNA (Burns et al., 2001). Abnormal interactions between FSH and circulating inhibin levels have been observed in adult human females with polycystic ovary syndrome (PCOS), showing increased production of Inhibin B and estradiol after an FSH challenge compared to normal ovaries (Wachs et al., 2006). It is apparent that FSH initiated signaling cascades are pivotal in the normal expression of these inhibin/activin signaling genes.

In light of this FSH directed signaling, the role of FSH in follicle assembly is unclear. When circulating FSH is neutralized with FSH anti-serum in a perinatal, prefollicular hamster, the formation of primordial follicles in impaired (Roy and Albee, 2000) and FSH augmentation of cultured hamster ovaries accelerates follicle assembly (Wang and Roy, 2004). Furthermore, FSH could control E_2 production in neonatal hamster ovaries by way of modulating aromatase expression and E_2 is vital for somatic cell survival and differentiation and normal follicle assembly (Wang and Roy, 2007). However, FSH has not been demonstrated to modulate follicle assembly in mice (Abel et al., 2000; Chen et al., 2007; Kezele and Skinner, 2003; Kumar et al., 1997), though it is necessary for female fertility. Mice display low circulating FSH levels during follicle assembly (Weng et al., 2006) and MOFs can be induced *in vitro* by DES in the absences of gonadotropins (Iguchi et al., 1990). While the ultimate role of FSH in follicle assembly is unknown, we have employed FSH treatments in the alligator as a tool to challenge ovaries and identify differences in responsiveness among females with different environmental contaminant exposure histories.

Induction of Multioocytic Follicles: A Hypothesis

Given the data presented above, we proposed the following hypothesis outlining a possible pathway to MOF formation due to environmental contaminant exposure capable of altering normal estrogenic signaling in a developing ovary (Fig 1-2). Before normal follicle assembly, appropriate concentrations of intra-ovarian steroids, including estrogens, maintain germ cell nest integrity. Within a specific developmental window, a decrease in steroid signaling allows the initiation of nest breakdown and an increase in activin production. These changes subsequently produce the critical activin milieu that modulates and stimulates (pre-)granulosa cells during follicle assembly. Elevated activins ultimately act as a negative feedback on themselves by increasing ESR expressions and increasing estrogen actions.

In post-hatchling alligators, we propose that all the elements of an estrogen-activin signaling system present and active and that these factors interact to orchestrate ovarian follicle assembly. Exogenous estrogenic factors are capable of acting on this pathway and lead to altered signaling that impedes follicle assembly, This is achieved through the actions of two possible mechanisms, Figure 1-2.

1. Environmental estrogens, from a variety of sources including various pesticides and industrial chemicals, are transferred to alligator egg yolks via maternal deposition. During embryonic and post-hatch development, these factors are mobilized from the yolk, enter circulation, and influence the developing ovary.

2. These environmental factors either alter or augment endogenous estrogenic signaling

3. At the ovary, these estrogenic factors interact with perinuclear estrogen receptors that act as DNA transcription factors altering normal gene expression, resulting in abnormal elevated expression levels of two activin-inhibitory factors: inhibin α subunit and follistatin.

4. Increased inhibin α subunit expression leads to an increase of gonadal inhibins, which in turn, alter the ovarian inhibin:activin ratio.

5. Inhibins act as local activin antagonists by competing with activins at their membrane receptors, facilitated by the inhibin co-receptor betaglycan. This decreases appropriate activin signaling in (pre-) granulosa cells.

6. Increased follistatin expression acts as a local activin antagonist by binding and inactivating extracellular activin molecules, again decreasing appropriate activin signaling in (pre-) granulosa cells.

7. Inhibition of activin signaling activity results in altered follicle assembly by way of impeded (pre-) granulosa cell proliferation and maturation.

8. Additionally, FSH signaling may modulate this pathway by altering activin, inhibin, follistatin, and aromatase expressions.

Research Goals

Our goals were to understand the normal timing and morphological changes associated with alligator follicle assembly and to investigate differences in follicle assembly related signaling in ovaries from female alligators from environments with differing exposures to contaminants. Furthermore, while the role of FSH in follicle assembly is unclear, we employed

FSH challenges to assess their effects on morphology and gene expression responsiveness in ovaries from animals of various ages and from environments with differing contamination levels. The synthesis and analysis of these results have produced a better understanding of alligator ovary development and of the conditions underlying the phenomena of MOF formation.

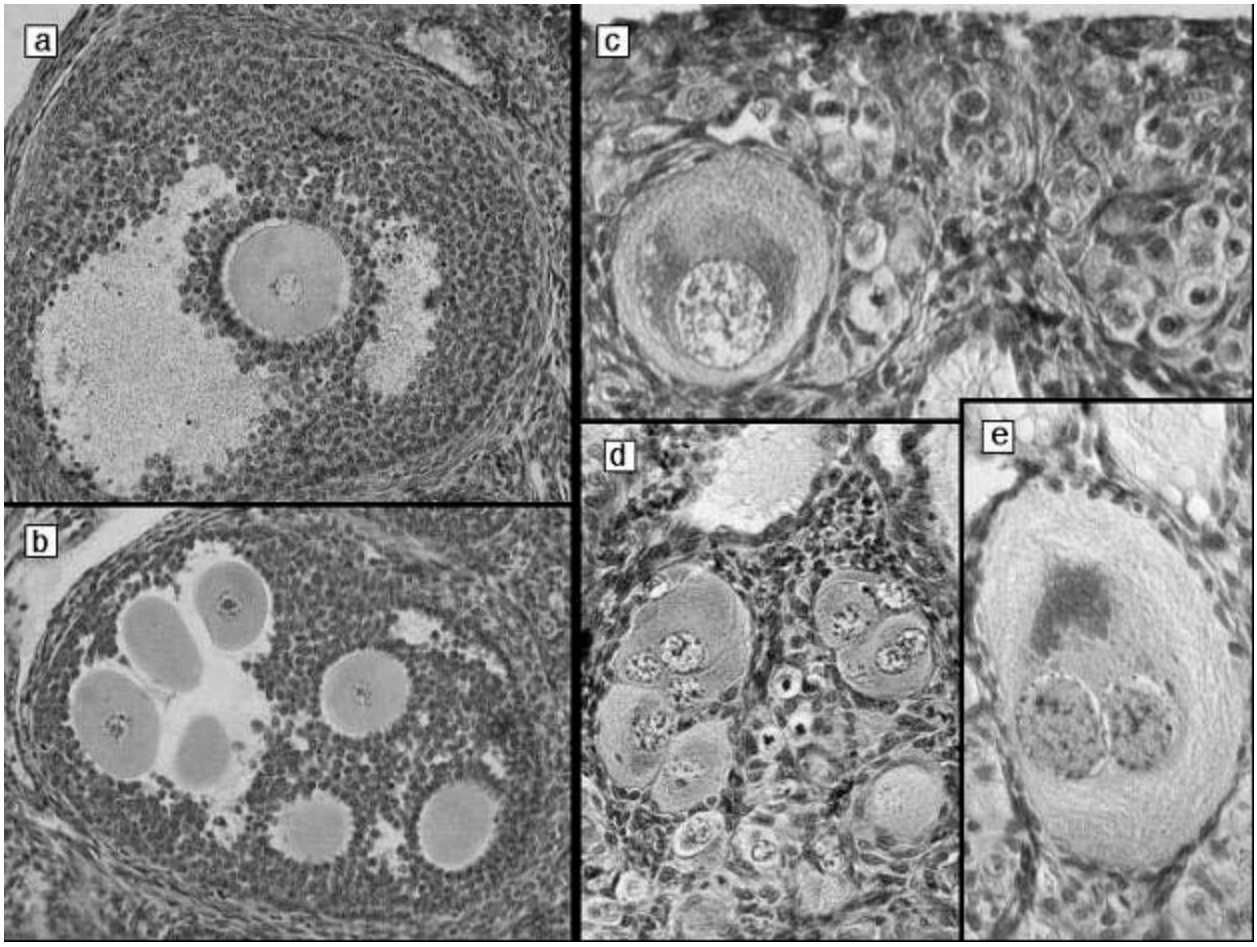

Figure 1-1. Mouse and alligator multioocytic follicles. A) Normal mouse follicle. B) Multioocytic mouse follicle. C) Normal alligator follicle. D) Multioocytic alligator follicle. E) Multinucleated alligator oocytes.

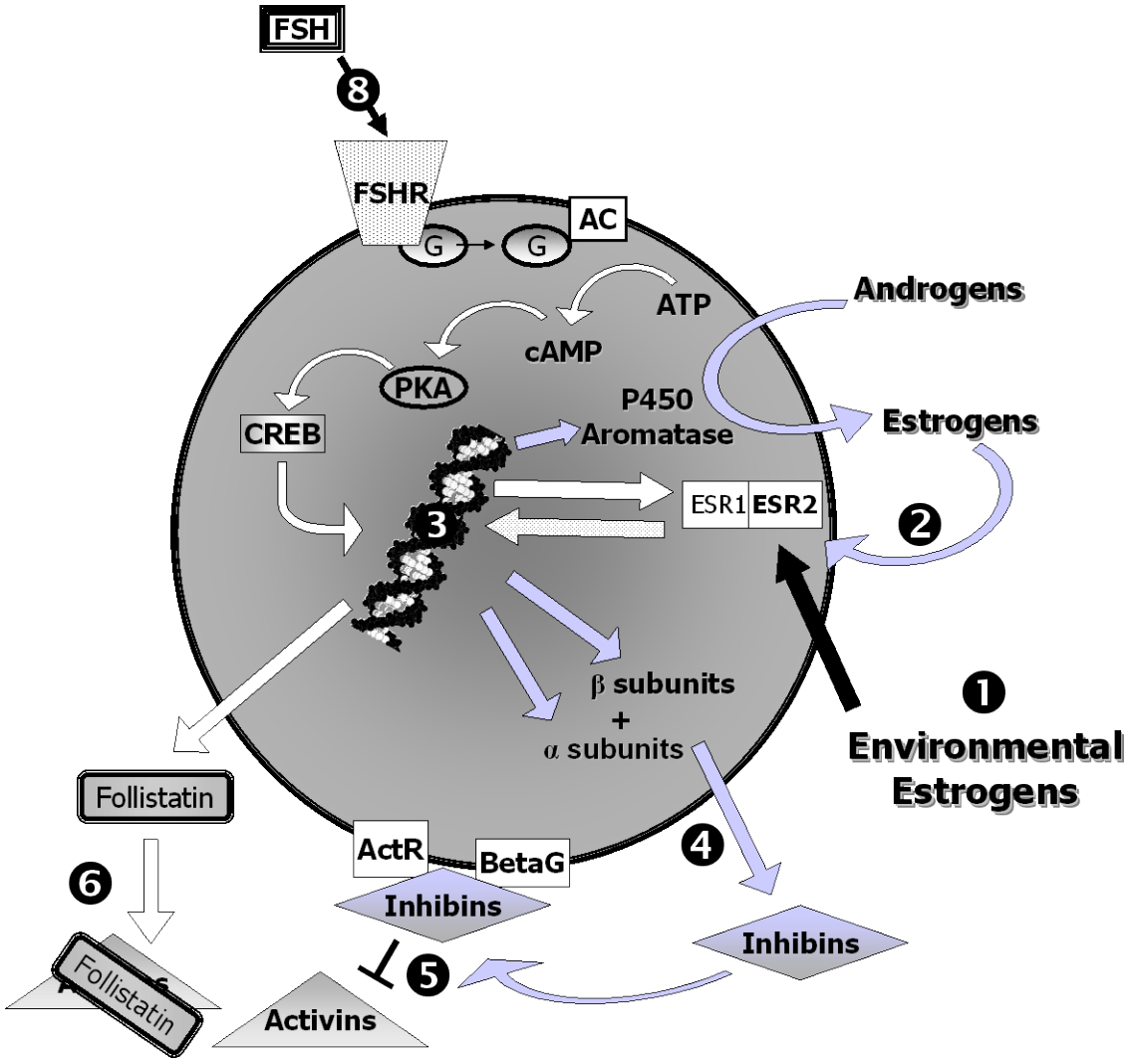

Figure 1-2. Proposed mechanism of MOF formation.

CHAPTER 2
INFLUENCES OF SEX, INCUBATION TEMPERATURE, AND ENVIRONMENTAL QUALITY ON GONADAL ESTROGEN AND ANDROGEN STEROID RECEPTOR mRNA EXPRESSION IN JUVENILE AMERICAN ALLIGATORS

Introduction

Steroid hormone signaling at the cellular and molecular level is regulated by the availability of ligands, the abundance of ligand-modifying enzymes, the abundance of receptors for these ligands, and molecular response elements, such as DNA response elements and subsequently recruited cofactors. Life stage, sex, and environment modulate steroid synthesis in the gonads and receptor expression, resulting in altered endocrine signaling. Across a wide range of animals, degraded environmental quality negatively influences endocrine activities through a variety of molecular mechanism, including nuclear receptor signaling (Iguchi and Katsu, 2008). In American alligator (*Alligator mississippiensis*) gonads, we investigated the effects of sex and the environment on sex steroid receptor mRNA expression.

The expression of steroidogenic factors and enzymes, steroid receptors (Kohno et al., 2008; Milnes et al., 2008), and circulating steroid concentrations (Guillette et al., 2000b; Guillette and Gunderson, 2001; Milnes et al., 2005) were compared between juvenile alligators from Lake Woodruff National Wildlife Refuge, an area of minimal anthropogenic influence, and highly polluted Lake Apopka. These data suggest that the gonads of juvenile alligators are physiologically active, sexually dimorphic at both hormonal and molecular levels of analysis, and can be influenced by environmental factors. For instance, the testes of 13-month-old alligators expressed higher levels of nuclear receptor 5A1 (*Nr5a1*, also named *SF-1*); steroidogenic acute regulatory protein (*STAR*); cytochrome P450 11A1 (*Cyp11a1*); hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*Hsd3b1*); and cytochrome P450 17A1 (*Cyp17a1*) than the ovaries of similar age animals (Milnes et al., 2008). Conversely,

ovaries of 13-month-old females expressed greater cytochrome P450 19A1 mRNA levels (*Cyp19a1*, or alternatively aromatase) than testes. These sexually dimorphic expression patterns were observed in animals hatched from eggs collected from Lake Woodruff National Wildlife Refuge and raised in a controlled laboratory environment. Within the same study, animals hatched from eggs collected from Lake Apopka did not exhibit the same pattern of sexual dimorphism. Lake Apopka males exhibited lower relative mRNA expression of *Nr5a1* and *STAR* compared to Lake Woodruff males, and no difference in relative abundance of these transcripts was detected between Apopka males and Apopka females. In addition, expression of *Cyp11a1* and *Hsd3b1* mRNAs were not different between Lake Apopka males and females. This lack of sexual dimorphism was associated with greatly increased post-hatching mortality in Lake Apopka animals, when compared to Lake Woodruff. These results support the hypothesis that the effects of poor environmental quality experienced by the mother can be transmitted through her eggs and thus, to her offspring who have never been directly exposed to the environmental contaminants.

Recently, our laboratory reported sexually dimorphic expression of estrogen receptor mRNA in the gonads of wild caught juvenile alligators in Lake Woodruff (Kohno et al., 2008). Juvenile alligators from Lake Apopka did not exhibit sexually dimorphic expression of estrogen receptor mRNA. It was unclear, however, if the absence of sexual dimorphism resulted from post-hatching environmental exposure to xenobiotics or developmental effects resulting from differences in egg incubation temperature or maternal contribution to the embryonic environment.

In the current study, we address a series of fundamental questions regarding the effects of egg incubation temperature, sex, and environmental quality on the expression of sex steroid

receptor mRNA in the gonad. The sex of all crocodilians is determined by incubation temperature during a critical window of embryonic development. Previous studies using eggs from these populations have shown that 30°C incubation produces only females, 33.5°C produces only males, and 32°C produces both males and females (Ferguson and Joanen, 1983). First we ask, what are the proportions of estrogen receptor α (*Esr1*), estrogen receptor β (*Esr2*) and androgen receptor (*Ar*) mRNA expression within gonads of each sex? Second, how do these proportions vary between ovaries and testes produced at different temperatures versus ovaries and testes produced at the same temperature? Third, we compare steroid receptor expression between ovaries and testes of alligators hatched from eggs collected at sites of differing environmental quality.

Finally, to expand our understanding of steroid signaling in these juvenile animals, we address associations between the mRNA expression of steroid receptors, regulators of steroidogenesis, and steroidogenic enzymes. To this end, we employ cluster analysis to identify novel, multivariate patterns in the data sets (Hand and Heard, 2005). This technique suspends the use of experimental independent variables and finds inherent patterns of mRNA expression in the data set, and forms de novo groups based on similarities in gene expression profiles. These de novo groupings of similar individual animals can then be employed to define and statistically test relationships not apparent when animals are clustered by experimentally defined independent variables alone. These patterns can then be used to understand a "gene expression landscape" within the experiment and to identify variability in response or "plasticity" found in various gene expressions.

Materials and Methods

Experimental Design and Animal Care

All fieldwork was conducted under permits from the Florida Fish and Wildlife Conservation Commission and the US Fish and Wildlife Service (Permit #WX01310). All work involving alligators was performed under the guidelines specified by the Institutional Animal Care and Use Committee at the University of Florida. Egg collection, handling, and incubation methods have been previously published in detail (Milnes et al., 2004b; Milnes et al., 2008). In brief, complete clutches of eggs were collected soon after oviposition from Lake Apopka and Lake Woodruff National Wildlife Refuge, Florida, USA. At least one egg per clutch was opened to confirm the embryonic stage of development according to published criteria (Ferguson, 1985). 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 eggs only from Lake Woodruff, in which 13 eggs were incubated at a female producing temperature, 30°C, and 17 eggs were incubated at a male producing temperature, 33.5°C. These groups were assembled from nine different clutches with a maximum of three eggs from any clutch at either incubation temperature. The imbalance in sample size 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 (Milnes et al., 2008).

Incubation of Lake Woodruff eggs at 30°C and 33.5°C resulted in 92% and 94% hatch rates, respectively. Hatching success and post-hatching mortality from eggs incubated at 32°C

has previously been published (Milnes et al., 2008). In summary, 90% of eggs from Lake Woodruff and 80% of eggs from Lake Apopka hatched. After hatching, alligators were web-tagged and 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. Sex, as determined by visual inspections of gonad morphology and the presence or absence of oviducts at necropsy, was true to incubation temperature expectations for all animals. At the time of necropsy, 100% (n = 12) of 30°C females and 81% (n = 13) of 33.5°C putative males survived 13 months. In the 32°C cohort, only 66% of the animals hatched from Lake Apopka eggs survived to 13 months of age, compared to 96% of animals hatched from eggs obtained from Lake Woodruff. This mortality was most extreme in females from Lake Apopka, which exhibited a 40% survival rate. At necropsy, Lake Woodruff was represented by 33 females and 19 males, whereas Lake Apopka was represented by 8 females and 19 males.

Prior to necropsy, body mass (BM), snout-vent length (SVL), and total length (TL) were measured. Condition indexes (CI) were calculated for each animal by dividing BM by SVL^3 (SVL CI) or TL^3 (TL CI). Morphometric data were log transformed to achieve homogeneous variances as needed.

Tissue Collection, RNA Isolation, and Quantitative Real-Time PCR

At necropsy, gonads were removed, flash frozen in liquid nitrogen, and stored at -80°C until RNA extraction was performed. RNA isolation and reverse transcription procedures have previously been published in detail (Milnes et al., 2008). Quantitative real-time PCR (Q-PCR) has been used to measure mRNA expression of each gene of interest in the American alligator (Gunderson et al., 2006; Katsu et al., 2004; Kohno et al., 2008), and primer sequence information, annealing temperatures, and accession numbers are reported in Table 2-1. Q-PCR

of steroid receptors (*Esr1*, *Esr2*, and *Ar*) was performed in the MyiQ single color detection system (BioRad, Hercules, CA) following manufactures 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 steroid receptor 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. Measurements of steroidogenic factors (*Nr5a1* and *STAR*) and steroidogenic enzymes (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*, and *Cyp19a1*) in these animals were performed using relative standard curves of serially diluted cDNA as previous reported (Milnes et al., 2008). Values for each measured steroidogenic factor or enzyme mRNA expression were normalized to a mean female expression level of one. All sample means were normalized using ribosomal protein L8 (*Rpl8*) expression (Katsu et al., 2004; Milnes et al., 2008).

Statistical Analysis

JMP for windows version 7.0.1 (SAS Institute, Cary, NC) was used for all statistical analyses. Gene expression ratios were arcsin transformed to achieve homogeneous variances as needed. Significance for all tests was set at $P < 0.05$. One-way ANOVA was used to compare relative expression of steroid receptors within a gonad. Unpaired Student's *t*-tests were used to compare means from males produced at 33.5°C to females produced at 30°C. 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. Two-way ANOVA was used to compare 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.

Hierarchical/agglomerative cluster analysis of mRNA expression data was performed using Ward's linkage clustering to explore relationships in mRNA expressions between alligators of each study. Before clustering, mRNA expression data were standardized so all variables had a mean of zero and a standard deviation of one. Elbow criteria were used to determine the number of clusters from scree plots (not shown). Cluster analyses produced matrixes of relative gene expression levels presented in grey-scale, colormetric gradients with dark squares denoting high expression levels and light squares denoting low expression levels. This expression matrix is in conjunction with adjacent distance dendrograms showing relationships based on mRNA expression similarities for individual animals in the horizontal axis and hierarchical clustering of similar gene expressions between all animals in the vertical axis. Clusters derived from the analysis were used as independent variables re-examining mRNA expression data using one-way ANOVA and followed by Tukey-Kramer post hoc comparisons when applicable. Pairwise linear correlation analyses were performed to further investigate specific relationships between gene expressions and are reported along with corresponding Pearson's r values.

Results

Body Morphometrics

Body mass, snout-vent length, total length, SVL CI and TL CI were not different between 30°C females and 33.5°C males (Fig. 2-1A,C,E,G, and I). Lake Apopka animals had greater BM, SVL, and TL than Lake Woodruff at 32°C, but no difference between sexes was detected (Fig. 2-1B,D, and F; lake effect $P < 0.001$ for each measurement). While SVL CI did not vary by lake or sex, TL CI was different between both lake and sex (Fig. 2-1H,J; lake effect $P = 0.01$, sex effect $P = 0.02$).

Steroid Receptor Expression

Detectable levels *Esr1*, *Esr2*, and *Ar* mRNA expression were measured in ovaries and testes. The mRNA expression levels of steroid receptors exhibited the following pattern, *Esr1* > *Ar* > *Esr2*, in all incubation groups except Lake Apopka males where *Esr1* = *Ar* > *Esr2*. The expression of *Esr1* mRNA was not different between 33.5°C males and 30°C females, whereas *Esr2* mRNA expression was greater in ovaries than testes, and *Ar* expression was greater in testes than ovaries (Fig. 2-2A-C; P = 0.2, < 0.001, and = 0.03, respectively).

The sex and lake of origin had a significant interaction on expression of *Esr1*, *Esr2* and *Ar* mRNA (Fig. 2-2D-F, P < 0.001 for each). *Esr1* and *Esr2* mRNA expression was greater in ovary than testis (Fig. 2-2D,E; sex effect P < 0.001 for both). Gonads of Lake Woodruff animals expressed greater *Esr2* mRNA levels compared to Lake Apopka animal gonads (Fig. 2E; two-way ANOVA, lake effect P = 0.002). Conversely, Lake Apopka animal gonads expressed greater amounts of *Ar* mRNA than Lake Woodruff animal gonads (Fig. 2-2F; two-way ANOVA, lake effect P = 0.05).

Steroidogenic Gene Expression

The expression of *Nr5a1* mRNA was not different between 33.5°C males and 30°C females (Fig. 2-3A; P = 0.07), whereas expression of *STAR*, *Cyp11a1*, *Hsd3b1*, and *Cyp17a1* was greater in testes than ovaries (Fig. 2-3B-F; P < 0.001 for each). On the other hand, *Cyp19a1* mRNA expression was much greater in ovary relative to testis in all comparisons. As previously reported (Milnes et al., 2008), laboratory-raised, Lake Woodruff males from 32°C had greater expression of *Nr5a1*, *STAR*, *Cyp11a1*, *Hsd3b1*, and *Cyp17a1* compared to Woodruff females from the same temperature. Expression of *Star* was greater in Lake Woodruff males compared to Lake Apopka males, and no difference was detected between 32°C males and females from Lake Apopka for *Nr5a1*, *STAR*, *Cyp11a1*, or *Hsd3b1*.

Multivariate Cluster Analysis of Individual Animals by mRNA Expression

Cluster analysis of females and males from 30°C and 33.5°C, respectively, yielded five clusters of animals (Fig. 2-4; clusters defined by horizontal lines on cluster matrix). The first node of the dendrogram of individual alligators separated males from females. Females further divided into two clusters, whereas males divided into three clusters (Fig. 2-4, Table 2).

Analysis of variance of the transcript levels data by cluster groups and subsequent post-hoc comparisons revealed differing expression patterns among the clusters (Table 2-2). The dendrogram of mRNA expression patterns can be divided into three groups (Fig. 2-4; groups defined by vertical lines on the cluster matrix). This analysis shows that *Esr2* and *Cyp19a1* mRNA expression levels display sexually dimorphic patterns, with females exhibiting elevated mRNA expressions. *Ar* and *Esr1* mRNA expression group had roughly sex-equivalent mRNA expressions. Compared to female clusters, *Hsd3b1*, *STAR*, *Nr5a1*, *Cyp11a1*, and *Cyp17a1* mRNA levels in male clusters exhibited considerably more variation. Transcript levels in cluster #3 were similar to those present in females. Clusters #4 and #5 showed variable mRNA expression of these steroidogenic actors.

Cluster analysis of males and females from 32°C yielded four clusters of animals (Fig. 2-5; clusters defined by horizontal lines on cluster matrix). The first two nodes of the dendrogram of individual alligators segregated all male alligators except two, and included one female. Clusters #3 and #4 were predominantly female (Fig. 2-5, Table 3). Male alligators from Lakes Woodruff and Apopka were represented in relatively equal proportions in clusters #1 and #2. The majority of Lake Woodruff females allocated to cluster #4, whereas Lake Apopka females distributed relatively equally between clusters #3 and #4.

Significant differences in mRNA expressions between clusters from 32°C were also observed, with post-hoc analysis showing a variety of sex-related expression dimorphisms (Table

2-3). In these cluster analysis, sexual dimorphisms of mRNA expression defined four groups (Fig. 2-5; groups defined by vertical lines on cluster matrix). *Esr1*, *Esr2*, and *Cyp19a1* grouped as female-elevated, sexually dimorphic expressions. The female-only cluster #4 had greater *Esr1*, *Esr2*, and *Cyp19a1* mRNA expression when compared to all other clusters, including cluster #3, which is composed of 82% females. Notably, the *Esr1* mRNA expression level for female-rich cluster #3 was lower than the mostly-male cluster #2 and not different from the all-male cluster #1. Expression levels of *Ar*, *STAR*, and *Nr5a1* mRNA varied between clusters, but not in a non-sexually dimorphic manner. Compared to female biased clusters, *Hsd3b1*, *Cyp11a1*, and *Cyp17a1* mRNA expression levels were greater in the male biased clusters. These steroidogenic enzymes had similar mRNA expression patterns in which the mostly-male cluster #2 displayed the greatest expressions, followed by the all-male cluster #1, and the lowest expression levels were observed in the all-female clusters.

Discussion

Alligators lack sex chromosomes (Valleley et al., 1994) and sex is determined by the temperature experienced during incubation in ovo (Ferguson and Joanen, 1983). Therefore, each embryo putatively has an equal potential to develop either testes or ovaries that produces sex-appropriate, gene expressions. The sexually dimorphic mRNA expressions of steroidogenic factors and enzymes from 30°C or 33°C incubated gonads of Lake Woodruff animals reported here are similar to those previously reported in Lake Woodruff animal ovaries and testes incubated at 32°C (Milnes et al., 2008). The one exception is a *Nr5a1* expression dimorphism was observed in the 32°C cohort, but not the 30°C/33°C cohort. These results support a hypothesis that variation in incubation temperature does not markedly effect the establishment of sex-specific mRNA expressions within gonads of a given sex.

In contrast to sexually dimorphic expressions of steroidogenic factors and enzymes observed in these gonads, the expression of estrogen and androgen receptor mRNA in ovary and testis did not present as pronounced, sex-specific differences. Measurement of gonadal steroid receptor mRNA expression levels in 13-month-old alligators revealed relatively consistent expression patterns, regardless of sex or incubation temperature (Fig. 2-2A-F). In testis or ovary, the level of *Esr1* mRNA expression was greatest, whereas *Ar* mRNA expression was intermediate, and *Esr2* mRNA expression was lowest. The exception to this pattern was Lake Apopka males incubated at 32°C: these males exhibited equivalent *Esr1* and *Ar* mRNA. Our laboratory has previously demonstrated a similar estrogen receptor mRNA expression pattern of *Esr1* > *Esr2* mRNA in gonads of wild-caught, Lake Woodruff juvenile alligators of approximately 5-7 years of age (Kohno et al., 2008). This study is the first to report gonadal *Ar* mRNA expression levels that are intermediate in relation to those of *Esr1* and *Esr2*. In support of our findings, a recent study of newborn mouse ovaries found ESR1 expression to be ~17 times greater that ESR2(Nakamura et al., 2008).

Taking into account this hierarchical ranking of estrogen and androgen receptors expression levels, we did observe sexually dimorphic steroid receptor expressions. In Lake Woodruff animals incubated at 30°C or 33°C, *Esr2* mRNA expression was greater in ovaries than testis; however. However, *Esr1* expression was not sexually dimorphic. Similar observations of sexually dimorphic *Esr2* expression has been observed in gonads of wild-caught Lake Woodruff animals (Kohno et al., 2008). Additionally, in this study, *Ar* mRNA expression was greater in testes. In the 32°C incubation cohort, irrespective of lake of origin, these ovaries expressed greater levels of both *Esr1* and *Esr2* than testes, while *Ar* did not differ by sex. Gonads from

Lake Woodruff animals, irrespective of sex, expressed higher levels of *Esr2* mRNA, whereas Lake Apopka gonads expressed higher levels of *Ar* mRNA.

Our findings support a hypothesis that sex, differing incubation temperatures, and environmental quality may influence steroid receptor expression, though to a lesser degree than to the relative overall *Esr1*, *Esr2*, and *Ar* levels observed in both ovary and testis. On average, estrogen receptor mRNA expressions were greater in ovaries than testes. Androgen receptors expression was sexually dimorphic between 30°C and 33.5°C gonads, but not between 32°C gonads. The elevated *Ar* and decreased *Esr2* mRNA expression levels in Lake Apopka gonads, in relation Lake Woodruff gonads, implies that the environment effects steroid receptor expressions in both ovaries and testes in a similar manner, possibly through similar mechanisms. The environmental exposures of these 13-month-old animals were only through maternal contribution to the egg, the oviducal environment, and a brief exposure to the nesting materials. Therefore, differences in mRNA expressions observed at dissection are contingent on factors present well before these tissues were collected and may be organizational alterations with long-term impacts on gonadal functioning (Guillette et al., 1995).

In this study, we measured receptor expressions from gonad homogenates. Currently, the distribution of these steroid receptors within alligator gonads is unknown; therefore, relatively small dimorphic expressions from whole gonad homogenates may translate into larger differences in expression in specific gonad compartments or cell types. In light of this provision, we propose that both estrogen and androgen signaling is active and equally necessary for appropriate function of juvenile alligator ovary and testis.

Literature evaluating steroid receptor expressions between sexes from non-mammalian vertebrate studies is sparse and usually focused on embryonic development (Ramsey and Crews,

2007; Rhen et al., 2007a). AR immunoreactivity has been observed in granulosa, theca, and fibroblast cells of chicken ovarian follicles (Yoshimura et al., 1993) and in Sertoli, Leydig, and myloid cells of chicken and duck testis (Dornas et al., 2008). During development, *Ar* mRNA is expressed in the left chicken ovary at higher levels than testis and *Ar* levels increase in both ovary and testis prior to hatching (Katoh et al., 2006). Antagonism of androgen signaling in embryonic chicken ovary by flutamide treatment results in a disorganized cortex; proper ovarian development is rescued by co-treatment with either testosterone or estradiol(Katoh et al., 2006). Flutamide treatment was shown to decrease aromatase expression, leading to a hypothesis that androgen-*Ar* signaling may regulate aromatase expression in the embryonic ovary. In post-hatchling quail, turkey, duck, and goose gonads, *Esr1* mRNA expression is not sexually dimorphic, in spite of concomitant ovarian aromatase expression orders of magnitude greater than testis expression (Koba et al., 2008a; Koba et al., 2008b). In light of this, our finding of a correlation between *Ar* (but not *Esr1* or *Esr2*) and sexually dimorphic *Cyp19a1* mRNA expression in the 32°C incubation ovaries is notable.

In mammals, the roles of both estrogens and androgens in maintaining gonadal health, regardless of sex, is becoming more apparent. In neonatal mouse testes, inactivation of Esr2 increases the number of gonocytes and testosterone production, while an inactivation of Esr1 hypertrophies Leydig cells and increases the expression of steroidogenic enzymes (Delbes et al., 2006). In contrast, neonatal estradiol benzoate treatment of rats alters testicular steroid receptor expression during subsequent postnatal/peripubital development, where mRNA expressions of *Esr1* and *Ar* decreases and *Esr2* increases (Tena-Sempere et al., 2000). Recent reviews highlight a growing understanding of the role androgen receptor activity plays in enhancing mammalian ovarian follicle development (Matsumoto et al., 2008; Walters et al., 2008). Female mice that

are androgen receptor deficient are subfertile, have impaired folliculogenesis, show accelerated follicle depletion similar to premature ovarian failure, and reduced levels of specific growth factors (Matsumoto et al., 2008; Shiina et al., 2006; Walters et al., 2008). Conversely, appropriate estrogenic signaling is vital to the establishment and maintenance of testis health (Akingbemi, 2005; Delbes et al., 2006; O'Donnell et al., 2001; Sierens et al., 2005). Adult Esr1 knockout mice have impaired spermatogenesis which is marked by decreased numbers of developing germ cells, decreased testis weights, larger Leydig cells, and increased levels of both circulating and testicular testosterone (Gould et al., 2007). Additionally, testes of Esr2 knockout adult mice have increased numbers of smaller Leydig cells and increased numbers of spermatagonia per testis. In summary, this research supports that both estrogen and androgen signaling play necessary roles in ovary and testis alike and demonstrates that extreme sexual dimorphic steroid receptor expressions in juvenile alligator gonads should not be expected.

If levels of steroid receptors are not expressed in highly sexually dimorphic manners, but other steroidogenic enzymes such as *Cyp19a1* and *Cyp11a1* show more pronounced sexually dimorphic expressions, what associations can be found both within and between sex steroid receptor and steroidogenic factor and enzyme expressions? Cluster analysis revealed that these expression patterns are more complex than can be explained simply by sex or environment. In cluster analyses mRNA expression data from both of the experimental groups, almost all males and females segregated at the earliest stages of hierarchical clustering. Subsequently, males and females segregated into primarily same-sex clusters of similar gene expression patterns. Analysis of mRNA expression levels across these clusters showed that only *Cyp19a1* expression is uniformly sexually dimorphic. In comparison, the steroidogenic factors, enzymes, and sex steroid receptors investigated here showed expression patterns that, when compared between

clusters, range from partial-sexual dimorphisms to no correlation between sex and gene expression. Expressions of steroidogenic enzymes *Hsd3b1*, *Cyp11a1*, and *Cyp17a1* were greater in all the male clusters of the 32°C incubation cohort, however, expression levels of these enzymes in some the 30°C/33°C cohort male clusters were equivalent to female expression levels. Pro-steroidogenic factors *Nr5a1* and *STAR* showed the highest expression in some male clusters, but the expression levels in other male clusters of both incubation cohorts were equivalent to female expression levels. Therefore, elevated *Cyp19a1* expression is a clear steroidogenic marker of ovary, while elevated *Hsd3b1*, *Cyp11a1*, and *Cyp17a1* mRNA expressions is indicative, but not diagnostic of testis. Pro-steroidogenic factors *Nr5a1* and *STAR* expression is male-biased, but displays substantial expression variability.

When examined across clusters, *Esr2* showed greater levels of sexual dimorphism and mRNA expression patterns more similar to *Cyp19a1* expression than *Esr1* expression in both incubation cohorts. A female cluster always showed the highest expression levels of *Esr1* or *Esr2*, however, *Esr1* and *Esr2* expression levels were equivalent between some predominantly male and female clusters. *Ar* mRNA expression patterns grouped with *Cyp19a1* in 30°C/33°C cohort, but more closely with *Hsd3b1*, *Cyp11a1*, and *Cyp17a1* in 32°C the incubation cohort. Furthermore, *Ar* mRNA expression did not display sexually dimorphic expression between male and female clusters in both studies. Therefore, while *Esr* expressions trended toward higher expression in ovaries, *Ar* mRNA expression showed greater intra-sex variability than inter-sex variability.

Here we demonstrated that both ovary and testis show a similar relative mRNA expression pattern of steroid receptors in which *Esr1* > *Ar* > *Esr2*. However, both sex and environment can modulate this expression pattern. Elevated *Cyp19a1* and *Esr2* mRNA expressions are more

diagnostic of alligator ovary, while elevated *Hsd3b1*, *Cyp11a1*, and *Cyp17a1* mRNA expressions are most indicative of alligator testis. Other gonadal mRNA expressions, such as *Ar*, *Esr1*, and *Nr5a1*, show variable expression patterns that are not entirely associated with sex.

Table 2-1. Steroid receptor quantitative real-time PCR primers

Gene	Forward Primer (5' - 3') Reverse Primer (5' - 3')	Anneal Temp (C^o)	Product size (bp)	Accession #
ESR1	AAGCTGCCCCTTCAACTTTTTA TGGACATCCTCTCCCTGCC	66.5	72	AB115909
ESR2	AAGACCAGGCGCAAAAGCT GCCACATTTCATCATTCCCAC	65.0	72	AB115910
AR	TGTGTTCAGGCCATGACAACA GCCCATTTCACCACATGCA	67.5	103	AB186356

Table 2-2. Steroidogenic factor and steroid receptor transcript expression cluster ANOVA from 30°C and 33.5°C incubation animals. P-values and Tukey-Kramer HSD post-hoc comparisons of mRNA expression data by clusters derived from hierarchical/agglomerative cluster analysis in figure 2-4 (left) and distribution of sex and numbers of animals across clusters (right).

	mRNA Transcript									Sex	
	Hsd3b1	***STAR***	***Nr5a1***	***Cyp11a1***	***Cyp17a1***	***Ar***	***Esr1***	***Esr2***	***Cyp19a1***	**Male**	**Female**
ANOVA P	<0.001	<0.001	<0.001	<0.001	<0.001	=0.047	<0.001	=0.002	<0.001		
	Tukey-Kramer HSD										
Cluster 1	B,C	C	B	B	C	A,B	A	A,B	A	-	3
Cluster 2	C	C	B	B	C	B	B	A	A	-	9
Cluster 3	B,C	B	B	B	C	A	B	B	B	4	-
Cluster 4	B	B,C	B	A	A	A,B	B	B	B	4	-
Cluster 5	A	A	A	A	B	A,B	B	B	B	5	-

Table 2-3. Steroidogenic factor and steroid receptor transcript expression cluster ANOVA from 32°C incubation animals. ANOVA P-values and Tukey-Kramer HSD post-hoc comparisons of mRNA expression data by clusters derived from hierarchical/agglomerative cluster analysis in figure 2-5 (left) and distribution of sex, lake of origin, and number of animals across clusters (right).

	mRNA Transcript									Sex			
	Ar	***Cyp11a1***	***Hsd3b1***	***Cyp17a1***	***Nr5a1***	***STAR***	***Esr1***	***Esr2***	***Cyp19a1***	**Male**		**Female**	
ANOVA P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
	Tukey-Kramer HSD									**Woodruff**	**Apopka**	**Woodruff**	**Apopka**
Cluster 1	B	B	B	B	B	B	B,C	B	C	10	12	-	-
Cluster 2	A	A	A	A	A	A	B	B	C	6	7	-	1
Cluster 3	C	C	C	C	B	C	C	B	B	2	-	6	3
Cluster 4	A	C	C	C	B	B,C	A	A	A	-	-	24	4

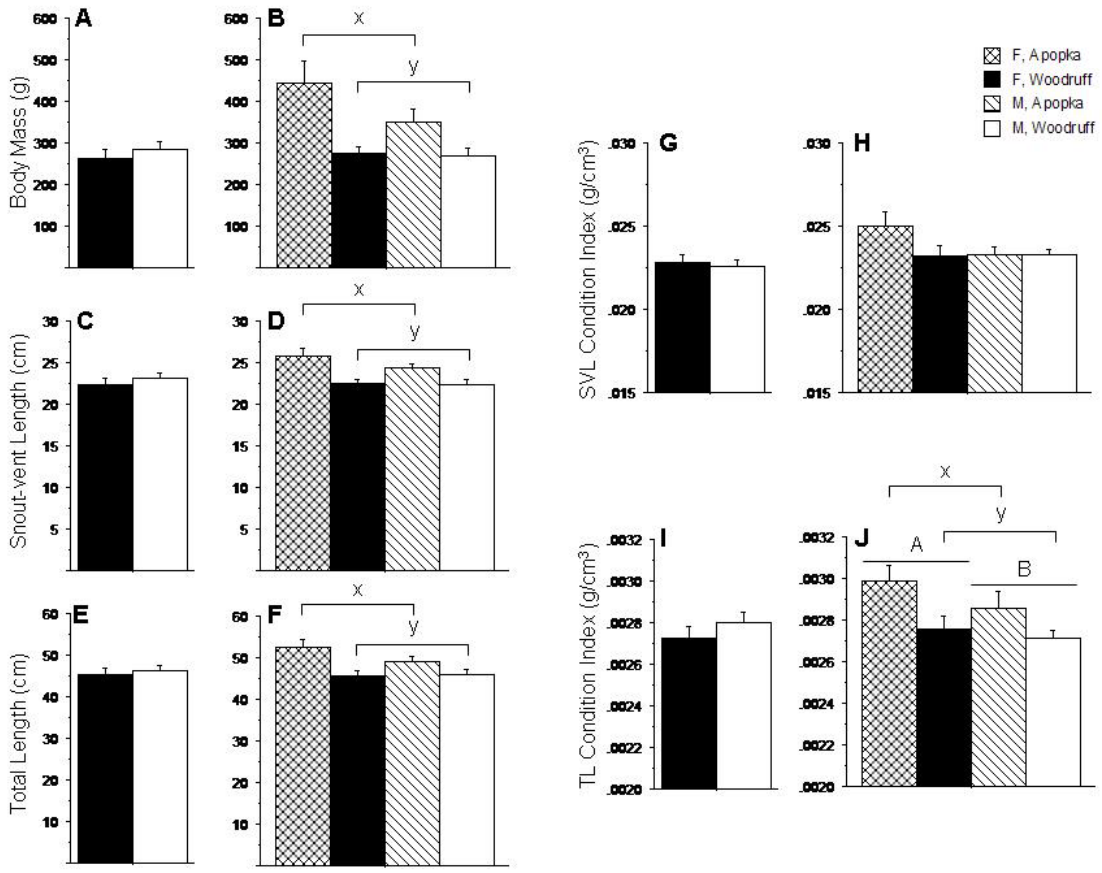

Figure 2-1. Body morphometrics measurements from 13-month-old alligators: 30°C/female or 33.5°C/male incubations (A, C, E, G, and I) and 32°C incubation (B, D, F, H, and J) temperatures. Crosshatched bars = Lake Apopka females, black bars = Lake Woodruff females, diagonal lined bars = Apopka males, white bars = Lake Woodruff males. Bars represent means (±SEM) for body mass-BM (A, B), snout-vent length-SVL (C, D), and total length-TL (E, F). Condition indexes (body mass/length measurement^3) calculated using either SVL (G, H) or TL (I, J). Lower case x,y over bars denotes significant ($P < 0.05$) for two-way ANOVA analysis showing difference between lake of origin, Apopka vs. Woodruff. Upper case A, B over bars denotes a significant sex dimorphism by two-way ANOVA.

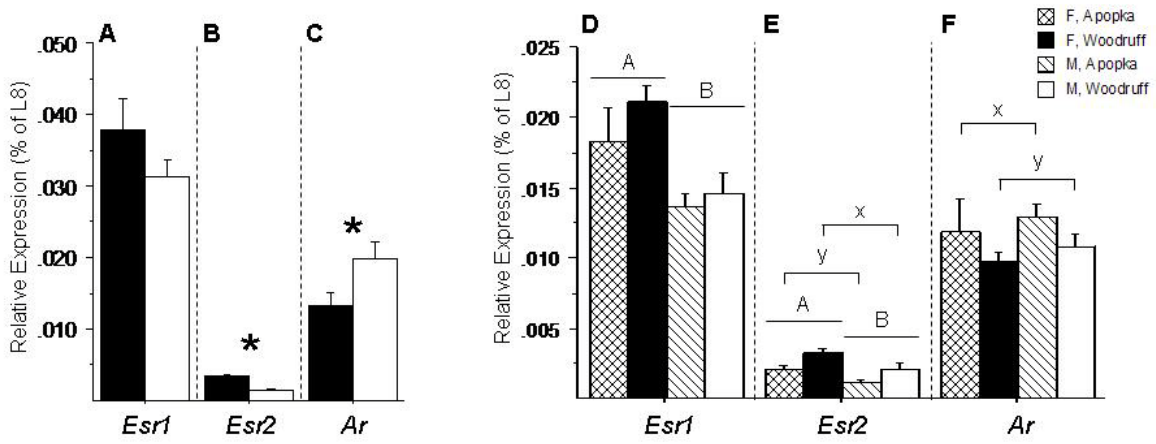

Figure 2-2. Steroid receptor mRNA expression in gonads from 30°C, 32°C, and 33.5°C incubated, 13-month-old alligators. Mean (±SEM) mRNA expression of *Esr1* (A, D), *Esr2* (B, E), and *Ar* (C, F). Alligators were incubated at 30/33.5°C (A-C) or 32°C (D-F). In A-C, black bars = females/30°C incubation, white bars = males/33.5°C incubation. Asterisks denote significant difference in expression between sexes at $P < 0.05$ by unpaired *t*-test. In D-F, crosshatched bars = Lake Apopka females, black bars = Lake Woodruff females, diagonal lined bars = 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 bars denotes sexually dimorphic expression. Lower case x or y over brackets denotes lake of origin dimorphic expression. In A-C and D-F, expression measurements separated by dotted lines are measured using absolute standard curves and are proportionally comparable across dotted lines.

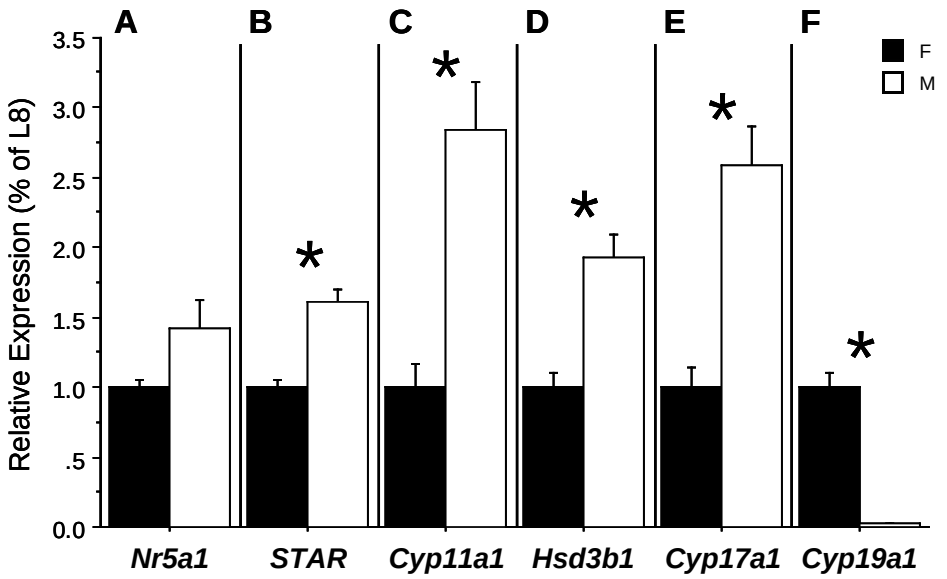

Figure 2-3. Steroidogenic factor mRNA expression in gonads from 30°C and 33.5°C incubated, 13-month-old alligators. Mean (±SEM) mRNA expression of (A) *Nr5a1*, (B) *STAR*, (C) *Cyp11a1*, (D) *Hsd3b1*, (E) *Cyp17a1*, and (F) *Cyp19a*1 in gonads of 13 month-old, 30°C/33°C incubated alligators. Black bars = females/30°C incubation, white bars = males/33.5°C incubation. Expressions within each section are standardized to a female expression value mean of one. Asterisks denote significant difference in expression between sexes at $P < 0.05$ by unpaired *t*-test. Expression measurements separated by solid black lines are not proportionally comparable.

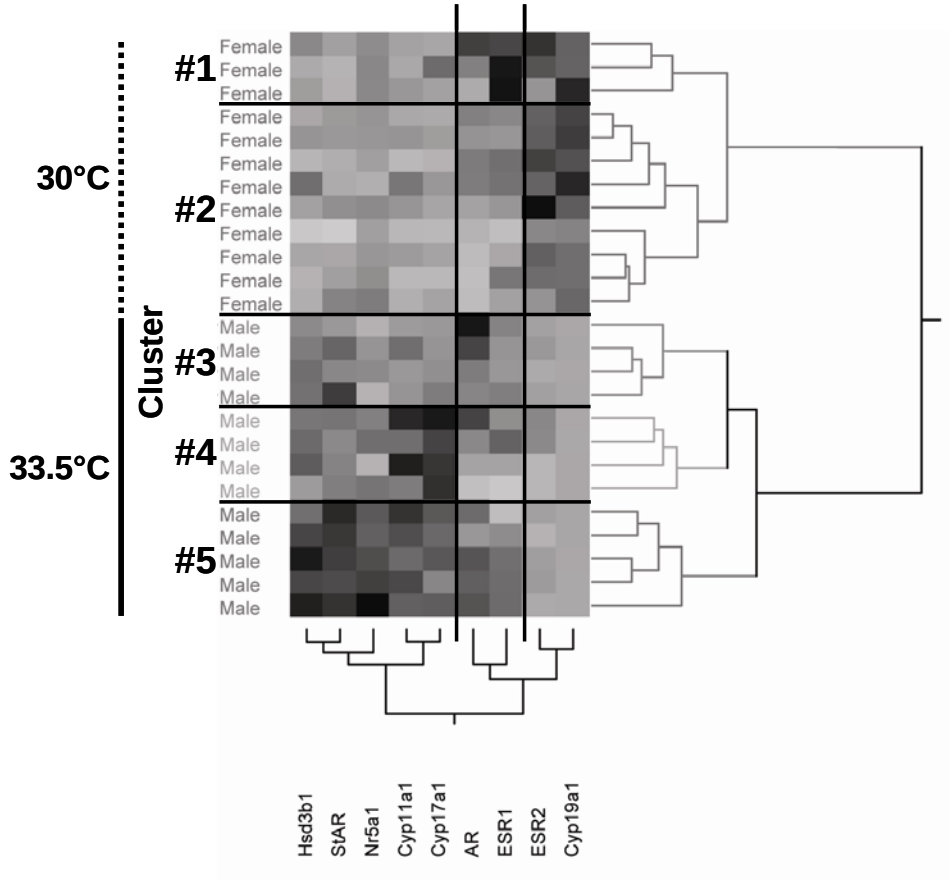

Figure 2-4. Cluster analysis of gonadal steroidogenic factor and steroid receptor expression from 13-month-old, 30°C or 33.5°C incubated Lake Woodruff alligators. Analysis uses Ward's linkage clustering. Color coded matrix with lower expression in lighter colors and higher expression in darker colors. Horizontal and vertical dendrograms show distance scales of joining distances for individual animals in the horizontal and individual, labeled mRNA expressions in the vertical. Sex of individual animals noted to the left of matrix. Numbered clusters, determined by scree plot criteria, are separated by horizontal lines. Groups of similar gene expression patterns are separated by vertical lines.

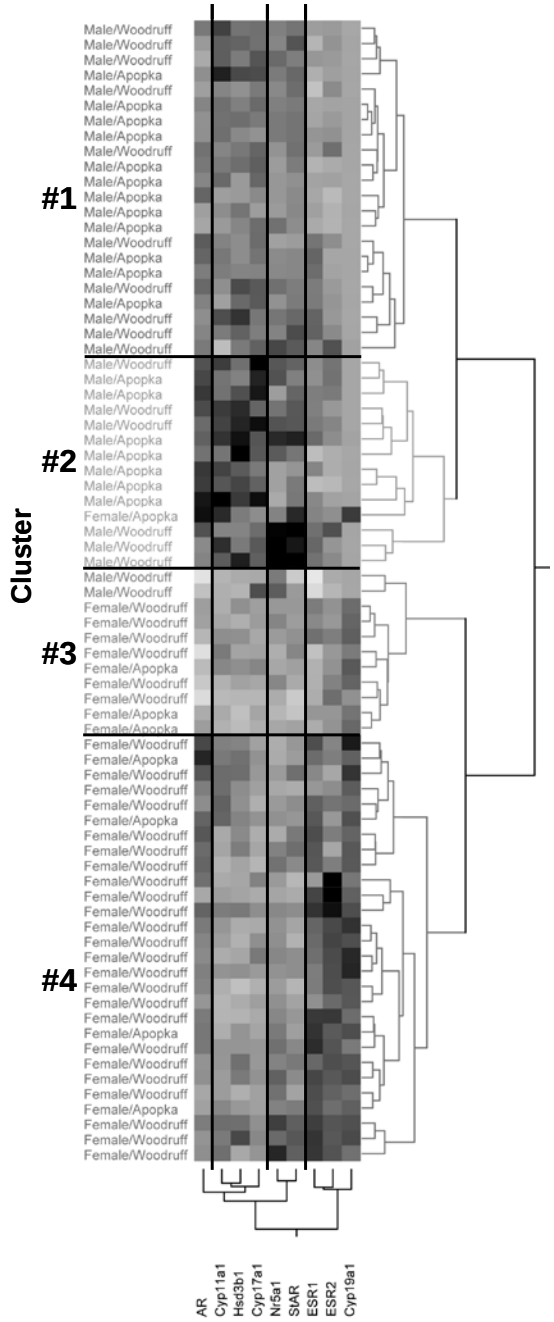

Figure 2-5. Cluster analysis of gonadal steroidogenic factor and steroid receptor expression from 13-month-old, 32°C incubated Lake Woodruff and Apopka alligators. Analysis uses Ward's linkage clustering. Color-coded matrix with lower expression in lighter colors and higher expression in darker colors. Horizontal and vertical dendrograms show distance scales of joining distances for individual animals in the horizontal and individual, labeled mRNA expressions in the vertical. Sex and lake of origin of individual animals noted to the left of matrix. Numbered clusters, determined by scree plot criteria, are separated by horizontal lines. Groups of similar gene expression patterns are separated by vertical lines.

CHAPTER 3
ALTERATIONS IN TGFB SIGNALING IN JUVENILE AMERICAN ALLIGATOR GONADS DUE TO ENVIRONMENTAL CONTAMINANT EXPOSURE

Introduction

Although the endocrine system has well-defined regulatory pathways, variation among individuals plays an essential role in the susceptibility or sensitivity of some individuals to environmental endocrine disruptors. Inbred and transgenic mouse lines have greatly aided our understanding of the underlying mechanisms of reproductive function. Engineered mouse lines maintain genetic homogeneity conferring chosen phenotypes and reduced likelihoods of genetic drift. This, in turn, has controlled experimental variability and increased experimental reproducibility. Outbred mouse stocks, in contrast, maintain heterozygosity with minimal genetic change in closed populations. These stocks are often selected for greater breeding performance, earlier sexual maturity, and larger litter sizes in laboratory settings, at the expense of genetic labiality and variation (Chia et al., 2005). However, even with the increased genetic diversity of outbred strains over inbred lines, outbred mouse strains can show profoundly different responses to endocrine disrupting exposures. Estrogenic exposures have resulted in differing phenotypes of testicular weight and spermatogenesis (Spearow et al., 1999), uterus and vagina cell proliferation and gene expression (Steinmetz et al., 1998), and ovarian follicle formation (Iguchi et al., 1987) by strain. Phenotypic impacts ranged from little treatment response in some strains to profound reproductive structural changes in others. Similar variation in environmental exposure sensitivity to ozone or cadmium have been observed among mouse strains (Chen et al., 2008; Vancza et al., 2009). When assessing an organism's vulnerability to an endocrine toxicant, observed responses in a genetically constrained population may not be transferable to understanding impacts occurring in wild populations (Spearow and Barkley, 2001). Even in zebrafish (*Danio rerio*) wild-type laboratory lines, genetic variation is decreased

compared to wild populations (Coe et al., 2009). When investigating multi-factorial processes, such as aging, some researchers have proposed using wild-caught mouse stocks to produce experimental results that can better be applied to understanding other wild-populations, including humans (Harper, 2008). Similarly, environmental exposure of wild populations to endocrine disrupting contaminants is often a multi-factorial phenomenon.

Wild populations living in heterogeneous, dynamic environments rely on genetic variation to adapt to changing selective pressures. The integration of genetic and environmental variance results in phenotypic variance. A population facing a selective pressure can respond in a way that changes the mean of a value that characterizes the phenotype, but that changes phenotypic variance of the trait across the population. Change of phenotypic variance has been observed in multiple studies of contaminant-exposed populations (Orlando and Guillette, 2001). Environmental contaminant exposures can effect sex-related endpoints, especially in non-mammalian species due to greater innate reproductive plasticity, resulting in profound impacts (Edwards et al., 2006a). In addition to morphological alterations, there is evidence for environmentally induced, phenotypic variation at the molecular level. For example, genetically male trout can be sex reversed using 17α-ethynylestradiol (Vizziano-Cantonnet et al., 2008). This treatment produced ovaries containing oocytes morphologically similar to those in control females and the expression of female-specific, ovarian differentiation-related mRNA in patterns similar, but not identical, to control females. However, morphological sex change did not suppress the expression of a suite of male-specific genes essential for testis-differentiation. Therefore, gene expression plasticity can produce multiple phenotypes. Additionally, changes in variance due to an environmental stress could result in differing phenotypic trait distributions (shifted, skewed, or multi-modal) across a population (Orlando and Guillette, 2001).

Recognizing both phenotypic mean values and variance in both reference and contaminant-exposed populations is crucial to understanding true exposure impacts on genetically diverse, wild populations.

To address this question, we investigated the effects and interactions of varying incubation temperatures and environmental quality on gonadal mRNA expressions in 13-month-old American alligators (*Alligator mississippiensis*). Here we compare gonadal mRNA expression between alligators hatched from eggs collected from two Florida lakes. Previous research from our laboratory has compared expression of various steroidogenic factors (Milnes et al., 2008) and steroid receptors (Kohno et al., 2008), as well as circulating steroid concentrations (Guillette et al., 2000b; Guillette and Gunderson, 2001; Milnes et al., 2005) between juvenile alligators from Lake Woodruff National Wildlife Refuge, an area of minimal anthropogenic influence, and the polluted Lake Apopka (Fujisaki et al., 2007; Guillette et al., 1999a; Heinz et al., 1991a). These data suggest that juvenile alligator gonads are physiologically active, show sexual dimorphism at both hormonal and molecular levels, but Lake Apopka animals display altered endocrine signaling compared to Lake Woodruff reference animals. Comparing population genetic structures of alligator from these lakes revealed little difference (Davis et al., 2002); these animals are genetically one population. Studying both ovary and testis allows us to identify phenotypic similarities and differences between sexes from Lake Woodruff (reference-site) and Lake Apopka (contaminant-exposed) alligators (Burger et al., 2007).

We measured the expression of a group of gonadal factors (Table 3-1), including transforming growth factor-β (TGFβ) superfamily ligand subunits, involved in paracrine signaling vital for male and female reproductive fitness (Drummond, 2005; Ethier and Findlay, 2001; Itman et al., 2006). Understanding of vertebrate gonadal TGFβ signaling has focused

primarily on mammals (Bristol-Gould et al., 2006; Yao et al., 2006; Yao et al., 2004b), chickens (Davis et al., 2001; Lovell et al., 1998; Onagbesan et al., 2004; Rombauts et al., 1995), and fish (Mousa and Mousa, 2003; Petrino et al., 2007; Wang and Ge, 2004), omitting many amniotes that exhibit non-genetic sex determination mechanisms, such as temperature-dependent sex determination (TSD) employed by some lizards, most turtles, and all crocodilians. In these TSD species, specific temperature ranges experienced during incubation produce male or female gonads in truly bipotential embryos that lack sex chromosomes (Modi and Crews, 2005a; Organ and Janes, 2008). We investigated if sexually dimorphic mRNA expression patterns of these regulatory factors observed in mammals and birds are present in 13-month-old alligator gonads from varying incubation temperature regimes. Furthermore, we examined if these expression patterns are similar in same-sex alligators produced at differing incubation temperatures. Lastly, we investigated if environmental contaminant exposure can influence these expressions.

Pollutants can influence steroid signaling milieus in a wide range of vertebrates (Hotchkiss et al., 2008); however, little research has investigated vulnerability of gonadal, non-steroidal protein ligand signaling mechanisms (Fowler et al., 2008; Loveland et al., 2007), especially in a wild population. To understand our results as part of a gene expression network vital for male and female reproductive fitness, rather than only as a series of discrete gene-by-gene expression measurements, we used a multivariate cluster analysis approach. This synthesis aids in an understanding of 'real world' variance and susceptibility at both the individual and population levels and identifies suites of the most vulnerable, and therefore the most valuable, endpoints for reproductive health assessment.

Materials and Methods

Experimental Design and Animal Care

All fieldwork was conducted under Florida Fish and Wildlife Conservation Commission 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 (Milnes et al., 2004a; Milnes et al., 2008). Complete clutches of eggs were collected soon after oviposition from Lake Apopka and Lake Woodruff National Wildlife Refuge, Florida, USA. To confirm the embryonic development stage according to criteria set forth by Ferguson (Ferguson, 1985) 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 (Ferguson and Joanen, 1983). 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 (Milnes et al., 2008).

Incubation of Lake Woodruff eggs at 30°C and 33.5°C resulted in 92% and 94% hatch rates, respectively. Hatching success and post-hatching mortality from 32°C incubated eggs has

previously been published (Milnes et al., 2008). In summary, 90% of eggs from Lake Woodruff and 80% of eggs from Lake Apopka hatched. Alligators were web-tagged and 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. Sex, as determined by visual inspections of gonad morphology and the presence or absence of oviducts at necropsy, was true to incubation temperature expectations for all animals. At the time of necropsy, 100% (n = 12) of 30°C females and 81% (n = 13) of 33.5°C males survived 13 months. In the 32°C cohort, only 66% of the animals hatched from Lake Apopka eggs survived to 13-months-old, as compared to 96% of animals hatched from eggs obtained from Lake Woodruff. Mortality was greatest in 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. Before necropsy, body mass (BM) and snout-vent length (SVL) were measured.

Tissue Collection, RNA Isolation, and Quantitative Real-Time PCR

At necropsy, gonads were 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 (Milnes et al., 2008). Quantitative real-time PCR (Q-PCR) has been used to measure mRNA expression of each gene of interest in alligators (Gunderson et al., 2006; Katsu et al., 2004; Kohno et al., 2008), and primer sequence information, annealing temperatures, and accession numbers are reported in Table 3-1. Q-PCR was performed in the MyiQ single color detection system (BioRad, Hercules, CA) following manufactures 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 (Katsu et al., 2004; Milnes et al., 2008).

Statistical Analysis

JMP for windows v7.0.1 (SAS Institute, Cary, NC) was used for statistical analyses. Morphometric data were log transformed and gene expression ratios were arcsin transformed to achieve homogeneous variances as needed. Significance for all tests was $P < 0.05$. Unpaired Student's *t*-tests compared means from females produced at 30°C to males produced at 33.5°C. 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. 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.

We performed hierarchical/agglomerative cluster analysis of mRNA expression data using Ward's linkage clustering to explore mRNA expression relationships among alligators of each incubation temperature regime. Before clustering, mRNA expression data were standardized so all variables had a mean of zero and a standard deviation of one. Elbow criteria determined the number of clusters from scree plots (not shown). Cluster analyses produced matrixes of relative gene expression levels presented in grey-scale, colormetric gradients with dark squares denoting high expression levels and light squares denoting low expression levels. This expression matrix is in conjunction with adjacent distance dendrograms showing relationships based on mRNA expression similarities for individual animals in the horizontal axis and hierarchical clustering of similar gene expressions between all animals in the vertical axis. Clusters derived from these

analyses were used as independent variables to re-examine mRNA expression data using one-way ANOVA followed by Tukey-Kramer post-hoc comparisons, when applicable.

Results

Body Morphometrics

Body mass (g) and snout-vent length (cm) were not different between 30°C females and 33.5°C males (mean ± S.E.; BM Female 262 ± 24, Male 283 ± 21; SVL Female 23.1 ± 0.6, Male 22.4 ± 0.7). At 32°C, Lake Apopka animals had greater BM than Lake Woodruff (mean ± S.E.; Woodruff female 278 ± 18, Woodruff male 269 ± 18, Apopka female 443 ± 53, Apopka male 351 ± 29) and SVL (mean ± S.E.; Woodruff female 22.6 ± 0.5, Woodruff male 22.4 ± 0.5, Apopka female 25.8 ± 0.9, Apopka male 24.3 ± 0.6) (two-way ANOVA, lake effect $P < 0.001$ for each measurement). No differences between sexes were detected. Measured mRNA expressions did not co-vary by BM or SVL.

Gonadal Regulatory Factor Expression

Gonads of Lake Woodruff animals incubated at 30° C (ovary producing) or 33.5°C (testis producing) displayed sexually dimorphic mRNA expression levels. Ovaries expressed greater mRNA levels of *Fst*, *Gdf9*, and *Foxl2* (Fig. 3-1D, E, and F; $P \leq 0.001$ for each) than testes, whereas testes expressed greater mRNA levels of *βB, Inhα*, and *Fshr* (Fig. 3-1A, C, and G; $P \leq 0.001$ for each) than ovaries. Expression of *βA* approached a significant difference (Fig. 3-1B; $P = 0.06$). The mRNA expression levels of *βB*, regardless of sex, were an order of magnitude greater than *βA* expression levels. Relative *βA* expression was greater than *Fst* and *Inhα*. Expression levels of *Inhα* mRNA in females were close to the detection limit of the assay. Ovary *Gdf9* mRNA expression was elevated and exhibited large variance in relation to minimal testis expression.

Gonadal mRNA expression levels of Lake Woodruff and Apopka animals incubated at 32°C displayed differences by sex, lake of origin, and by interactions between sex and lake. Lake Woodruff male expression of the *βB* was greater than similar expression levels observed in Lake Apopka males and females from either lake (Fig. 3-2A; P = 0.019). Expression of *βA* mRNA (Fig. 3-2B) showed differences by sex (testis expression greater than ovary; P < 0.001) and lake (greater Lake Woodruff expression compared to Lake Apopka expression; P = 0.03). Expression of *Inhα* also showed a sex by lake difference (Fig. 3-2C). Lake Apopka male expression was greater than Lake Woodruff male expression, while both male expression levels were greater than female expression levels (P = 0.048). Lake Woodruff female *Fst* mRNA expression was greater than expression in Lake Apopka females and males from either lake (Fig. 3-2D; P = 0.042). Relative expression levels of activin/inhibin subunits and *Fst* mRNA were similar to those observed in 30°/33.5°C gonads with βB expression levels an order of magnitude greater than *βA* expression levels and *βA* expression levels greater than *Fst* and *Inhα* expression levels. Female expression levels of *Inhα* mRNA were close to the detection limit of the assay.

Expression of *Gdf9* mRNA was approximately five fold greater in Lake Woodruff ovaries compared to similar levels observed in both Lake Apopka ovaries and testes from either lake (Fig. 3-2E; P < 0.001). Both *Foxl2* and *Fshr* mRNA expressions were sexually dimorphic with ovaries expressing far greater levels of *Foxl2* than testes (Fig. 3-2F; P < 0.001) and testes expressing greater levels of *Fshr* mRNA than ovaries (Fig. 3-2G; P < 0.001).

Multivariate Cluster Analysis of Individual Animals by mRNA Expression

We included aromatase mRNA levels from a previous study using the same cDNA in the cluster analysis (Milnes et al., 2008) due to its vital role in gonad regulation. Cluster analysis of gonadal mRNA expression levels for individual alligators from Lake Woodruff, incubated at 30° or 33.5°C, yielded three clusters (Fig. 3-3A, clusters defined by the horizontal lines on the

matrix). The first node of the dendrogram segregated all males from females: females further divided into clusters #1 and #2, whereas all males composed cluster #3 (Table 3-2A). Mean body masses were not different among clusters (data not shown).

Analysis of variance of mRNA expression data by clusters and subsequent post-hoc analyses revealed differing expression patterns among clusters (Table 3-2A). The dendrogram of mRNA expression patterns divided into two major groups (Fig. 3-3A, groups defined by the vertical line on the matrix). Female clusters expressed greater levels of *Fst*, *Foxl2*, *Cyp19a1*, and *Gdf9* than the male cluster, whereas differing expression levels of *Fst*, *Cyp19a1*, and *Gdf9* separated the two female clusters. The male cluster expressed greater levels of *βB* and *Inhα* when compared to both clusters of females. Male cluster expression levels of *βA* and *Fshr* were greater than female cluster #1, but similar to female cluster #2.

Cluster analysis of gonadal mRNA expression levels of Lake Woodruff and Apopka alligators incubated at 32°C yielded eight clusters (Fig. 3-4, clusters defined by the horizontal lines on the matrix). The first node of the dendrogram segregated males from females. Lake Woodruff male alligators mainly populated clusters #1 and #2, whereas male alligators from Lake Apopka mainly populated clusters #1 and #4 (Table 3-2B). Female Lake Woodruff alligators distributed amongst clusters #5-8, whereas Lake Apopka females occupied clusters #5 and #6 only. Mean BM were different among clusters (Table 3-3); however, BM did not correlate with mRNA expression levels (data not shown).

Significant differences among clusters revealed sex-related dimorphisms (Table 3-2B) that defined two groups of mRNA expression. Most female clusters expressed elevated *Fst, Foxl2*, *Cyp19a1*, and *Gdf9* levels, whereas most male clusters expressed elevated *βA*, *βB*, *Fshr*, and *Inhα* levels (Fig. 3-4, groups defined by the vertical line on the matrix). Expression levels of

Foxl2, *Cyp19a1*, *Gdf9*, and *Inhα* mRNA were completely sexual dimorphic. Clusters #5 and #8, composed mostly of Lake Woodruff females, had greater expression levels of the female-elevated mRNAs. In contrast, the majority of Lake Apopka females allocated to cluster #6 exhibited lower expression levels of most female-elevated mRNAs compared to other female clusters. Clusters #2 and #3 had the highest levels of the male-elevated mRNA expressions. Cluster #4, mostly composed of Lake Apopka males had lower expression levels of male-elevated factors compared to most other male clusters.

Discussion

Our data reveal sexually dimorphic mRNA expressions in the gonads of 13-month-old alligators that were similar to patterns established from other vertebrates. That is, alligator gonads from Lake Woodruff animals (reference-site animals) incubated at differing temperatures and thus generating different sexes, produced patterns of gene expression that were similar to those established in species with genetic sex determination. While incubation temperature did not have an overt effect on gene expression levels within Lake Woodruff alligators of the same sex, the lake of origin (e.g., differing exposure to endocrine disrupting contaminants) did alter some mRNA expressions that are crucial for reproductive function, such as *βB*, *Fst*, and *Gdf9*. The loss or diminishing of sexually dimorphic mRNA expression in yearling alligators from Lake Apopka (contaminant-exposed animals) could be associated with the previously reported loses in sexual dimorphism in Lake Apopka animals, such as phallus length (Guillette et al., 1996; Guillette et al., 1999b), gonadal steroidogenic factor mRNA expression(Milnes et al., 2008; Moore et al., Submitted), and circulating steroid levels (Crain et al., 1997; Guillette et al., 1994a; Milnes et al., 2005). Therefore, our study demonstrates the first evidence of an environmental impact on a larger suite of reproductive endpoints, including gonadal TGFβ signaling, within the population of Lake Apopka alligators.

Three of the mRNA factors measured in this study dimerize to produce activin and inhibin, TGFβ ligands. Activins are either homo- or heterodimers of two β subunits, βA or βB, to form activin A (βAβA), activin B (βBβB), or activin AB (βAβB). Inhibins, antagonistic ligands to activin receptor binding and activation, are heterodimers composed of a β subunit (either βA or βB) and an α subunit (Inhα) forming either inhibin A (βAα) or inhibin B (βBα). 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). Ovarian activin signaling plays many roles, including regulation of follicle formation and initial follicle pool size (Bristol-Gould et al., 2006). Female tissue-specific βA knock-out mice are subfertile, whereas βA/βB double knockouts are infertile (Pangas et al., 2007). In testes, activin A plays a paracrine role in regulating Sertoli cell number in the developing testis (Buzzard et al., 2003) and a loss of activin signaling in mice results in decreased numbers spermatogonia and sperm production (Kumar et al., 2001). In gonads from animals incubated at 32°C, the *βA* mRNA expression was sexually dimorphic, but a similar pattern was not observed in gonads obtained from animals incubated at 30°C or 33.5°C. Furthermore, in gonads from animals incubated at 32°C, mRNA expression levels of *βA* were greater in gonads from reference-site, Lake Woodruff animals versus those of Lake Apopka irrespective of sex.

Inhibins and follistatin regulate activin signaling. Ovarian inhibin expression is minimal prior to puberty (Raivio and Dunkel, 2002), but is vital for testicular formation and maintenance (Loveland et al., 2007). In humans, inhibin B measurement is a clinical tool to assess prepubital testicular development and Sertoli cell health (Lahlou and Roger, 2004). A post natal peak of inhibin B is hypothesized to be caused by proliferation of Sertoli cells (Meachem et al., 2001).

In the developing mouse gonad, *βB* expression is greater in testes than in ovaries. In males, elevated *βB* levels, along with elevated *Inhα* expression, produce inhibin B, a key factor in modulating the formation of testis-specific vascularity (Yao et al., 2006). In young cockerels (Lovell et al., 2000) and post natal rats (Buzzard et al., 2004; Meachem et al., 2001), testicular inhibin B production is greater than inhibin A. In our 13-month-old reference-site alligators, both incubation regimes generated sexually dimorphic testicular *Inhα* mRNA expression that is associated with *βB* mRNA levels that are an order of magnitude greater than *βA* levels. This infers greater inhibin B production than inhibin A, which would be correspond with observations from other vertebrate species studied to date. However, testes obtained from Lake Apopka males incubated at 32°C expressed *Inhα* at a greater level than testes obtained from Lake Woodruff males, but only female-equivalent *βB* expression levels. These changes could result in decreased inhibin B production for the contaminant-exposed, Lake Apopka males. This is notable in the light of the necessity of inhibin B in proper testicular development and maintenance (Yao et al., 2006) and that circulating inhibin B levels positively correlate with circulating FSH and testosterone levels during sexual development (Andersson et al., 1997; Lovell et al., 2000).

Follistatin, an antagonist that binds and neutralizes TGFβ ligands including activins, is a somatic-cell produced factor that is expressed in greater concentrations in embryonic mouse ovaries than testes (Menke and Page, 2002), but plays roles in both ovary and testis development (de Kretser et al., 2004; Meehan et al., 2000; Yao, 2005a; Yao et al., 2004b). In the developing ovary it is a vital antagonist to the development of testis-specific vasculature and promotes the survival of meiotic germ cells in the ovarian cortex (Yao et al., 2004b). Conditional Fst knockout female mice have fertility defects similar to premature ovarian failure (Jorgez et al., 2004). Alligator ovaries obtained from reference-site, Lake Woodruff females exhibited

elevated *Fst* levels compared to testes obtained from males from the same lake. In contrast, at 32°C incubation, Lake Apopka ovaries expressed *Fst* levels lower than Lake Woodruff ovaries, equal to Lake Woodruff testes, but still greater than Lake Apopka testes. While expression is sexually dimorphic comparing animals from each lake separately, the possible impact of diminished *Fst* expression in contaminant-exposed, Lake Apopka ovaries warrants further investigation.

Growth differentiation factor 9 (Gdf9) is a vital, germ cell secreted TGFβ ligand. It regulates folliculogenesis through modulating somatic cell proliferation and differentiation (Carabatsos et al., 1998; Dong et al., 1996; Johnson et al., 2005). Though expressed at greater levels 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 chicken ovaries, *Gdf9* mRNA expression is greater in follicles less than 1 mm in diameter than in larger, more developed follicles (Johnson et al., 2005). In contrast to studies of other vertebrates (Fitzpatrick et al., 1998; Nicholls et al., In Press), *Gdf9* has not been detected in chicken testes. However, we detected *Gdf9* mRNA in all alligator testis samples, though at levels lower than ovaries.

Mice lacking Gdf9 have ovarian defects, including increased frequencies of multi-oocytic follicles (MOFs) (Yan et al., 2001), are infertile due to failure of ovarian follicular development, and displays compromised attachment of somatic cells to oocytes (Carabatsos et al., 1998). Furthermore, one layer follicles of *Gdf9* deficient ovaries express elevated α subunits levels, whereas *Fst* and *βB* expressions are decreased ovary-wide (Elvin et al., 1999). Gonads from our reference-site, Lake Woodruff, displayed sexually dimorphic *Gdf9* expression under both incubation temperature regimes. However, mRNA expression of *Gdf9* in ovaries obtained from

contaminant-exposed, Lake Apopka females incubated 32°C was equivalent to testicular levels, which were five times lower than observed in ovaries from Lake Woodruff females. Previously, our laboratory has shown that yearling Lake Apopka alligators ovaries exhibit increased frequencies of MOF (Guillette et al., 1994a). Therefore, an etiology of Lake Apopka ovary MOF formation could involve interactions of both somatic cell (*Fst*) and germ cell (*Gdf9*) produced factors.

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). It is vital for primordial follicle pool formation (Schmidt et al., 2004), granulosa cell differentiation (Schmidt et al., 2004), and regulation of aromatase (*Cyp19a1*) expression (Hudson et al., 2005; Pannetier et al., 2006). Expression of *Foxl2* mRNA has been detected in chicken (Govoroun et al., 2004) and duck (Koba et al., 2008c) ovaries at orders of magnitude greater levels than in testes, similar to the dimorphism we reported here for in juvenile alligator.

We measured follicle stimulating hormone receptor (*Fshr*) mRNA expression levels to explore possible differences in gonadal gonadotropin signaling. Follicle stimulating hormone (FSH) stimulates the production of *Inhα* (Tuuri et al., 1996), *Fst* (Tuuri et al., 1996), and *βA* in vitro (Tuuri et al., 1996; Welt and Schneyer, 2001) and in vivo (Welt et al., 1999b) in mature granulosa cells. Furthermore, FSH induces alterations in gene expression for inhibin/activin subunits in ovarian granulosa cells cultured from 4-day-old rat ovary (Findlay et al., 2001). Follicle stimulating hormone augments the effects of activin A and/or follistatin on testicular development (Buzzard et al., 2003; de Kretser et al., 2004; Itman et al., 2006). Further, embryonic chicken testes produce inhibin in response to FSH stimulation (Rombauts et al., 1995,

1996). Changes in ovarian *Fshr* receptor expression levels have been measured during duck prepubertal development (Ni et al., 2007). However, no publication has compared FSHR expression levels between sexes in post-hatching birds gonads to date. Here we show greater testicular *Fshr* mRNA expression levels compared to ovaries irrespective of incubation temperature regime.

Cluster analyses examined mRNA expression patterns in these studies, exploring both similarities and variances across gonads. We observed mRNA expression patterns segregated by sex at the earliest stages of each cluster analysis. Within sexes, multiple gene expression motifs formed clusters of similar expression patterns. Comparing gene expression levels between these clusters, some mRNA expressions showed complete sexual dimorphic expression, while others displayed incomplete dimorphic expression with most clusters being sexually dimorphic, but other showed no difference by sex. We observed complete dimorphic expressions in ovary-elevated levels of *Foxl2*, *Fst*, *Cyp19a1*, and *Gdf9* and in testis-elevated levels of *Inhα*, irrespective of incubation temperature or contaminant exposure. While both similarities and differences in mRNA expressions levels occurred between clusters within a given sex, male and female clusters did not share similar mRNA expression levels. Expression of the *βB* subunit was sexually dimorphic between 30°C and 33.5°C gonads, however showed incomplete sexually dimorphic expression levels among 32°C clusters. Expressions of βA and *Fshr* mRNA were similar between many clusters of both sexes within both incubation regimes. These differing patterns of sexual dimorphism for mRNA expression from reference-site animals provides assistance in defining ovary- or testis-specific gene expression patterns that have greater likelihoods of variable expressions due to environmental perturbation.

Clustering of mRNA expression levels from the 32°C incubation revealed mRNA expression pattern differences in contaminant-exposed, Lake Apopka gonads, compared to reference-site gonads. The majority of Lake Apopka males segregated into two of the four male clusters. One of these two clusters (Cluster #4 that contained ~80% Apopka males) expressed the lowest levels of *βA*, *βB*, *Fshr*, *Inhα,* and *Fst* among all male clusters. Similarly, the majority of Lake Apopka females segregated to a single cluster (Cluster #6) that expressed only lower levels of *Fst*, *Foxl2*, *Cyp19a1*, and *Gdf9* as compared to other female clusters. In developing chicken (Govoroun et al., 2004; Hudson et al., 2005), duck (Koba et al., 2008c), and goat (Pannetier et al., 2006) ovaries, *Foxl2* expression patterns spatially and temporally correlate with *Cyp19a1* expression. In support of these findings, post-hoc levels of *Foxl2* and *Cyp19a1* were identical between clusters in 32°C incubation cohort gonads. Taken together, these analyses demonstrate that some contaminant-exposed, Lake Apopka male and female animals segregate into clusters that express suites of gonadal mRNA at lower levels than observed in clusters of Lake Woodruff animal of similar sexes.

When testing the potential for an environmental compound to disrupt reproductive development, researchers have called for the inclusion of susceptible genotypes (Spearow and Barkley, 2001). Cluster analysis has demonstrated that unexposed, juvenile alligator gonadal gene expression shows sexual dimorphisms and within- and between-sex variances. We also observed that a large proportion of contaminant-exposed animals fell into clusters expressing lower levels of genes vital to male or female reproductive health. We propose wild population constitute a component of "susceptible genotypes" that need to be addressed when assessing the impact of endocrine disrupting compounds.

Recent research has demonstrated interactions between contaminant exposure and altered TGFβ signaling. Long-term embryonic exposure to sewage sludge used for pasture fertilization decreased the number of *Gdf9*-positive oocytes and follicle density in fetal sheep ovaries (Fowler et al., 2008). Maternal smoking elevates serum inhibin A levels during the second trimester (Bersinger and Odegard, 2007; Ferriman et al., 1999). Particulate pollutants induce genes involved in fibrogenesis and airway wall fibrosis by way of a TGFβ mediated signaling mechanism (Dai et al., 2003). Decreased testis size and inhibin B levels in Danish boys is hypothesized to have an environmental component (Main et al., 2006). We hypothesize that environmental alterations in gonadal in TGFβ signaling could be more prevalent than reflected in the current literature and present an important, new research frontier. Our approach, using quantitative RT-PCR and cluster analysis is an initial attempt to begin to identify not only the expression of specific genes that might be affected but provide a better understanding of how regulatory pathways, such as the gonadal inhibin-activin regulatory pathway discussed here, are potentially affected by environmental perturbations, such as endocrine disrupting contaminants.

Table 3-1. Growth factor quantitative real-time PCR primers

Gene	Forward Primer (5' - 3') Reverse Primer (5' - 3')	Anneal Temp (°C)	Product size (bp)	Accession #
Inhibin α	ACAATCCACTTGTCCCAGCC CAACTGCCACCGCGC	70.0	68	DQ010151
Activin βA	ACCCACAGGTTACCGTGCTAA GCCAGAGGTGCCCGCTATA	63.8	67	DQ101152
Activin βB	GGGTCAGCTTCCTCCTTTCAC CGGTGCCCGGGTTCA	64.7	70	DQ010153
Follistatin	CGAGTGTGCCCTCCTCAAA TGCCCTGATACTGGACTTCAAGT	66.5	65	DQ010156
FoxL2	ATCAGCAAGTTCCCCTTCTAC GCCTTTCTCGAAAATGTCCTC	65.0	171	EU848473
GDF9	TCAGTTTCCTCCTCTTCTCCAATT ACACACTTGGCTAGAAGGATCATTC	63.0	78	DQ015675
FSHR	GAAATTACCAAACGAGGTTTTTCAA GGGCAGGAAACTGATTCTTGTC	60.0	81	DQ010157

Table 3-2. ANOVA and post-hoc comparisons of mRNA expression levels and body mass data by clusters and distribution of alligators by sex and lake of origin that were incubated at A) 30℃ or 33.5℃ B) 32℃

		mRNA Transcript								Body Mass	Sex			
		βA	*βB*	*Fshr*	*Inhα*	*Fst*	*Foxl2*	*Cyp19a1*	*Gdf9*		Male		Female	
A	**P**	= 0.039	< 0.001	< 0.001	= 0.004	< 0.001	< 0.001	< 0.001	< 0.001	= 0.78				
		Tukey-Kramer HSD									Woodruff	Apopka	Woodruff	Apopka
Cluster	**1**	B	C	B	B	B	A	B	A	-	0	-	5	-
	2	A,B	B	A,B	B	A	A	A	B	-	0	-	7	-
	3	A	A	A	A	C	B	C	C	-	13	-	0	-
B	**P**	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	= 0.001				
		Tukey-Kramer HSD												
Cluster	**1**	B,C	B	A	B	D	C	C	C	B	11	8	0	0
	2	A	A	A,B	B	C	C	C	C	A,B	4	0	0	0
	3	A,B	B,C	A	A	D	C	C	C	A,B	1	2	0	0
	4	B,C,D	D	C	B	E	C	C	C	A	2	9	0	0
	5	D	C,D	B,C	C	B	A	A	B	A,B	0	0	8	2
	6	C,D	C,D	C	C	C	B	B	B	A,B	0	0	9	6
	7	B,C	D	B	C	B	B	B	A	B	0	0	7	0
	8	B,C	B,C,D	A,B	C	A	A,B	A,B	A	B	0	0	7	0

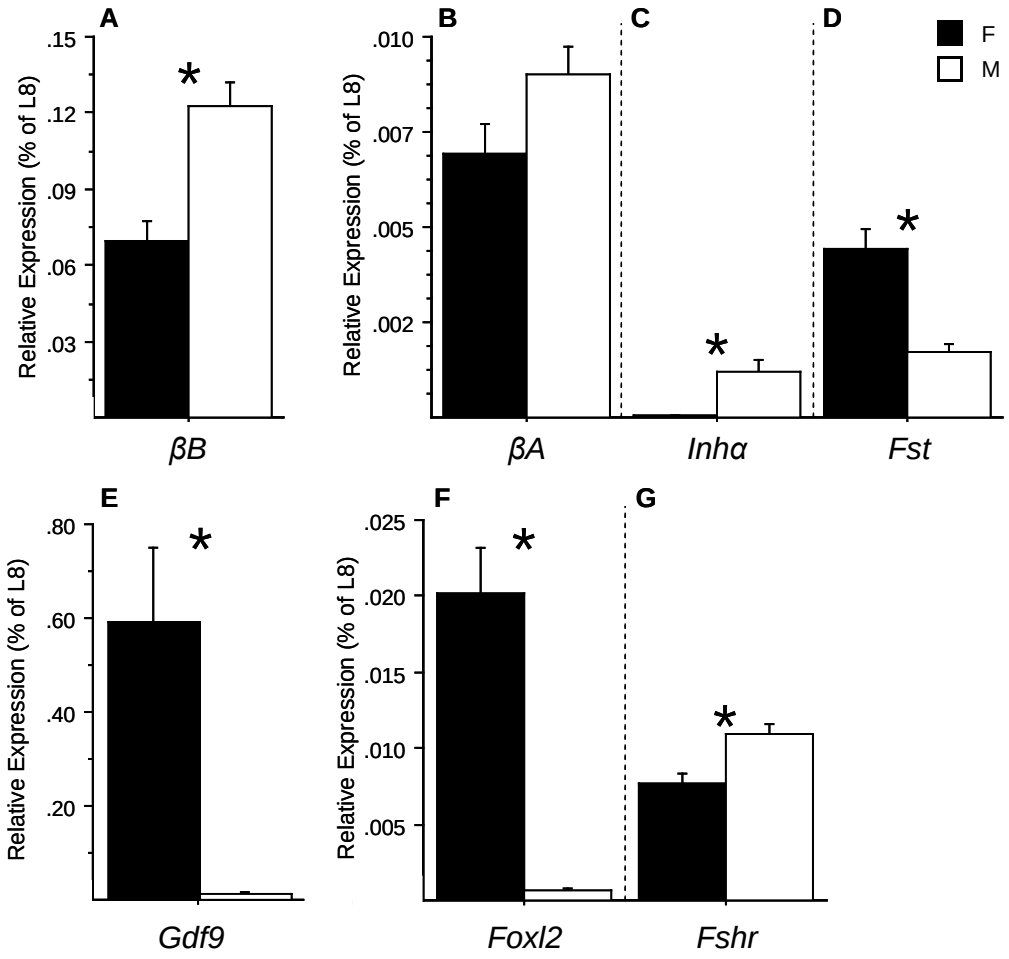

Figure 3-1. Growth factor mRNA expression in gonads from 30°C and 33.5°C incubated, 13-month-old alligators. Mean (±SEM) expression of (A) *βB*, (B) *βA*, (C) *Inhα*, (D) *Fst*, (E) *Gdf9*, (F) *Foxl2*, and *(G)Fshr*. Black bars = females/30°C incubation, white bars = males/33.5°C incubation. Expressions within each section are standardized to a female expression value mean of one. Asterisks denote significant difference in expression between sexes at $P < 0.05$ by unpaired *t*-test.

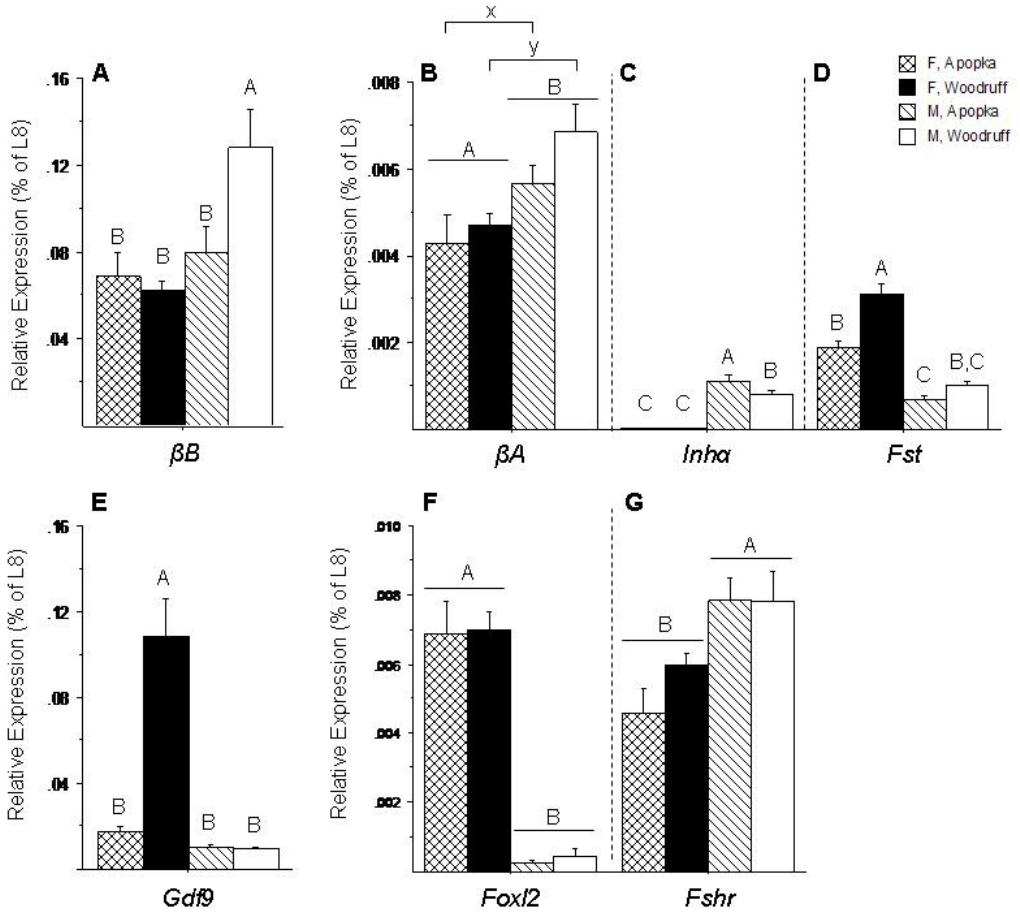

Figure 3-2. Growth factor mRNA expression in gonads of 32˚C incubated, 13-month-old alligators. Mean (±SEM) mRNA expression of (A) *βB*, (B) *βA*, (C) *Inhα*, (D) *Fst*, (E) *Gdf9*, (F) *Foxl2*, and *(G)Fshr*. Crosshatched bars = Lake Apopka females, black bars = Lake Woodruff females, diagonal lined bars = Apopka males, white bars = Lake Woodruff males. Superscripts denote differences between groups by two-way ANOVA. P-values of two-way ANOVA factors in sub-table, bold text is significant at $P < 0.05$. Capital A or B over bars denotes sexually dimorphic expression. Capital letters over individual error bars denotes post-hoc expression differences in lake by sex analysis.

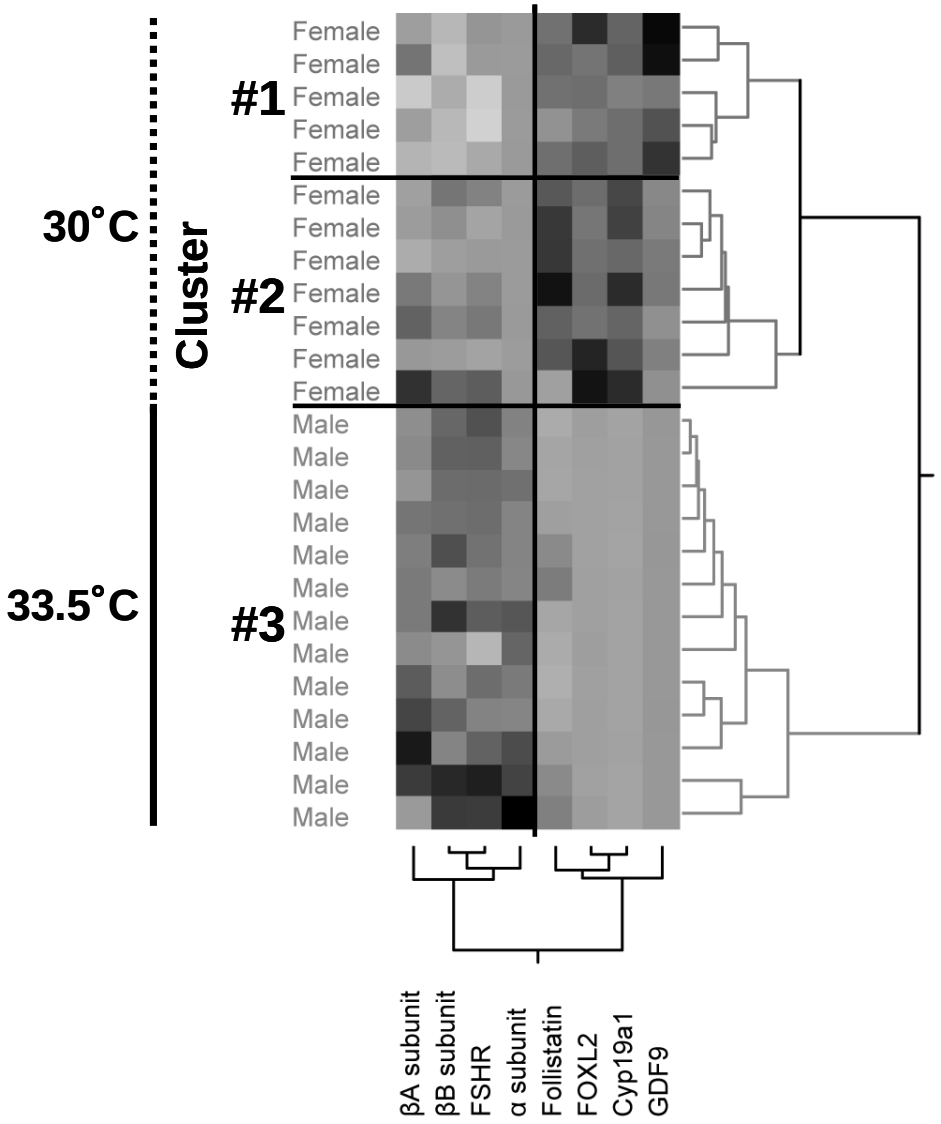

Figure 3-3. Cluster analysis of gonadal growth factor expression from 30℃ or 33.5℃ incubated, 13-month-old Lake Woodruff alligators. Analysis uses Ward's linkage clustering. Color coded matrix with lower expression in lighter colors and higher expression in darker colors. Horizontal and vertical dendrograms show distance scales of joining distances for individual animals in the horizontal and individual, labeled mRNA expressions in the vertical. Sex of individual animals noted to the left of matrix. Numbered clusters, determined by scree plot criteria, are separated by horizontal lines. Groups of similar gene expression patterns are separated by vertical lines.

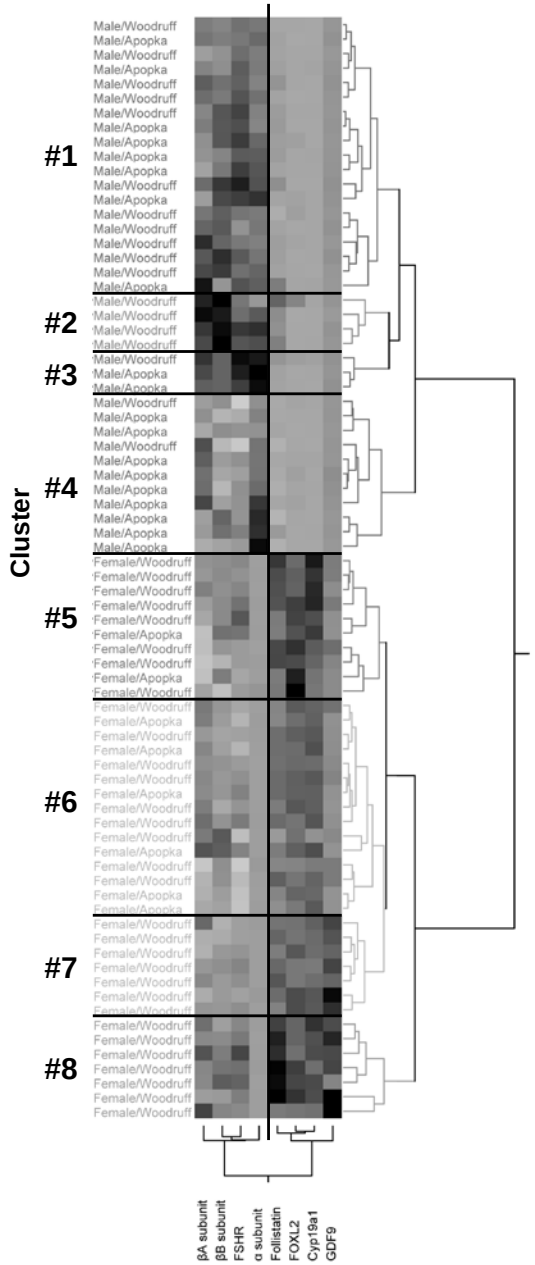

Figure 3-4. Cluster analysis of gonadal growth factor expression from 32°C incubated, 13-month-old Lake Woodruff and Apopka alligators. Analysis uses Ward's linkage clustering. Color coded matrix with lower expression in lighter colors and higher expression in darker colors. Horizontal and vertical dendrograms show distance scales of joining distances for individual animals in the horizontal and individual, labeled mRNA expressions in the vertical. Sex and lake of origin of individual animals noted to the left of matrix. Numbered clusters, determined by scree plot criteria, are separated by horizontal lines. Groups of similar gene expression patterns are separated by vertical lines.

CHAPTER 4
POST-HATCHING DEVELOPMENT OF ALLIGATOR OVARY AND TESTIS

Introduction

A fundamental component of gonadal differentiation and development is the formation of a sex-specific extracellular matrix (ECM). Alligator gonads show diverging, sex-specific structural protein formation during embryonic development (Smith and Joss, 1995). At the end of the period of temperature-dependent sex determination (TSD), embryonic ovaries have an expanded cortex of germ and somatic cells lacking laminin-immunoreactive (IR), overlying a medulla of fragmenting laminin-IR primary sex cords. Within the ovarian medulla, epithelial cells rest on laminin-IR basement membranes lining expanding lacunae. Conversely, alligator gonads that develop a testicular morphology show a regressed cortex and a medulla of seminiferous cords invested with germ cells and defined by laminin-IR basement membranes. Both ovary and testis juxtapose a mesonephric kidney with laminin-IR glomeruli and renal tubules. Here we expand on these pre-hatching observations through an investigation of the post-hatching development of the alligator gonad. We employ histochemical techniques to investigate further ontogenetic changes in ECM distribution and structure and relate these findings to morphological changes of the gonad, including germ cell maturation.

Ovarian follicle assembly occurs with the assembly of its basement membrane. This definitive ECM feature separates the oocyte and granulosa from the surrounding stromal and/or thecal elements of an ovarian follicle. Basement membranes are primarily composed of collagen interwoven with laminin (Rodgers and Rodgers, 2002). Additional components such a fibronectin and other proteoglycans, and polysaccarides augment this complex structure. Previously, we have demonstrated that follicle assembly is a post-hatching event in the American alligator (Moore et al., 2008). Initial stages of alligator oocyte maturation do not display either a

complete, encircling complement of follicular (pre-granulosa) cells or evidence of a basement membrane separating the germ cell from stromal tissues. Oocytes surrounded by a complete complement of follicular cells and enclosed within a basement membrane were not observed until four months after hatching under laboratory rearing conditions (Moore et al., 2008).

Both mammalian granulosa and thecal cells express mRNA for basement membrane components, including collagen and laminin (Zhao and Luck, 1995). However, it is hypothesized that granulosa cells produce the majority of basement membrane components (Rodgers et al., 2003). Therefore, initiation of follicular basement membrane synthesis around alligator oocytes should be associated the organization of follicular cells. While morphological changes associated with follicle assembly have been characterized in the American alligator (Moore et al., 2008; Uribe and Guillette, 2000), here we expand the scope of our observations from three to five months post-hatching and examine the ontogeny of follicular basement membrane assembly in association with post-hatching ovary development.

A limited body of research has addressed alligator testis morphology during the post-hatching period (Forbes, 1940; Guillette et al., 1994a; Smith and Joss, 1995). Hatchling alligator testes present seminiferous cords invested with Sertoli and germ cells. Flattened stroma cells separate cords and differentiated Leydig cells are not observed. Sertoli cells contain ovoid nuclei placed adjacent to the basement membrane and project a large volume of apical cytoplasm into the cord interior. Using a series of manuscripts from previous laboratory-based experiments, we can describe a rough developmental sequence. Seminiferous cords of four-month-old testes present “activity and growth, as evidenced by crowding of the cords, numerous mitotic figures, and some anastomosis of the cords” (Forbes, 1937). Six- and 12-month-old tubules contain only spermatogonia (Forbes, 1940) whereas spermatocytes were observed in 15- and 18-months-old

testes, but spermatogenesis was not noted (Forbes, 1938, 1940). Thickening of the tunica albuginea, increased number of spermatogonia, and development of the seminiferous cord basement membrane occurred during this period. Husbandry practices and body morphometrics were mostly unreported in these studies; therefore, body conditions are unknown at given ages.

Here, we focus on the first five months of testicular development and contrast changes in ECM, especially in seminiferous cords and the testicular capsule, to those observed in the ovary. The lamina propria is the vascularized layer of connective tissues in contact with Sertoli cells and spermatogonia that delimits the seminiferous tubules. It is composed of two layers; an inner acellular basement membrane composed of primarily laminin and collagen fibrils (adding tensile strength) and an outer multicellular layer containing peritubular myoid and lymphatic cells (Siu and Cheng, 2004a; 2004b). Basic similarities have been demonstrated between reptilian, mammalian, and avian lamina propria, including the types and arrangements of both cells and cellular and extracellular fibers (Unsicker and Burnstock, 1975). Mammalian peritubular myoid and Sertoli cells cooperate in the production of lamina propria ECM (Maekawa et al., 1996; Weber et al., 2002). Collagen- and laminin-IR of seminiferous cord lamina propria increases over the first 10 post-natal days in rats (Gelly et al., 1989). Similarly, alligator lamina propria development has been observed to continue during post-natal morphological differentiation (Forbes, 1940). A focus of this research will characterize alligator lamina propria in terms of the basement membrane development and myoid cell morphology during the post-hatching period.

The ECM has traditionally been described to act as a physical barrier compartmentalizing tissues, supplying rigid or elastic mechanical tissue support, filtering fluids passing through the matrix, or passively regulating osmotic forces. However, ECM has also been shown to establish "microenvironments" which can directly modulate cell behaviors, including growth, maturation,

and differentiation (Rodgers and Rodgers, 2002). For example, ECM components have been shown to directly enhance survival and proliferation of granulosa cells *in vitro* (Huet et al., 2001). Similarly, collagen itself has been demonstrated to be a biologically active molecule in testis (Siu and Cheng, 2004a). ECM is now recognized as an active regulator of endocrine signaling and steroidogenesis (Berkholtz et al., 2006b) and can act as a barrier against growth factors or bind growth factors and serve as a reservoir (Rodgers and Rodgers, 2002; Wang et al., 2008). The ovarian follicle basement membrane of the chicken stores biologically active molecules such as growth factors and binding proteins and maintains normal granulosa cell morphology during *in vitro* culture (Asem et al., 2000a). Changes in transcription factor expression involved in sex differentiation have been associated with changes in structural protein expression, including laminin and collagen, in pre- and post-natal rat gonads (Pelliniemi and Frojdman, 2001). Increased levels of steroidogenic enzyme expression in both theca and granulosa cells have been shown to be associated with, and possibly directly modulated by, changes in ovarian ECM (Huet et al., 1997; Huet et al., 2001). In contrast, type IV collagen down regulates gonadotropin induced steroidogenesis in Leydig cells (Diaz et al., 2005). Isolated basement membrane components increased progesterone production in granulosa cells isolated from chicken ovarian follicles (Asem et al., 2000b). Therefore, this investigation of ECM distribution and development in hatchling alligator gonads not only elaborates structural maturation of these gonads, but likely also predicts concomitant physiological, biochemical, or endocrine changes.

Materials and Methods

American alligator (*Alligator mississippiensis*, Daudin, 1801) eggs were obtained from nests in the Lake Woodruff National Wildlife Refuge (Permit #WX01310) in June, 2006.

American alligator sex determination is temperature dependent (Ferguson and Joanen, 1983). Eggs were obtained prior to the period of sex determination, returned to the University of Florida, and candled for viability. Eggs (n=105) from seven clutches were 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, eggs were systematically arranged in trays of damp moss and trays were rotated throughout incubation to minimize possible clutch 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).

All animal procedures conformed to an IACUC approved protocol. Following hatching, animals were housed in tanks within a temperature-controlled animal room (~20 neonates / 0.7 m^3), experienced a 16:8 photoperiod with heat lamps for basking, and ambient room temperatures ranged from 27°C to 31°C. Alligators hatch with a relatively large residual yolk mass; thus hatchlings were fed *ad lib* starting 10 days after hatching with a small pellet size, commercial alligator chow (Burris Mills, LA).

Subsets of animals were killed using a lethal dose of sodium pentobarbital at: one week (30°C n = 12, 33°C n=10), one month (30°C n = 11, 33°C n=9), three months (30°C n = 10, 33°C n=8), and five months post hatching (30°C n = 11, 33°C n=10). Gonads and underlying mesonephric and adrenal tissues were removed as a single sample and fixed in Bouin's fixative for 24 hours. After fixation, tissues were washed in distilled water, dehydrated in a series of graded alcohols, cleared, paraffin embedded, and serially sectioned parasagittally at 6 μm. Tissue sections were stained with Masson's trichrome (Humason, 1979), periodic acid (PAS)-Alcian blue (pH 2.5) with hematoxylin counterstain, or Gomori's periodic acid-methionine silver (PAMS) with an omission of gold toning and nuclear fast red counterstain. Tissues were

examined with an Olympus BH-2 light microscope and photographed with a Pixelink PL-B623CU 3.0 megapixel digital camera.

PAS stains periodate reactive molecules containing a high proportion of carbohydrate and glycoconjugate macromolecules (glycogen, glycoproteins, and proteoglycans), typically found in connective tissues, mucus, and basement membranes. Laminin is a PAS-positive glycoprotein component of basement membranes that attaches non-connective tissue cells to collagenous fibers. PAMS staining detects argyrophilic molecules including collagenous reticular fibers (thin structural elements that provide supporting framework to many organs) and components of basement membranes. This technique has a broad specificity (Puchtler and Waldrop, 1978) and therefore direct inference of specific PAMS staining targets, especially in alligator, cannot be made at this time. However, silver staining techniques have successfully been used to stain glomerular mesangial matrix and Bowman's capsule of mammals (Adler et al., 2000; Harvey et al., 1998) and alligators (Moore and Hyndman, 200X), both collagen-rich structures (Abrass et al., 1988).

Microscopic examination of gonads showed all animals incubated at 30°C had ovaries whereas 68% of animals incubated at 33°C had testes. Of the females produced from the 33°C incubation, 92% came from three of the seven egg clutches collected for the experiment. Only results from gonads of 30°C females and 33°C males are presented in this manuscript. Snout-vent lengths (SVL) and body masses (BM) were recorded prior to dissections (average age of each group in days at measurement: one week = 4.5 d, one month = 28.1 d, three months = 88.5 d, and five months = 145.6 d) and body measurements were statistically compared by dissection ages and sex using two-way ANOVA and Newman-Keuls post-hoc analysis (JMP 7, SAS Institute). Samples sizes are presented in Fig. 4-1B. To assess possible growth trajectory

differences due to experimental conditions, we compared these laboratory-raised animals to data taken from a cohort of field-captured, Lake Woodruff juvenile animals (field-captured cohort: female $n = 40$, male $n = 49$).

Examining the ovarian histology, morphological and histochemical changes in both cortex and medulla were observed. States of oogenesis and folliculogenesis, along with the presence and distribution of PAS- and PAMS-positive ovarian material, were characterized at each dissection time point. Chromatin and cytoplasmic morphologies were used to determine various stages of prophase I of oogenesis. Additionally, oocyte stages were identified according to a system that incorporates both meiotic characteristic and extent of folliculogenesis (Uribe and Guillette, 2000). Stage-1 oocytes exhibit meiotic germ cells at a pre-diplotene stage often with fibrillar chromatin and in loose association with follicular cells. Stage-2 oocytes display an incomplete follicular layer of somatic cells and diplotene nuclei that often display chiasma, lampbrush chromosomes, and several nucleoli. Stage-3 oocytes, also diplotene, are distinctly larger in diameter than Stage-2 oocytes, often displaying lampbrush chromosomes, and possess a complete follicular layer of granulosa as well as a developing layer of surrounding thecal cells.

Testicular histology, including morphological and histochemical changes in the seminiferous cords, interstitial spaces, and the testicular capsule was observed. Germ cell maturation was characterized by cellular morphology and location within tubules, with insight from the recent characterization of adult alligator germ cell maturation (Gribbins et al., 2006).

Results

Somatic Growth, Gonad Placement, and Surrounding Tissues

During the first five months after hatching, alligators displayed robust growth in body mass and length (Fig. 4-1A-C). Average body masses for both males and females more than quadrupled between months one and three and then doubled between months three and five.

Female BM and SVL were greater than male at months one and three, no differences were observed at one week or five months. Laboratory conditions increased BM growth in relation to SVL over measurements from field-captured animals (Fig. 4-1C).

Hatchling alligator gonads are thin, laminar tissues located on the caudal, dorsal peritoneal cavity wall. During post-hatch development, the anterior-medial aspect of the gonad is found lateral to the adrenal gland, whereas the medial- posterior aspect of the gonad is ventral to the degenerating mesonephric kidneys and large lymphatic vessels. Morphologically, the adrenal glands present intermingled aggregates of highly basophilic chromaffin cells and less basophilic intrarenal cells with each cell type clearly demarcated by PAS-positive (Figs. 4-2A, 5B) and PAMS-positive (not shown) extracellular matrix. The mesonephros contains renal corpuscles and associated ducts. Glomeruli show strong PAS (Fig. 4-2A, 4-4A) and PAMS reactivity (Fig. 4-2C, 4-4C). In one-week-old animals, the separation between gonad and adrenal gland or mesonephros is thin and irregular. Ovarian medullary tissues are not clearly demarcated from underlying mesonephric tissues. Testicular tubules are often separated from kidney structures by thin segments of connective tissue (Fig. 4-4A). During the first five months post-hatching, the connective tissues between gonad and adrenal gland (Fig. 4-5B) or mesonephrose thicken and separation between the organs becomes more pronounced.

Ovary

At 10 days after hatching, the ovarian cortex is composed of a series of interconnected germ cell nests (Fig. 4-2 A,C) containing oogonia clusters and Stage-1 oocytes. Stage-1 oocytes present meiotic chromosomes and begin to show physical interactions with adjacent somatic cells. Nests are separated by trabecula, peninsula-like extensions of the medullary connective tissue into the cortex layer. Some trabeculae, when sectioned at oblique angles, appear in cross-section as islands of PAS/PAMS reactive tissues within germ cell nests (Fig. 4-2A,C).

Unlike the cortex, the ovarian medulla presents a fine meshwork matrix of PAS and PAMS-positive materials. These materials encircle the lacuna (Fig. 4-1A,C) and define the basement membranes on which low, cuboidal epithelial cells of the lacunae rest. Lacunae spaces contain PAS/PAMS reactive secretory materials. On the edges of the lacuna spaces, round voids in this secretory material suggest the previous presence of lipophilic materials that histological clearing of the tissues removed. The cortex/medulla boundary is clearly defined by the limit of medullary PAS and PAMS-positive materials. The medullary region directly underlying the cortex, including the trabeculae, display greater PAMS reactivity compared to the remainder of medullary tissues.

One month after hatching, the ovarian cortex has greater numbers of oocytes with meiotic chromatin, but only Stage-1 oocytes (Fig. 4-2B,D). The cortex continues to be separated from the medulla by a distinct boundary of PAS and PAMS reactive materials. PAMS reactivity throughout the medulla, especially at the cortex/medulla boundary, is more robust than at one week after hatching. Sporadic somatic cells on the coelomic epithelium of the cortex show PAS reactivity. In the medulla, lacuna diameter increased while the amount of interstitial tissues decreased. Concomitant with the change in lacunae diameter, lacunae epithelial cells are more often squamous than low cuboidal. Secretory materials in the expanded lacunae continue to be both PAS- and PAMS-positive and continue to show signs of lipid droplets along the margin of the lacunae spaces.

At three months after hatching, the ovarian cortex is thickened and is populated with oogonia and Stage-1 to -3 oocytes (Figs. 4-3A,C and Figs. 4-6A-C). Germ cell nests present single and multinucleated oocytes with condensed, meiotic chromatin and juxtanuclear Balbiani bodies in their ooplasms (Fig. 4-6A,C). Stage-2 oocytes have large spherical nuclei containing

lampbrush chromosomes (Fig. 4-6B), and PAS-positive Balbiani bodies (Fig. 4-7B) and are incompletely surrounded by follicular cells. Follicle assembly around Stage-2 oocytes is associated with a thin PAS-positive basement membrane (Fig. 4-7B), although it does not always fully surround the follicle. The basement membrane forming around Stage-2 oocytes is not PAMS-positive (Figs. 4-3D and 4-6B). An increased number of squamous somatic cells on the coelomic epithelium of the cortex show PAS reactivity (Fig. 4-3A) compared to one month after hatching (Fig. 4-2A).

Complete follicles enclose Stage-3 oocytes. Granulosa are bounded by a complete, monolayer of granulosa cells and present PAS/PAMS-positive basement membranes and oocyte cytoplasms have prominent PAS/PAMS-positive Balbiani bodies (Figs. 4-3A,C and 4-6C). Fibrous thecal layers are first observed surrounding Stage-3 follicles. PAS/PAMS-positive fibers intertwine thecal layers and segregate the follicles from the remainder of the germinal nests of the cortex. Stage-3 oocytes are always found in contact with some aspect of the cortex/medulla interface and the PAS/PAMS-positive fibrous materials of the basement membranes are often contiguous with morphologically similar medullary fibers (Figs. 4-2A,C and 4-5C). Most epithelial cells of the lacunae are squamous and often directly juxtapose epithelial cells of adjacent lacunae due to the lack of interstitial tissues (Figs. 4-2A,C and 4-5B,C).

At five months after hatching, proliferation of Stage-2 and -3 oocytes contributes to the continued thickening of the cortex (Figs. 4-3B,D and 4-7A,B). The expanded sizes of these oocytes results in convolution of germ cell nests in the cortex. Nests now wrap around the enlarging follicles, disrupting their previous, relatively uniform arrangements. PAS-positive materials around somatic cells located at the coelomic epithelium are more prevalent (Figs. 4-3B and 4-7A,B). Multi-oocytic follicles, two or more oocytes in a follicle encompassed by a single

basement membrane, are infrequently observed (Fig. 4-7A). The morphology of medulla at five month after hatching is similar to that observed at three months.

In the most posterior aspect of some ovaries of various ages is a small, distinct region of tissue, previously termed the "medullary rest" (Forbes, 1937). If present, this medullary rest is posterior to the termination of the cortex and adjacent to the posterior end of the medulla. The medullary rest lies under a thick tunica albuginea and contains poorly formed tubules, defined by PAS-positive basement membranes (Fig. 4-7C). The interior of these tubules contain cells with germ cell-like morphologies. Squamous cells intercalate within the tubule basement membranes. Around the tubules, interstitial tissues contain cells with Leydig-like morphologies.

Testis

One week after hatching, single layers of Sertoli cells and spermatogonia resting on a basement membrane line seminiferous cords (Fig. 4-4A,C). Sertoli cytoplasmic projections fill the cords and lumens are not observed. Cords are demarcated by fine PAS- and PAMS-positive basement membranes that are internal to simple squamous layers of peritubular myoid cells. Interstitial tissues are sparse. Leydig cells are not observed and interstitial cells present irregularly shaped nuclei. Alligator testes are not divided into lobules by way of fibrous septa. Within a thin testicular capsule seminiferous cords branch and anastomose freely. A tunica serosa of squamous cells lies over a fibrous, PAS-positive tunica albuginea (Fig. 4-4A) and the coelomic aspect of the testicular capsule is not PAMS reactive (Fig. 4-4C).

One month after hatch, the tunica albuginea is thicker than at one week after hatching and is both PAS- and PAMS-positive (Fig. 4-4B,D, respectively). In the tunica albuginea, squamous cells are interspersed with fibers running parallel to the coelomic epithelium. The volume of interstitial tissue is greater than at one week and small clusters of cells now exhibit Leydig-like morphology with round nuclei and larger cytoplasms. A more robust PAMS-positive layer

bounds seminiferous cords. Within the cords, spermatogonia, with large round nuclei, are sporadically found lying off the basement membrane (Fig. 4-4B). These putative spermatogonia type B did not display meiotic chromatin.

At three months after hatching, testes exhibit distinct morphological changes. Seminiferous cords observed at one month after hatching are now seminiferous tubules containing germ cells in a variety of meiotic states (Fig. 4-5A,C). Toward the center of the tubules are primary spermatocytes with increased nuclear and cytoplasmic sizes and condensed chromatin. Thin PAS- and PAMS-positive basement membrane and surrounded by simple, squamous layers of peritubular myoid cells continue to define seminiferous tubules. However, these myoid cells show an increased frequency of overlapping cytoplasms giving them a pseudo-stratified appearance. The volume of interstitial tissues has further increased and presents clusters of Leydig cells with relatively large well-defined cytoplasm, surrounded by PAS- and PAMS-positive fibrous ECM. The tunica albuginea continues to thicken. Fenestrations in the tunica albuginea that join with seminiferous tubules are observed (Fig. 4-5A,C). At these fenestrations, the PAS- and PAMS-positive basement membranes of the seminiferous tubules merge with fibrous components of the testicular capsule.

Morphological changes initially observed at three months after hatching continue at five months after hatching. The tunical albuginea thickens and continues to exhibit infrequent fenestrations to the coelomic cavity. Interstitial tissue volumes expand and present large clusters of Leydig cells, often found within a matrix of PAS and PAMS-positive fibers (Figs. 4-5B,D and 4-8A-C). Seminiferous tubules contain developing germ cells including primary spermatocytes and round spermatids (Figs. 4-5B,D and 4-8A-C). Round spermatids present dense nuclei without visible chromosome fibers. Apical Sertoli cell cytoplasmic projections do not fully fill

some tubules containing early, round spermatids, suggesting the formation of a luminal compartment in these tubules (Figs. 4-5B,D and 4-8A-C). Infrequently, clusters of round cells lay on the coelomic side of the tunica albuginea. These cells do not resemble squamous cells of the tunica serosa and putatively are germ cells that are a remnant of incomplete cortex regression during gonadal differentiation (Fig. 4-8C).

Discussion

During the first five months after hatching, we observed profound morphological changes in alligator gonads. In ovaries, germ cells entered into meiotic progressions, follicular cells organized around oocytes, steroidogenic cells organized around follicles, and medullary regions transformed into a matrix of expanded lacunae. In testes, we observed meiotic germ cells, clusters of steroidogenic cells became apparent, interstitial tissues expanded, and both seminiferous tubules and the testicular capsules matured. Intercalated within these gonadal architectural changes were pronounced developments in the ECM that we visualized using histochemical stains. PAS-reactivity clearly delineated the ovarian cortex and medulla, marked the formation of follicular basement membranes, defined the lamina propria of seminiferous tubules, and showed the thickening of the testicular capsule. PAMS-reactivity marked the maturation of follicles in both their basement membrane and Balbiani bodies and also testicular composition of the lamina propria, interstitial space, and capsule. In light of these observations, we propose that the gonadal ECM is playing a role beyond that of acting only as a structural framework for the organ.

Follicle assembly and early maturation observed in this study agrees with previous observations in which one-month-old alligator ovaries exhibit only Stage-1 oocytes, whereas three-months-old ovaries had Stage-1 to -3 oocytes (Moore et al., 2008). Here we have demonstrated that in five-month-old ovaries, the frequency of Stage-2 and-3 oocytes increases

compared to three months. Additionally, we have observed the formation and modification of ovarian ECM during this developmental period.

Ovarian follicular ECM is a spatially and temporally dynamic structure that remodels in accordance to the developmental state of the follicle (Berkholtz et al., 2006a; Irving-Rodgers and Rodgers, 2006; Mazatid et al., 2005). A distinct temporal pattern of laminin and collagen formation around mouse follicles has been observed (Berkholtz et al., 2006a). During folliculogenesis, between the vascularized theca and the avascular granulosa a basement membrane is deposited. The basement membranes of primordial follicles, composed of laminin, encompass the exterior of the granulosa. Later, follicles acquire type I collagen during the transition to primary follicles and type IV collagen and fibronectin as the granulosa expands in secondary follicles. Type IV collagen is also found predominantly in thecal layers and, to a lesser extent, in granulosa and stroma. As follicular maturation commences, type IV collagen increases around the theca and thickens between granulosa and stroma.

Postnatal follicle assembly is similar in pigs and rabbits. In one-day-old pig ovaries, germ cell nests are encircled by laminin-positive basement membranes and contain mitotic oogonia and meiotic oogonia (Lee et al., 1996). Follicle assembly in these nests commences where they juxtapose the medulla, forming primordial follicles that exhibit laminin-positive basement membranes. Similar to the pig, five-day-old rabbit ovaries display germ cell nests circumscribed by a laminin-positive matrix, whereas collagen fibers are not detectable (Lee et al., 1996). At 14 days post-natal, basement membranes encircle rabbit primary follicles. In post-natal ovaries of both pig and rabbit, laminin-positive materials are observed spanning from the ovarian surface epithelium to the ovarian rete and define the apical and basal aspects of germ cell nests. Additionally, collagen is not observed until the initiation of follicle maturation.

The changes in ovarian ECM associated with alligator follicle assembly are in agreement with these observations. From one week to one month after hatching, a PAS-positive ECM along the cortex/medulla boundary and at the cortex /coelomic boundary bound germ cell nests by distinct aggregates. This localization of PAS-reactivity is similar to laminin-IR observed previously at hatching (Smith and Joss, 1995). Three months after hatch, PAS-positive material at the cortex/coelomic boundary is more pronounced and the cortex/medulla boundary becomes increasingly convoluted with the initiation of follicle assembly. Stage-2 oocytes begin to exhibit PAS-positive basement membranes concomitant with increasing interaction with follicular cells. Stage-3 oocytes enclosed in follicles with complete complements of follicular cells and a theca layer present a PAS- and PAMS-positive follicular ECM layer, indicative of the presence of both laminin and type IV collagen.

The formation of a thecal layer concomitant with the detection of PAMS-positive materials suggests an interaction of collagenous ECM development and an alteration in the follicles steroidogenic capacity. Stage-3 follicles are always observed with some direct contact to the cortex/medulla boundary. In chicken ovaries, granulosa cells of small follicles are steroidogenically inactive (Nitta et al., 1993) and steroidogenic cells recruit to theca layers from the medulla (Narbaitz and Derobert, 1968; Pedernera et al., 1988a; Sekido and Lovell-Badge, 2007). The direct connections of PAMS-positive fibers of the medulla and those of the theca layers of Stage-3 oocytes supports the hypothesis that the recruitment of steroidogenic cells into the alligator cortex layer is similar to that observed in chickens (Moore et al., 2008). A small percentage of follicles contained more than one Stage-3 oocyte. These multioocytic follicles (MOFs) did not present similar PAS- or PAMS-positive material between the multiple oocytes as was observed between the oocytes and follicular cells. A similar morphology has been observed

in six-month-old ovaries in which Stage-3 follicles and a few multi-oocytic follicles "extend more deeply into the medulla" (Forbes, 1940).

Additionally, PAS-positive Balbiani bodies that are observed in Stage-1 through -3 oocytes gain PAMS reactivity in Stage-3 oocytes. Balbiani bodies of birds are reactive to silver stains (Guraya, 1962); however, how this shift in reactivity relates to development or maturation of Balbiani bodies is unknown.

The ovarian medulla undergoes substantial remodeling during the post-hatching period. At 10 days after hatching, the ovarian medulla is primarily PAS-positive and shows signs of fragmentation. PAMS reactivity is observed primarily in the basement membrane underlying epithelial cells of the lacunae. Within the lacunae, PAS- and PAMS-positive secretory materials presented peripheral spherical spaces indicative of lipid deposits removed during histological clearing. In hatchling chickens, steroidogenic cells derived from the primary sex cords reside in the medulla (Gonzalezmoran et al., 1985; Narbaitz and Derobert, 1968). If these steroidogenic cells are also present in the alligator medulla, these putative lipid droplets could supply substrates for steroidogenesis.

During this post-hatching period, PAMS reactivity in the medulla increases as lacunae expand and interstitial tissues diminish. At one year of age, the alligator medulla consists of numerous thin strands of laminin-IR connective tissue which form the walls of lacunae (Ferguson and Joanen, 1983; Smith and Joss, 1995). The progression of morphological developments observed in this study over the first five months post-hatching is in agreement with the observed morphology in one-year-old alligator medulla. We hypothesize that the alligator ovarian medulla is a responsive, endocrine tissue and is capable of influencing ovarian

development (Edwards et al., 2006b; Moore et al., 2008). Therefore, these morphological changes most likely influence the physiology of the entire ovary.

At the posterior aspect of ovaries throughout the growth period, we observed small adjunct tissues previously referred to as a 'medullary rest'. These tissues have been previously observed in ovaries of 18- and 21-month-old alligators and were noted to resemble testis of 6-month-old alligator (Ferguson and Joanen, 1983; Forbes, 1937, 1938) and our current observations support these previous studies.

During the first five months after hatching, profound changes occurred in testicular morphology. From a 10-day-old testis containing spermatogenic cords invested with spermatagonia resting against the basal lamina, we observed subsequent maturation resulting in spermatogenic tubules containing meiotic and post-meiotic germ cells in the tubule interiors and putative lumen formation. This germ cell maturation was concomitant with increased interstitial tissues, the establishment of prominent Leydig cell clusters, and increased PAS and PAMS reactivity of the tunica and interstitial regions. We observed the greatest maturation during the prodigious body mass doubling observed between three and five months post-hatching. Therefore, to put these morphological observations in context, an examination of interactions between alligator body size or condition, sexual maturation, and seasonality is necessary.

The minimum size for sexual maturity in male alligators has been reported as ~100 cm SVL (Joanen and McNease, 1980). In wild-caught adult alligators, initiation of spermatogenesis was observed in mid-February (Gribbins et al., 2006; Lance, 1989) and sperm has been found in the penile sulcus of wild-caught alligators from May to June (Joanen and McNease, 1980). Spermiation ceased in late June at the conclusion of the breeding season and was followed by testicular regression (Gribbins et al., 2006; Joanen and McNease, 1980). However, alligators

held in controlled environments have shown deviations from seasonal reproductive and growth cycles. Sperm has been observed in the penile sulcus of animals kept in a heated enclosure in mid-winter (Cardeilhac, 1981). Alligator growth can be limited by resource availability, habitat suitability, population density, growing season length, and salinity (Saalfeld et al., 2008), but temperature is a primary factor modulating growth (Lance, 2003). Alligator growth is more rapid in heated, controlled environments than in the wild (Coulson and Hernandez, 1983; Herbert et al., 2002; Joanen and McNease, 1989). If animals are raised in heated enclosures (~31°C) with ambient photoperiod, alligator growth can be decoupled from seasonal patterns and exhibit steady increasing body mass and length during the first year of growth (Herbert et al., 2002). Conversely, low temperatures or poor nutrition can impede sexual maturity (Lance, 2003).

Our group has demonstrated seasonal elevations of circulating testosterone concentrations in wild male alligator that have attained a SVL > 38 cm (Rooney et al., 2004). In wild juvenile Florida alligators, a strong correlation has been observed between SVL and BM (Guillette et al., 2000a) and also SVL and age (Milnes et al., 2002). These studies were performed using animals from a defined geographic range of Florida that share similar environmental conditions. In contrast, animals in this study experienced rearing conditions that were quite different from those experienced by wild animals: stable elevated temperature, lack of environmental variability, lack of predator pressures, and constant access to food. This resulted in alteration of the growth trajectory with our captive-raised animals, attaining greater body masses per unit SVL than field-caught animals. Furthermore, field-caught alligators with a SVL of 38 cm have body masses (~1100 g) that are similar to those of five-month-old, laboratory-raised alligators with SVLs of ~32 cm. We propose that an initiation of sexual maturation marked by increases in circulating testosterone concentrations observed in field animals > 38 cm

could be more related to an attainment of a threshold body mass or condition index than of a specific length, in light of the testis maturation observed in five-month-old laboratory animals with similar body masses, but shorter SVLs.

In light of this prodigious body growth and putative precocious gonadal maturation, we observed a testicular maturational pattern more similar to domestic chickens than wild alligators. In hatchling chicken testes, seminiferous tubules do not exhibit lumens and are surrounded by compact interstitial tissues of undifferentiated mesenchyme and few Leydig cells (Gonzalez-Moran, 1997). Spermatogonia proliferate concomitantly with an increase in tubule diameter at 5 weeks of age; spermatocytes are observed at week six (Kumaran and Turner, 1949). With the initiation of meiosis, spermatids are observed between weeks 8-12 post-hatch (Bergmann and Schindelmeiser, 1987; Kumaran and Turner, 1949). Leydig cell growth accompanies tubule expansion. This morphological progression in chicken testis over three months has temporal and morphological similarities to our observations in alligator testes over the five months post-hatching and is clearly in conflict with a maturational timeline of wild, male alligators. But it should also be noted, that the traditional description of testicular maturation/sexual maturation at 1m SVL in wild animals is also unlikely as wild juvenile males of 38 cm snout vent length or greater exhibit seasonal variation in plasma testosterone concentration and their testes are likely to be similar in development to that described in the present study.

A comparison of the morphological development observed in these laboratory-raised alligator testes to other vertebrates can provide a more refined characterization of the level of maturation in these animals. Comparing the morphological development observed in these laboratory-raised alligator testes to other vertebrates, we can better characterize the level of maturation observed in these animals. In many vertebrates, male germ cell development begins

in the basal epithelial compartment of the seminiferous tubule, where they are sheathed in the cytoplasmic folds of Sertoli cells (Siu and Cheng, 2004b). As development continues, germ cells migrate inward, toward the medial aspect of the tubule, and eventually exit the blood-testis barrier (BTB) formed by tight junctions between Sertoli cells and enter the apical/adluminal compartment. The BTB is an immunological barrier segregating post-meiotic germs cells from the systemic antibodies of the host organism. Germ cells developed up to preleptotene spermatocyte stage (diploid) are found within the BTB whereas pachytene spermatocytes (haploid) and later developmental stages are found outside of the BTB. A BTB is formed either before puberty (mouse, man), during pubescent initiation of meiosis in others (dog, rabbit), or after the onset of meiosis (reptiles, birds) (Bergmann et al., 1984; Siu and Cheng, 2004b). In chicken testis, BTB formation occurs around eight weeks of age, after the initiation of meiosis, and concomitant with early spermatid (haploid germ cell) formation (Bergmann and Schindelmeiser, 1987). Chicken tubules containing these early spermatids and BTB do not possess lumens. We observed round spermatids (haploid) and evidence of lumen formation in the testes of five-month-old laboratory-raised alligators. If alligator testis maturation is similar to chicken, then these animals should have produced a BTB. Further research is needed to characterize BTB ontogeny in crocodilian testes.

Recent characterization of alligator spermatogenesis (Gribbins et al., 2006) has allowed new insight into post-hatching alligator testicular development. A previous investigation of six-month-old alligator testes by our group showed seminiferous tubules containing both spermatogonia and meiotic germ cells and interstitial spaces containing Leydig cell clusters (Guillette et al., 1994a). Within this study, animals hatched from eggs collected from Lake Woodruff, a national wildlife reserve, and raised with supplemental heat displayed testes

containing germ cells developed up to round spermatid stage. The testes of five-month-old, Lake Woodruff animals described in this manuscript share many of the morphologies observed in the six-month-old animals from the previous study, including Leydig cell development and evidence of spermatogenesis. In contrast, six-month-old testes from animals hatched from eggs collected from Lake Apopka, a highly-contaminated environment (Guillette et al., 1999a), showed germ cells in seminiferous tubule centers that displayed "bar-shape nuclei". In light of the morphological maturation during alligator spermatogenesis (Gribbins et al., 2006), these cells are probably later-stage spermatids initiating nuclei elongation. Before dissection, these animals underwent an ovine luteinizing hormone (LH) challenge with daily injections for two days. This treatment significantly increased circulating estradiol-17β (E_2) levels in Lake Apopka animals (~3.3 times greater), but did not alter Lake Woodruff animal E_2 levels. Recent research has demonstrated that both LH and E_2 modulate the transition of round spermatids to elongating spermatids. Additionally, treatment with the aromatizable androgen testosterone propionate has induced the formation of elongated spermatids in juvenile crocodile testes (Ramaswam and Jacob, 1965).

In LHβ-subunit knockout mice, spermatogenesis is halted at the round spermatid stage (Ma et al., 2004). LH has been demonstrated to regulate aromatase expression in rat Leydig cells (Genissel et al., 2001) and epididymis (Shayu and Rao, 2006), while Sertoli and germ cells may also produce estrogens (Carreau et al., 2006). Mice with disrupted aromatase expression are infertile due to failure to elongate round spermatids (Robertson et al., 1999). Taken together, an LH challenge resulting in increased circulating E_2 levels provides a hypothesis explaining the production of elongating spermatids in six-month-old alligator testes. Why this response was observed in Lake Apopka animals, but not in Lake Woodruff animals is unknown, however,

research has demonstrated altered gonadotropin signaling and testis morphology resulting from herbicide exposure (Shariati et al., 2008).

During the five-month post-hatching period, we observed thickening of an originally thin and discontinuous testicular capsule. The alligator tunica is similar to testicular capsules of birds being composed of a thin tunica serosa, an outermost layer formed from the peritoneum, overlying the tunica albuginea composed of a thick layer of smooth muscle-like cells, fibroblasts, collagen bundles, and elastic fibers (Aire and Ozegbe, 2007). We observed both PAS- and PAMS-positive staining of the tunica albuginea, but not the tunica serosa. The capsule encompassed the entire testis and did not divide the sex cords into lobules. In contrast, testes of pituitary extract treated 21-month-old alligators displayed a thickened tunica and septa dividing testis lobules, though septa were not observed in control animals (Forbes, 1937). Our observations are in agreement with a lack of septa in juvenile alligator testes, similar to the morphology of bird testes (Aire and Ozegbe, 2007).

In association with tunica development, we observed two intriguing and possibly associated morphologies. Clusters of cells resembling germinal epithelium were infrequently found on the coelomic aspect of the tunica. Putative germ cells on the peritoneal side of the tunica albuginea have been previously observed in post-hatching alligator testes (Forbes, 1937, 1940). Furthermore, these clusters were more frequently observed at six months than at three months, initiating a hypothesis that these cortical cells could proliferate during this time period (Forbes, 1940). In this study, these clusters were more often observed in older animals in which we noted the initiation of spermatogenesis and also openings in the tunica where the medullary cords directly interfaced with the peritoneum. These fenestrations connected medullary tubules containing meiotic germ cells to the external aspect of the tunica, a morphology previously

observed in 6, 15, 18, and 21-month-old alligators (Forbes, 1937, 1938, 1940). Furthermore, clusters of germ cells have been previously observed passing through these openings in the tunica albuginea and forming "isolated cortical proliferations". With *in vivo* estrone treatment these cortical projections proliferated and expanded over the peritoneal tunica (Forbes, 1938), therefore, demonstrating these cells show endocrine responsivity. Research describing primitive sex cord formation in turtles showed cord formation resulted from an invagination of the coelomic epithelium with the basal lamina of sex cords being continuous with basal lamina of the coelomic epithelium (Yao et al., 2004a). We propose the fenestration observed in alligator testes may be developmentally related to the invaginations observed in turtle testes, with the coelomic epithelium giving rise to somatic cells of the sex cords.

From our current and previous observations, hypotheses regarding these cortical cells can be formulated. First, these cortical cells may be remnants of the regressing cortex during sex differentiation that become more prominent during later post-hatching development. Second, these cortical cells are derived from the medulla through either an incomplete tunica during early testicular development or from medullary tubules opening through pronounced fenestration in the tunica. Lastly, cortical cells could be derived through a combination of both mechanisms. Regardless of the mechanism, the presence of germ cells on the testis coelomic epithelia and the medullary rest morphology observed in ovaries shows that post-hatching alligator gonads display distinctive morphologies that deviate from archetypical testis and ovary.

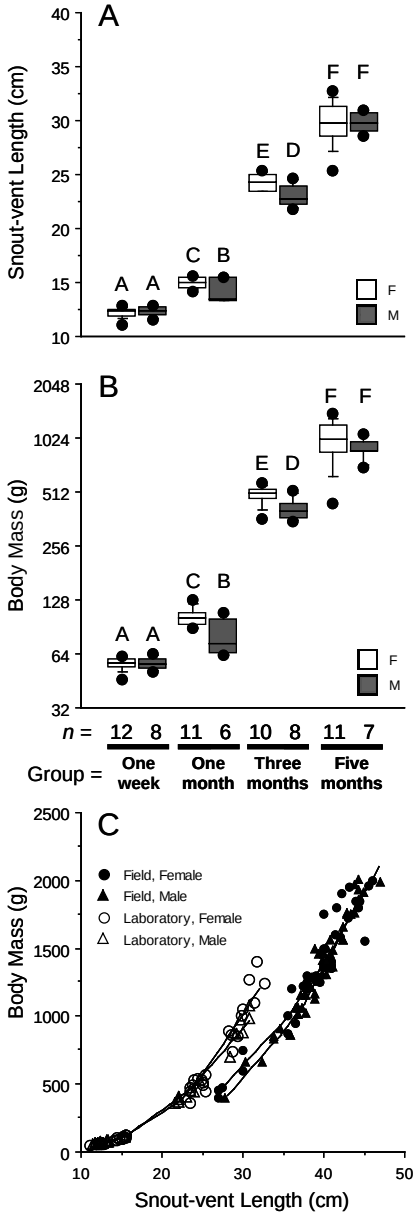

Figure 4-1. Alligator growth over first five months post hatching. A,B) Box plot diagrams of snout-vent length (A) and body mass (B, note log2 scale) for dissected animals grouped by age and sex (open boxes = female, grey boxes = male). Boxes define 75^{th} to 25^{th} percentiles and are bisected by the mean values. Whiskers define 90^{th} and 10^{th} percentiles with outliers (circles) above and below. Superscripts over boxes denote differences between groups ($P > 0.05$). Sample sizes for each group are set below graphs. **C)** A scatterplot diagram of snout-vent length and body mass for laboratory-raised animals (open figures) and field-captured, Lake Woodruff juvenile animals (solid figures). Males = triangles, females = circles. Individual locally weighted scatter plot smoother (LOWESS) curves (tension = 50) are plotted for each group.

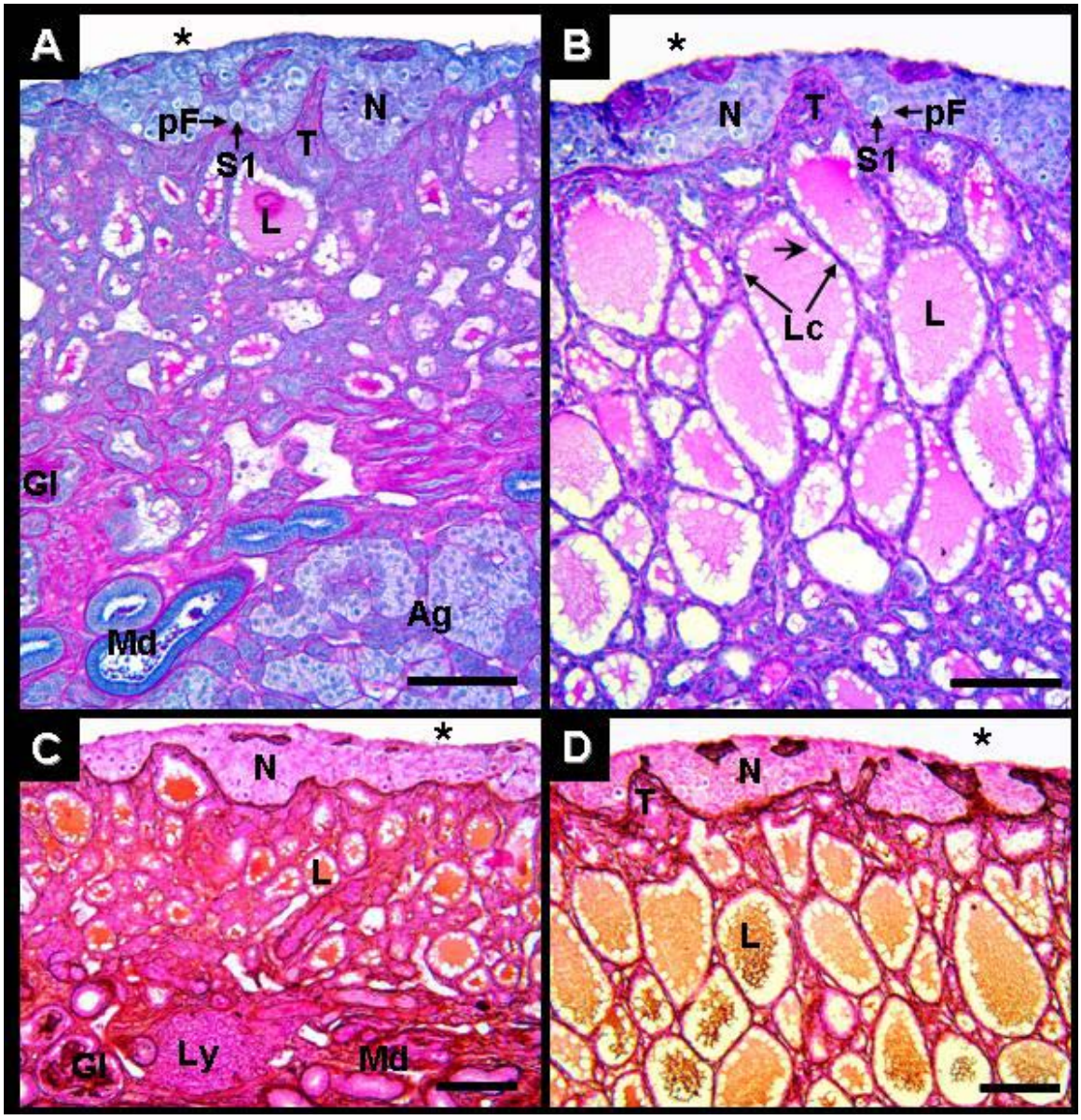

Figure 4-2. Ovary of *Alligator mississippiensis* at one week (A,C) and one month (B,D) after hatching in parasaggital view (A,B - PAS/AB; C,D - PAMS). The ovarian cortex contains nest of germ cells (N) defined by connective tissue trabeculae (T) along the coelomic (*) border. These nests contain Stage-1 oocytes (S1) in close interaction with prefollicular cells (pF). Extracellular components of the medulla are robustly PAS- (A,B) and PAMS- (C,D) positive, clearly delimiting the reactive medulla from cortex of germ cell nests. At one week after hatching (A,C), degenerating medullary cords form lacunae (L). At one month after hatching (B,D), the medullary lacunae are expanded, contain PAS/PAMS-positive material, and show characteristic remnants of lipid droplets (arrowhead). Squamous and low cuboidal epithelial cells (Lc) line lacunae. The gonads overlie adrenal tissue (Ag) and mesonephros tissue containing glomeruli (Gl), mesonephric ducts (Md), and lymphatic aggregates (Ly). Scale bars = 100 *um*.

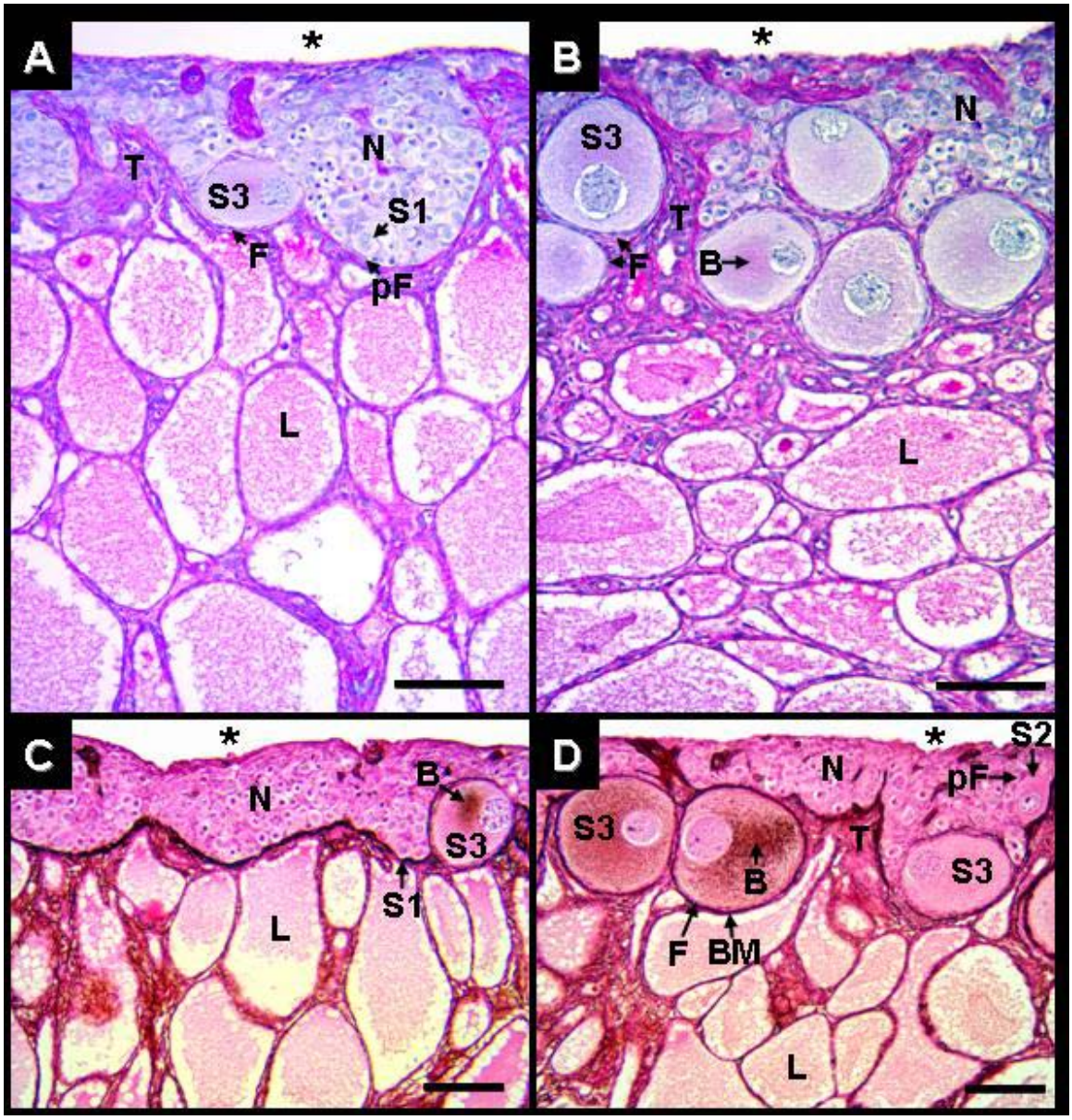

Figure 4-3. Ovary of *Alligator mississippiensis* at three months (A,C) and five months (B,D) after hatching in parasaggital view (A,B - PAS/AB; C,D - PAMS). Ovarian cortex contains oogonial nests (N) and Stage-1 through -3 oocytes. Compared to Stage-1 oocytes (S1), Stage-2 oocytes (Fig. 2 C,D, respectively) have larger ooplasms and nuclei and a more complete complement of prefollicular cells (pF). Stage-3 oocytes (S3) display large ooplasms containing Balbiani bodies (B), are contained in follicles defined by contiguous rings of follicular cells (F), and a PAS/PAMS-positive basement membrane (BM). PAS/PAMS-positive connective tissues and trabecula (T) radiating from underlying medullary tissues containing lacunae (L) clearly delimits the cortex. Scale bars = 100 *um*.

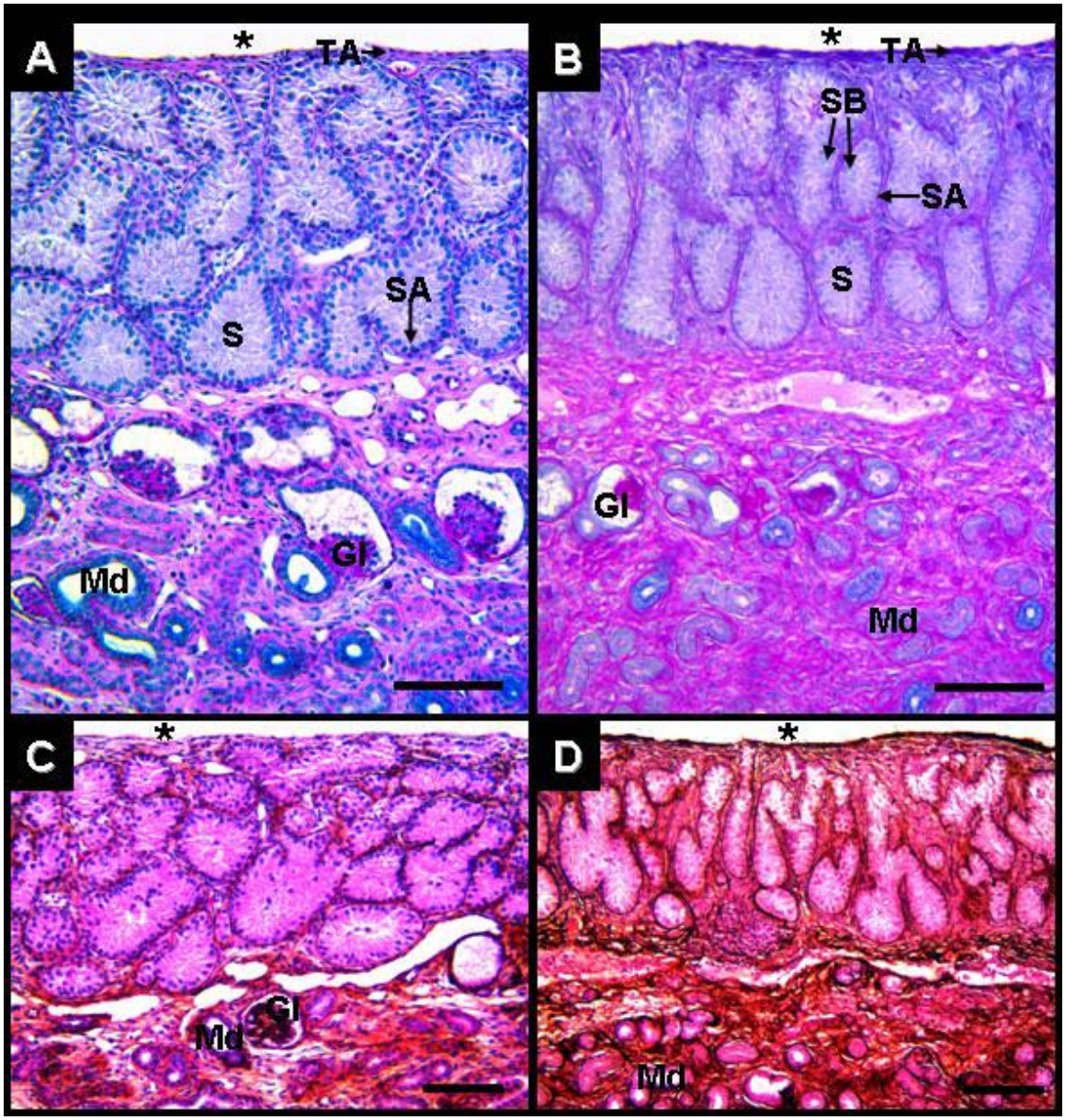

Figure 4-4. Testis of *Alligator mississippiensis* at one week (A,C) and one month (B,D) after hatching in parasaggital view (A,B - PAS/AB; C,D - PAMS). The coelomic (*) border is defined by a thin tunica albuginea (TA). The tunica is PAS-positive at both one week and one month after hatching (A,B), while a PAMS reactive tunica is observed in one month old animals (D) The gonads contain well defined seminiferous cords (S) lined with spermatogonia A (SA). At one month after hatching, occasional spermatagonia B (SB) with enlarged cytoplasms are observed. The gonad overlies mesonephros tissue containing glomeruli (Gl) and mesonephric ducts (Md). Scale bars = 100 *um*.

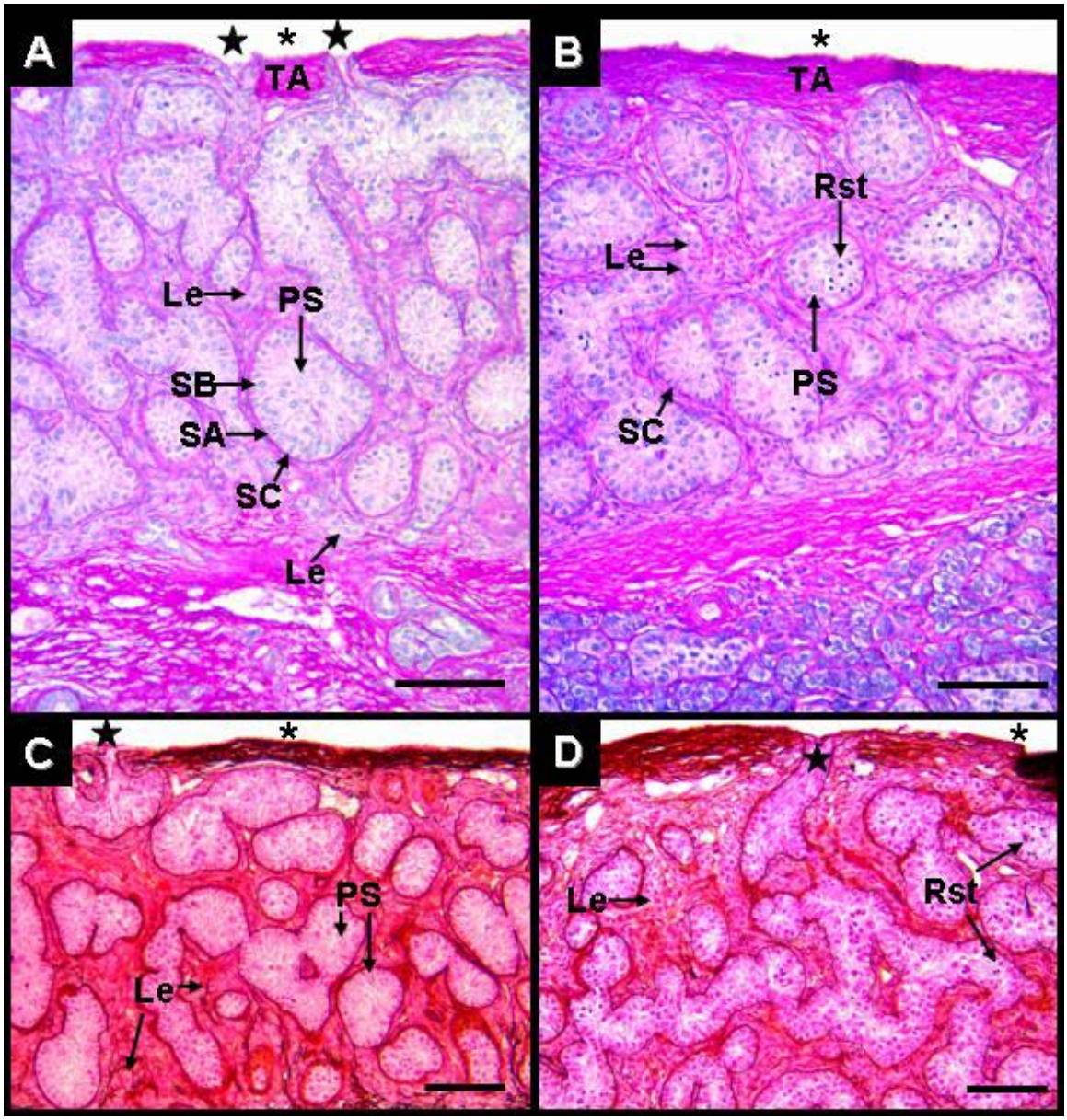

Figure 4-5. Testis of *Alligator mississippiensis* at three months (A,C) and five months (B,D) after hatching in parasaggital view (A,B - PAS/AB; C,D - PAMS). Compared to one week and one month testes (Fig. 3), distinct morphological changes are apparent. The tunica albuginea (TA) thickens and becomes intensely PAS/PAMS-positive, though has occasional fenestrations (stars). Interstitial tissues (I) between seminiferous tubules expand and present Leydig cells (Le). Seminiferous tubules present Sertoli cells (SC), spermatogonia A and B (SA and SB, respectively) and primary spermatocytes (PS) at three months after hatching and present round spermatids (Rst) at five months after hatching. Scale bars = 100 *um*.

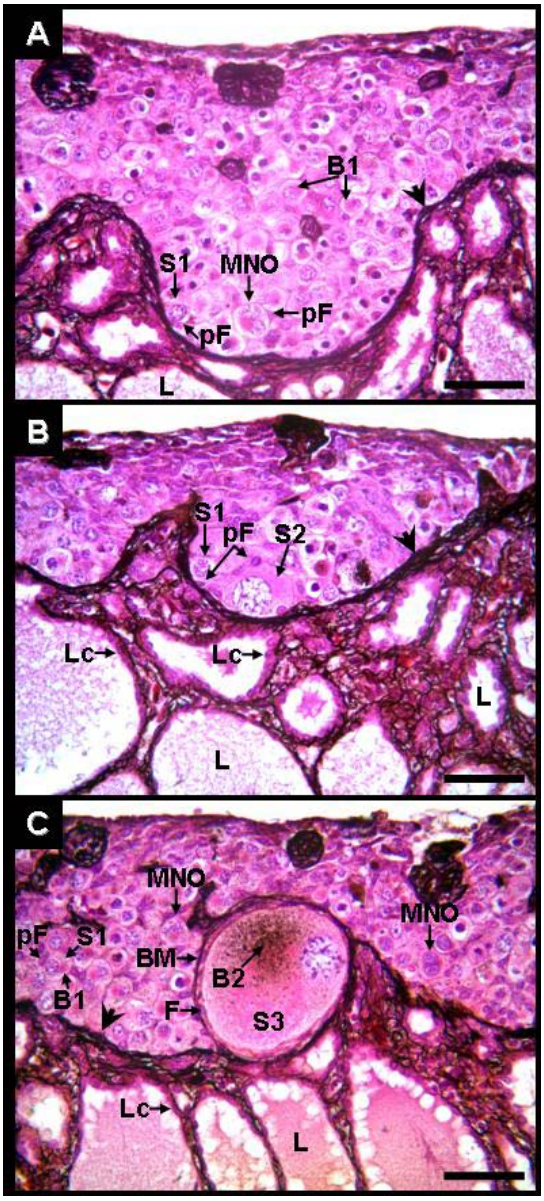

Figure 4-6. Ovary of *Alligator mississippiensis* at three months after hatching (**A-C**) in parasaggital view (PAMS) showing nests of germ cells in various stages of oocyte maturation. Cortical germ cell nests are clearly delimited by PAMS-positive fibers (large arrowheads) which are contiguous fibers within the ovarian medulla. Lacunae epithelial cells (Lc) lay upon a basement membrane composed of PAMS-positive fibers. Stage-1 oocytes (S1) with early Balbiani bodies (B1) are in association with prefollicular cells (pF). Some Stage-1 oocytes with diplotene chromatin are multi-nucleated (MNO). Stage-2 and -3 oocytes are in cortical regions directly adjacent with the medulla. Stage-2 oocytes (S2) display enlarged ooplasm, but lack a complete layer of cells and surrounding basement membrane. Stage-3 oocytes (S3) are completely encircled by follicular cells (F) and a PAMS-positive basement membrane (BM) and often display a PAMS reactive Balbiani body (B2). Scale bars = 100 *um*.

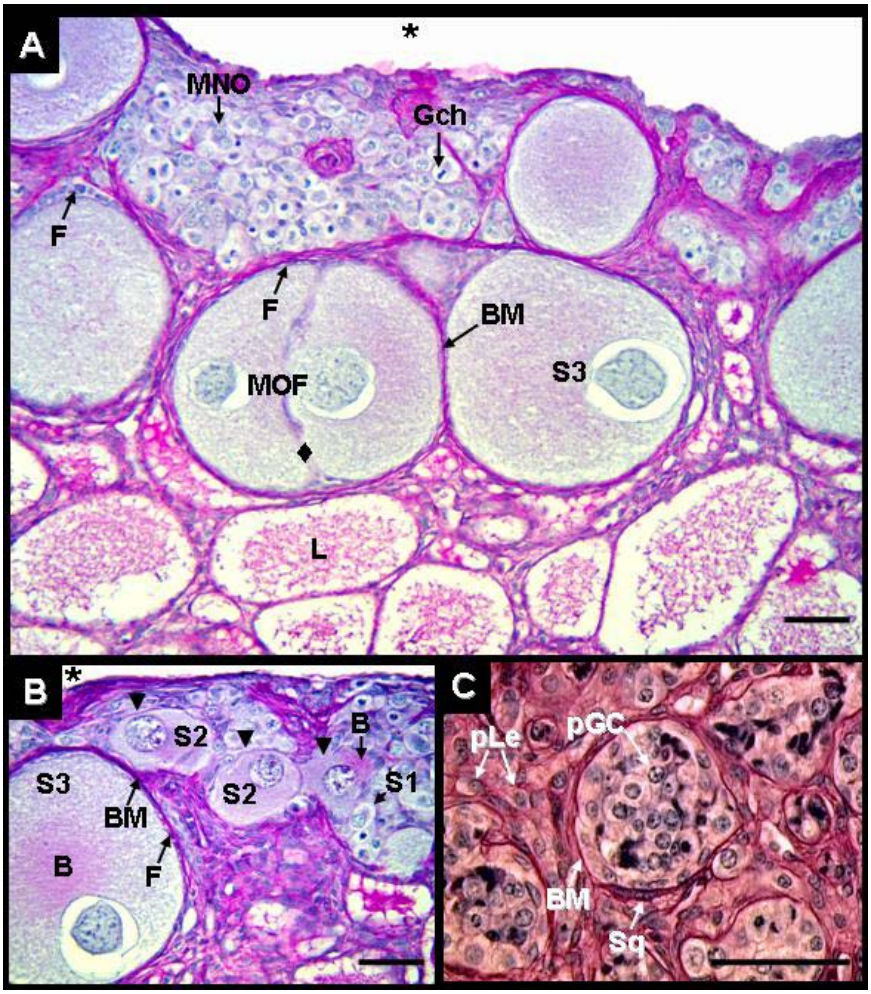

Figure 4-7. Ovary of *Alligator mississippiensis* at five months after hatching (**A-C**) in parasaggital view (PAS/AB) showing nests of germ cells in various stages of oocyte maturation and location. **A-B)** Cortical germ cells with mitotic chromosomes (Gch) and uni-nucleated (S1) and multinucleated (MNO) Stage-1 oocytes with diplotene chromatin are observed in germ cell nests. Stage-2 and -3 oocytes are observed in association with regions with direct connection to an adjacent medulla. Stage-2 oocytes (S2) display enlarged ooplasms, PAS-positive Balbiani bodies (B), and a thin, irregular PAS-positive basement membrane (black triangles), but lack a distinct full complement of encircling follicular cells. Stage-3 oocytes (S3) display a Balbiani bodies (B) which are PAS-positive. In contrast to Stage-2 oocytes, Stage-3 oocytes have a complete ring of follicular cells (F) and a distinct, encompassing PAS-positive basement membrane (BM). Stage-3 oocytes may be contained in a multioocytic follicle (MOF) with a lack of follicular cells and basement membrane demarcation between germ cells (diamond). Coelom = (*). **C)** Putative germ cells (pGC) are observed in tubules within the medullary rest of the alligator ovary. A PAS-positive basement membrane (BM) associated with squamous, myoid-like cells (Sq) encompasses an aggregate of putative germ cells with large cytoplasms and displaying nuclei with fibrous chromatin. Interstitial tissues display cells with Leydig cell morphology (pLe). Scale bars = 50 *um*.

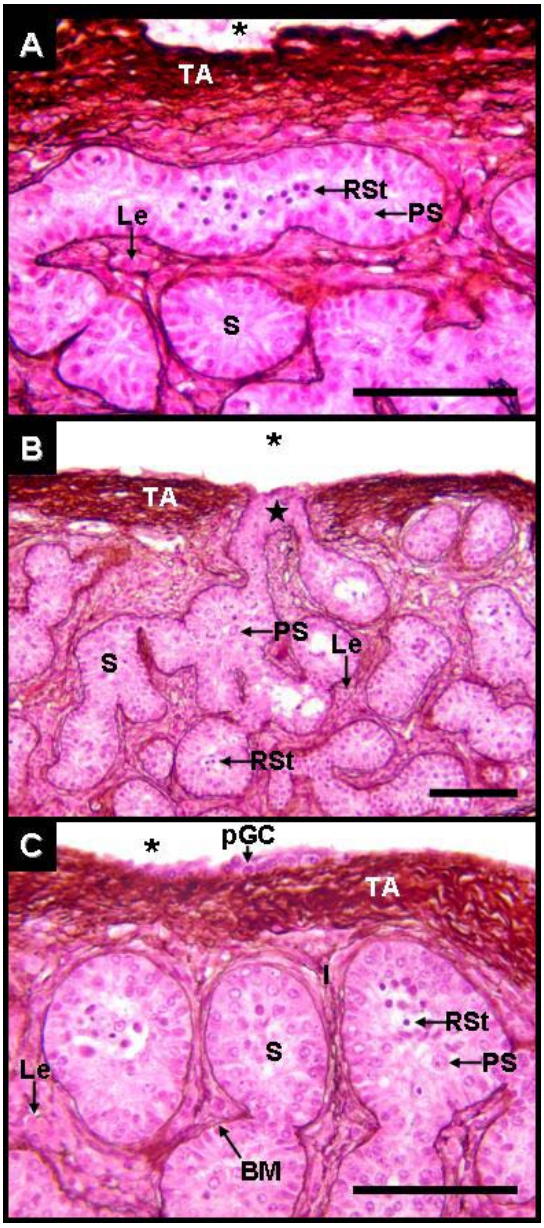

Figure 4-8. Testis of *Alligator mississippiensis* at five months after hatching (**A**-**C**) in parasaggital view (PAMS). Seminiferous tubules (S) lie within a robust tunica albuginea (TA) of PAMS-positive fibers bordering the coelom (*). Occasional a fenestration connect seminiferous tubules to the coelomic cavity (star). Primary spermatocytes (PS) and round spermatids (RSt) are within some tubules. Tubule basement membranes (BM) and surrounding interstitial tissues show PAMS-positive fibers. Clusters of Leydig cells (Le) are interspersed within the interstitial tissues. Putative germ cells are occasionally observed on the coelomic side of the tunica (pGC). Scale bars = 100 *um*.

CHAPTER 5
GONADAL MRNA EXPRESSION LEVELS OF TGFB SIGNALING FACTORS CORRESPOND WITH POST HATCHING MORPHOLOGICAL DEVELOPMENT IN AMERICAN ALLIGATORS

Introduction

The timing of vertebrate gametogenesis shows striking differences. Most amniote female germ cells 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 five 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 is considerable difference in the timing of initial follicular somatic-germ cell interactions. As oogonia enter into meiotic prophase I, cytoplasmic bridges between germ cell syncyntia break down (Matova and Cooley, 2001; Pepling, 2006; Pepling et al., 1999). 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 post-natal immature period (e.g., rabbit, dog). Non-mammalian vertebrates show similar variability with birds assembling follicles during the post-hatching period (Callebaut, 1968a; 1968b). Some reptiles form follicles as embryos (Forbes, 1956), whereas follicle assembly occurs prior to hatching in many turtle species (Pieau and Dorizzi, 2004). In the American alligator, we have demonstrated that oogenesis and follicle assembly occur over an

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 five months post-hatching and investigate concomitant mRNA expression changes of factors involved in ovarian maintenance and 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. Little research has investigated juvenile or alligator testicular development; though it is assumed that wild alligators have a prolonged 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 (Guillette et al., 1999b; 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 degeneration of the germinal epithelium (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). We have observed that ovarian development accelerates in animals held under optimum, laboratory conditions (JMorph paper). 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 various 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) ligand subunits dimerize to produce signaling factors, such as activins and inhibins, vital to reproductive maturation and function (Barakat et al., 2008; de Kretser et al., 2004; Drummond, 2005; Pangas et al., 2007). 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 two β subunits, βA (Inhba) or βB (Inhbb), form activin A (βAβA), activin B (βBβB), or activin AB (βAβB). 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 results in decreased 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 (βAα) or inhibin B (βBα). Ovarian inhibin expression is minimal prior to puberty (Raivio and Dunkel, 2002), however, it is vital to testis 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 plays both ovarian and testicular development roles (de Kretser et al., 2004; Yao, 2005a; 2005b; Yao et al., 2004b).

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 an oocyte-secreted TGFβ ligand that regulates folliculogenesis through directing granulosa cell proliferation and differentiation (Carabatsos et al., 1998; Dong et al., 1996; 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; Ottolenghi et al., 2007b; Yao, 2005a) 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; Rhen et al., 2007b; Uhlenhaut and Treier, 2006). 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, trays were rotated 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°C to 31°C. Alligators hatch with a relatively large residual yolk mass; thus *ad lib* hatchling feeding starting 10 days after hatching with a small pellet size, commercial crocodilian diet (Mazuri, #5MG1).

Subsets of animals were killed using a lethal dose of sodium pentobarbital at: one week (30°C n = 12, 33°C n=10), one month (30°C n = 11, 33°C n=9), three months (30°C n = 10, 33°C n=8), and five 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 hours, underwent standard paraffin histology, were sectioned parasagittally at 6 μm, and stained with periodic acid (PAS)-alcian blue (pH 2.5) with hematoxylin counterstain. Tissues 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= 30°C/33°C: one week 12/8, one month 11/6, three months 10/8, and five months 11/7). Body masses (BM) were recorded prior to necropsy (average age of groups at measurement in days: one week = 4.5 d, one month = 28.1 d, three months = 88.5 d, and five months = 145.6 d).

While examining ovarian histology, we observed morphological changes in both cortex and medulla. States of oogenesis and folliculogenesis were characterized at each time point in our study. Chromatin and cytoplasmic morphologies were used to determine 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. Germ cell maturation was characterized by cellular morphology and location within tubules, with insight from the recent characterization of adult alligator germ cell maturation (Gribbins et al., 2006).

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 (Gunderson et al., 2006; Katsu et al., 2004; 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, Hercules, CA) following manufacture'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. We calculated expression levels of mRNA 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 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 regressed by sex and age group.

Results

Growth and Gonadal Morphology

Under our laboratory animal room conditions, alligator growth was prodigious over the period of this study (age- mean male BM (g) ± SEM, female BM ± SEM): one week- 56.9 ± 4.4, 56.7 ± 4.5; one month- 80 ± 19, 103 ± 12; three months- 412 ± 56, 493 ± 58; five months- 901 ± 118, 985 ± 81. At five months, the average alligator BM was more than 15 times greater than that recorded at one week of age. Female BM was greater than male at one and three months (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 5-1A-D). Mesonephric and adrenal tissues juxtapose gonadal tissues (Fig. 5-1A, 5-2A,D). During the five months after hatching, we observed cortex expansion and follicle assembly. At one week and one month after hatching, the cortex is composed of oogonia nests, S1 oocytes, and somatic pre-follicular cells (Fig. 5-1A,B). The medulla fragments and lacunae expand during this period. Cortex thickness increases at three and five months after hatching, concomitant with enlarging germ cell nests and follicle assembly around S2 and S3 oocytes (Fig. 5-1C,D). Cytoplasms of S2 and S3 oocytes exhibit Balbiani bodies. Follicles containing S3 oocytes have a full complement of granulosa cells and, externally, a thin layer of fibroblast forming thecal layers (Fig. 5-1G,H). At five months, S3 oocytes were more numerous than at three months and we continued to observe oogonia with mitotic chromatin within germ cell nests (Fig. 5-1H).

From one week to one month after hatching, alligator testes are characterized by seminiferous cords lined with Sertoli cells and spermatogonia A that are separated by sparse interstitial tissues (Fig. 5-2A,B,E and F). At three 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. 5-2C,G). At five months after hatching, seminiferous tubules contained centrally located round spermatids along with spermatogonia and spematocytes (Fig. 5-2 D,H). Interstitial tissues and Leydig cell clusters continued to enlarge.

Gene Expression

Expression levels of *Rpl8* mRNA did not different by sex, age, or sex by age. Levels of *Inhba*, *Inhbb*, *Inha*, *Fst*, *Gdf9*, *Pcna*, and *Cyp19a1* mRNA expression showed significant sex by age interactions (Fig. 5-3A-L,O,P; $P = 0.003$, $= 0.004$, < 0.001, < 0.001, $= 0.42$, $= 0.039$, and $= 0.008$, respectively). Female *Foxl2* mRNA expression levels were greater than male expression levels, which were at the limit of detection (Fig. 5-3M,N; $P > 0.001$). We observed additional sexually dimorphic expressions that were uniform across all ages. Testicular expression levels of *Inhbb* and *Inha* mRNA were greater than ovarian levels (Fig. 5-3C-F). Low female *Inha* expression levels were at the limit of detection (Fig. 5-3E). Testicular levels trended to decrease as age increased (Fig. 5-3D, F). Conversely, ovarian *Fst* and *Cyp19a1* mRNA expression levels were greater than that observed in testicular tissue (Figs. 5-3G,H and 5-3O,P, respectively). Testicular *Inhbb* levels were over an order of magnitude greater than *Inhba* levels (Fig. 5-3B,D). Ovarian *Inhba*, *Inhbb*, *Fst*, and *Cyp19a1* mRNA expression levels shared a similar expression pattern with decreased expression levels between one week and one month, followed by a trend to increase at three months and then decrease levels at five months (Fig. 5-3A,G, and O). In contrast, ovarian *Gdf9* and *Pcna* mRNA expression levels increased with age between one and three months (Fig. 5-3I, K). Testicular *Gdf9* mRNA expression was greater in month three and five than at one week and one month (Fig. 5-3J) and *Pcna* mRNA expression levels showed a increasing trend between three and five months after hatching (Fig. 5-3L).

Linear regression of ovarian *Foxl2* by *Cyp19a1* mRNA expression by age groups showed significant linear relationships at one week ($P = 0.005$, $R^2 = 0.56$) and three months ($P = 0.001$, $R^2 = 0.71$), however one month ($P = 0.40$, $R^2 = 0.02$) and five months ($P = 0.18$, $R^2 = 0.19$) did not showed correlations.

Discussion

During the first five months after hatching, we observed prodigious body mass growth, 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 by our laboratory, post-hatchling alligator gonads are not quiescent (Guillette et al., 1994a; 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 adult fertility.

Appropriate activin signaling is vital for both ovarian and testicular maturation (Loveland et al., 2007; Pangas et al., 2007). We observed sexually dimorphic expressions of *Inhbb*, *Inha*, and *Fst* in post-hatchling alligator gonads. In mammals and birds, ovarian *Inha* mRNA expression prior to puberty is minimal (Billiar et al., 2003; da Silva et al., 2004; Onagbesan et al., 2004; Raivio and Dunkel, 2002); 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 (Bristol-Gould et al., 2006; da Silva et al., 2004). 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 (Barakat et al., 2008; Buzzard et al., 2004; Meachem et al.,

2001). Sertoli cells proliferate for seven weeks after hatching in chickens (Bozkurt et al., 2007). Our observations of post-hatching elevated testicular *Inha* and *Inhbb* mRNA levels are inline with these observations. We hypothesize that decreasing testicular *Inha* and *Inhbb* mRNA levels over time could represent cessation of Sertoli cell proliferation or, alternatively, decreased concentration of Sertoli cell-derived mRNA in cDNA samples due to increased mRNA from increasing numbers of germ cells and interstitial cells.

In the developing mouse testes, *Inhbb* 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., 2004b). Conversely, *Fst* mRNA over expression leads to Leydig cell hyperplasia, an arrest of spermatogenesis, and seminiferous tubular degeneration (Guo et al., 1998). Taken together, elevated 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 three and five month old ovaries. This increase coincided with the assembly of complete follicles around S3 oocytes. This is in agreement with the reported roles of Gdf9 in primary and primordial follicle growth as it has been reported to promote granulosa and possibly thecal cell proliferation and differentiation (Trombly et al., 2009b). 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 three and five months after hatching. Further, rat testicular Gdf9 mRNA expression was greatest in round spermatids (Nicholls et al., In Press) and we observed the greatest mean expression of *Gdf9* mRNA in alligator testes at five months, at the time round spermatids appeared.

If *Gdf9* mRNA expression promotes somatic cell proliferation, we hypothesized that we could detect 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). We observed increased ovarian *Pcna* mRNA expression at three and five months after hatching compared to one month. During these later 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 lacked statistical significance.

Among vertebrates, *Foxl2* expression is vital for ovarian development (Ottolenghi et al., 2007b) and has high amino acid sequence identity in the functional forkhead domain (Oshima et al., 2008). In Foxl2 knockouts, *Fst* mRNA expression is decreased, meiotic prophase oocytes form, whereas primordial follicles do not assemble (Ottolenghi et al., 2005; Uda et al., 2004). As expected, we observed highly dimorphic *Foxl2* mRNA expression in juvenile alligator gonads. In pre-follicular chicken ovaries, *Foxl2* and *Cyp19a1* mRNA expression co-localize in the medulla (Govoroun et al., 2004) and show positively correlated expression levels during

embryonic ovarian development in turtles (Rhen et al., 2007b) and chicken (Govoroun et al., 2004). Finally, Foxl2 binds to and regulates the Cyp19a1 promoter (Pannetier et al., 2006; Wang et al., 2007) and Cyp19a1 transcript expression could potentiate a positive feedback on Foxl2 expression via estradiol-17β (Hudson et al., 2005).

Similar to *Foxl2*, juvenile alligator gonadal *Cyp19a1* mRNA expression was sexually dimorphic. We found evidence of Foxl2 regulation of Cyp19a1 in positive correlations at one week and three months, however not at one month or five months. We hypothesize that because one week encompasses the period of greatest post hatching medullary fragmentation and three months marks the initiation of follicle formation around S3 oocytes, greater estrogenic signaling could characterize 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, then rises during the following months (Belaid et al., 2001). Estrogens impede testicular cord formation and possibly maintains female medullary morphology and gene expression after sex differentiation (Belaid et al., 2001; Pieau and Dorizzi, 2004). We hypothesize elevated *Cyp19a1* mRNA levels at one week after hatching, driven by *Foxl2* mRNA expression, plays a roll in medullary restructuring.

In hatchling chickens, steroidogenic cells migrate from the medulla into the cortex and surround assembling follicles as theca (Drummond, 2005; Gonzalez-Moran et al., 1985; Narbaitz and Adler, 1966; Narbaitz and DeRobertis, 1968). In small chicken follicles, the single thecal layer synthesize estrogens, not the granulosa (Nitta et al., 1991a; 1991b). 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 three months after hatching concomitant with increased *Cyp19a1* expression compared to one month after hatching. This increase in *Cyp19a1* mRNA 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. To clarify the signaling underlying this morphological development, additional research is required.

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., 2009b). We observed mRNA expression patterns of *Inhba, Inhbb*, and *Fst* mRNA that were similar to *Cyp19a*1. This coordinated change in expression levels is likely related to integrated estrogen-activin signaling that is observed in neonatal and juvenile rodent ovaries.

Table 5-1. Quantitative real-time PCR primers

Gene	Forward Primer (5' - 3') Reverse Primer (5' - 3')	Anneal Temp (°C)	Product size (bp)	Accession #
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

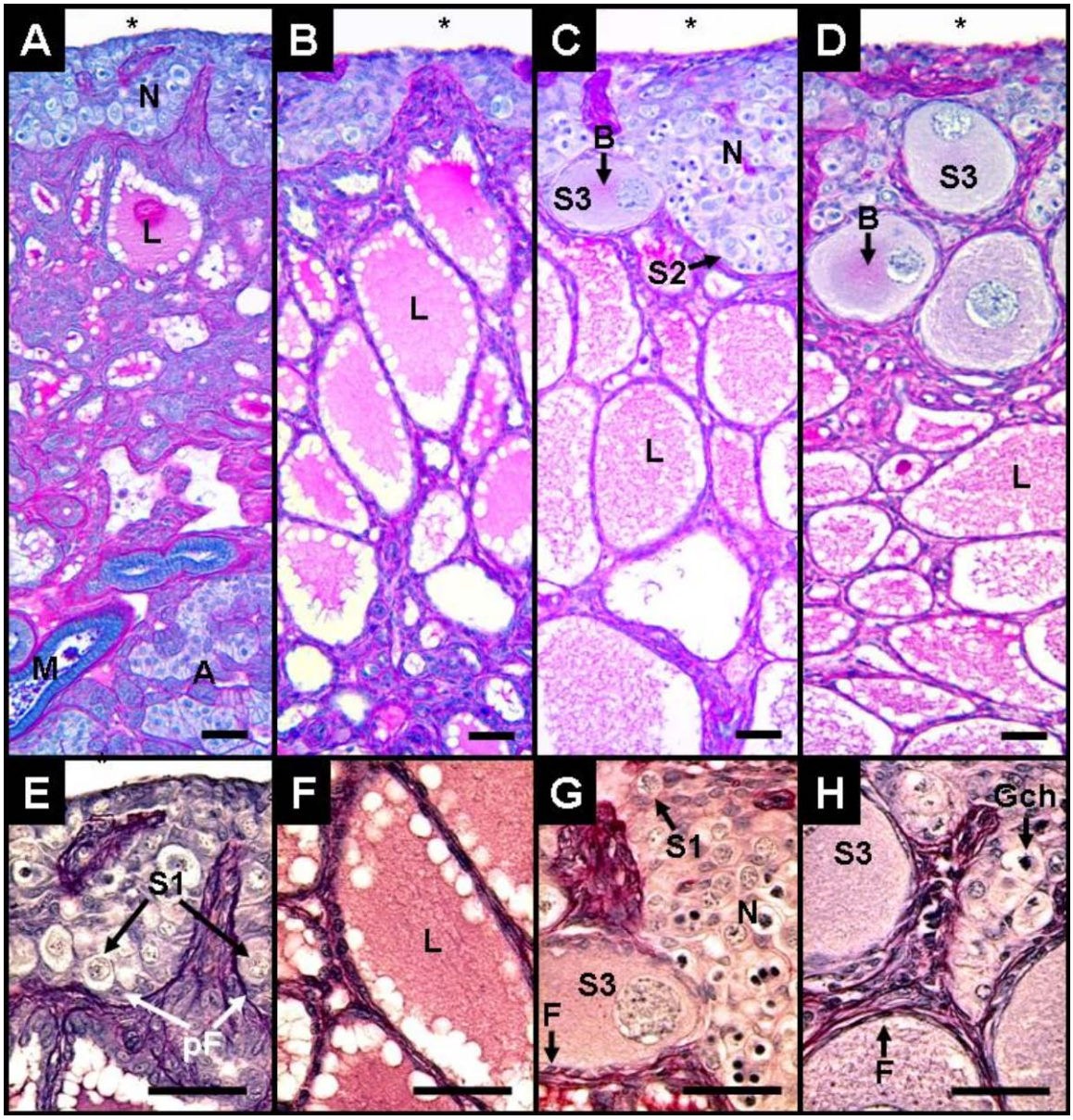

Figure 5-1. Ovary of *Alligator mississippiensis* at one week (A,E), one month (B,F), three months (C,G), and five months (D,H) after hatching in parasaggital view (A-D) and enlarged detail (E-H). At one week after hatching the ovarian cortex contains nest of germ cells (N) along the coelomic (*) border. 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 one month after hatching, the medullary lacunae (L) have expanded. At three and five months after hatching, the cortex contains Stage-2 (S2) and -3 oocytes (S3). Stage-3 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 five months after hatching. Scale bars = 50 *um*.

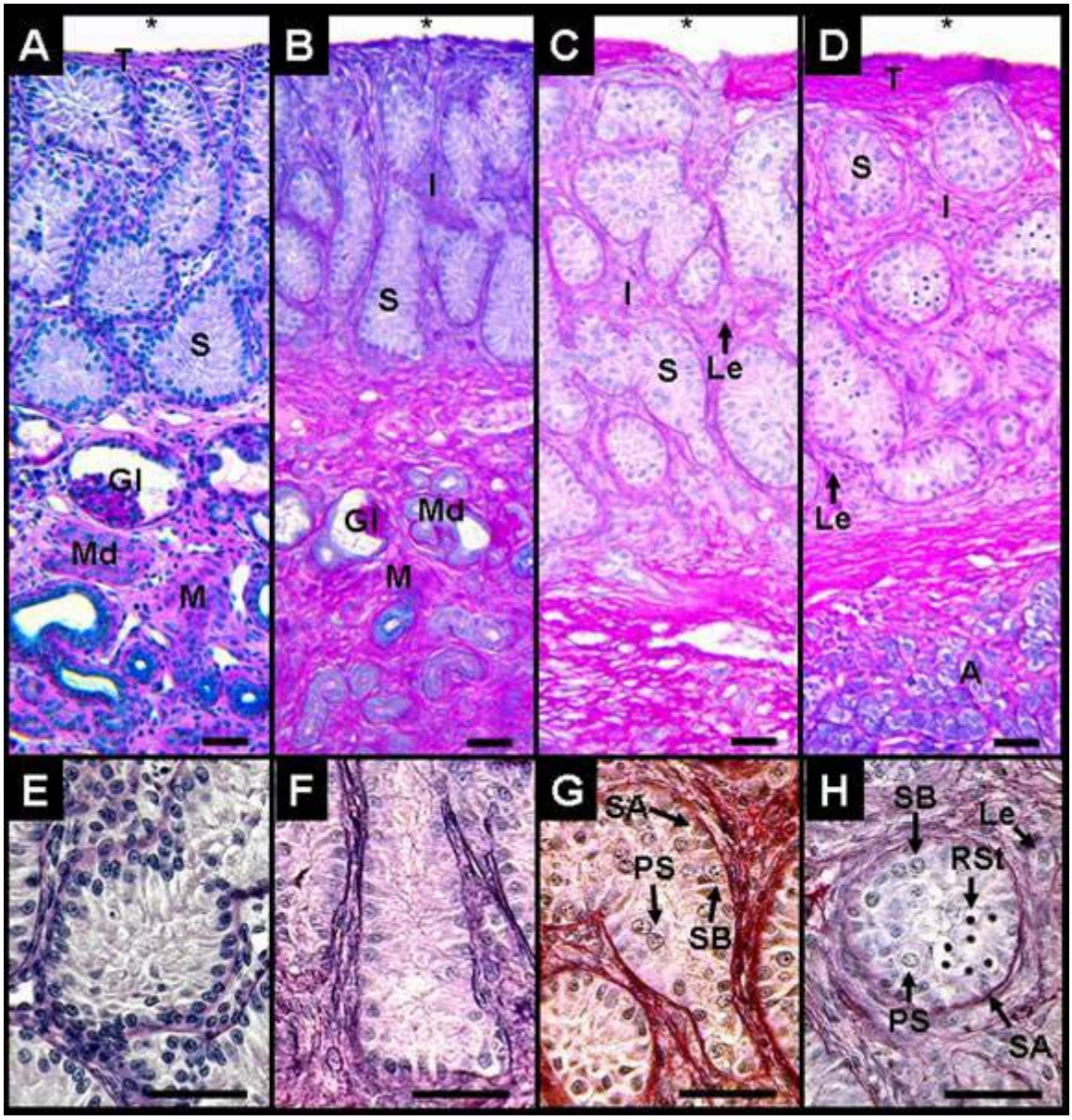

Figure 5-2. Testis of *Alligator mississippiensis* at one week (A,E), one month (B,F), three months (C,G), and five months (D,H) after hatching in parasaggital view (**A-D**) and enlarged detail (**E-H**). The coelomic (*) border is defined by the tunica (T) that thickens with age. The gonads contain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

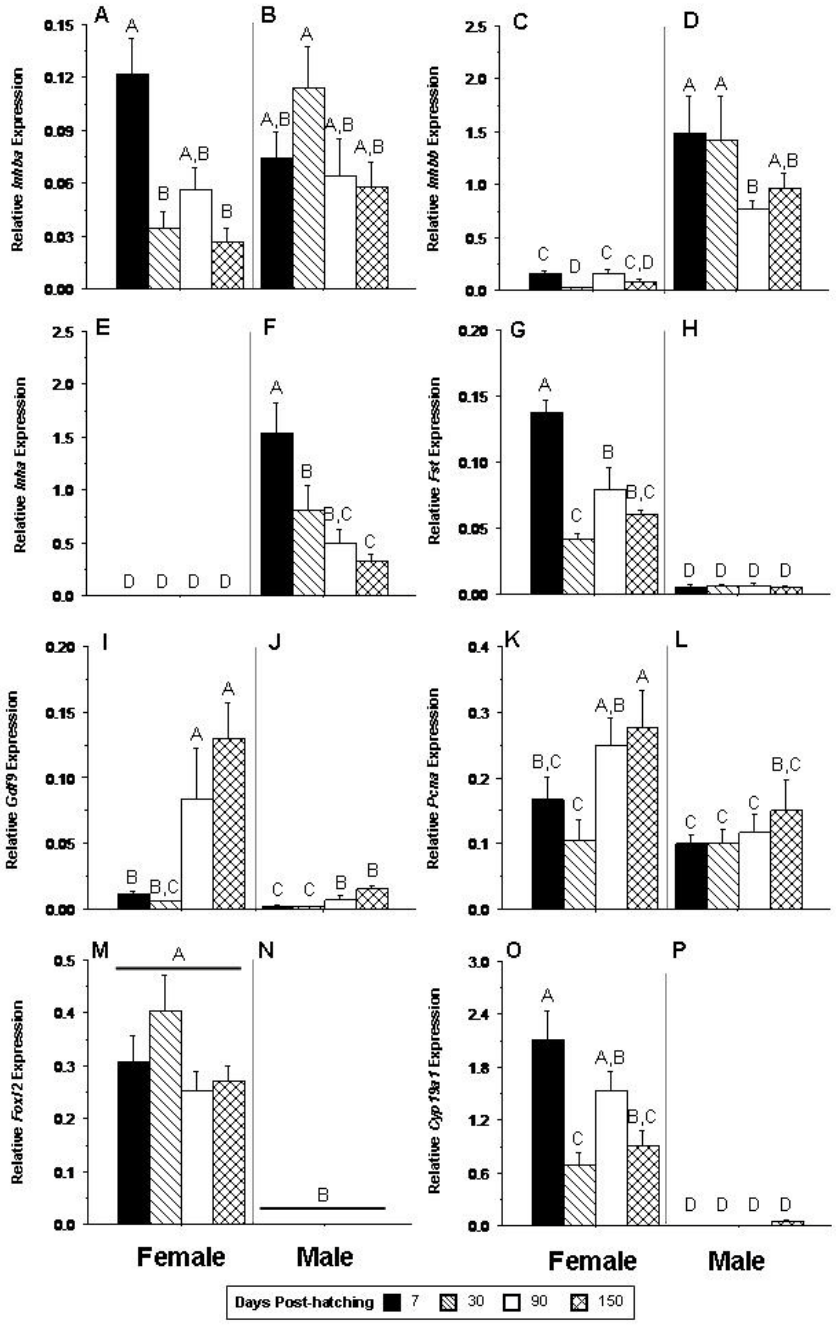

Figure 5-3. Mean (±SEM) gonadal mRNA expression of *βB* (A, B), *βA* (C, D), *Inhα* (E, F), *Fst* (G, H), *Gdf9* (I, J) *Pcna* (K, L) *Foxl2* (M, N), *Cyp19a1* (O, P) in female (A, C, E, G, I, K, M, and O) and male (B, D, F, H, J, L, N, and P) alligators. Days after hatching: black bars = 7, diagonal 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 indicate statistical significance at $P < 0.05$.

CHAPTER 6
ALTERED SEX HORMONE CONCENTRATIONS AND GONADAL MRNA EXPRESSIONS IN POST HATCHLING ALLIGATORS FROM CONTAMINATED AND CONTROL LAKES IN FLORIDA

Introduction

Morphological malformations are often manifestations of underlying genetic, endocrine, or physiological abnormalities. Multioocytic follicles (MOFs, alternatively called polyovular follicles) are two or more oocytes surrounded by a common follicular envelope of granulosa cells. These malformed follicles are hypothesized to result from oogonial clusters that do not properly dissociate and remodel during normal ovarian follicle assembly (Iguchi and Takasugi, 1986; Iguchi et al., 1986; Kipp et al., 2007b; Mayo et al., 2007). Normally this morphology is rare. However, increased frequencies of MOFs can be generated in animals by experimental or medicinal prenatal and neonatal exposure to estrogens, such as the natural endogenous estrogen, estradiol-17β (E_2) (Nakamura et al., 2008), pharmaceutical estrogens like diethylstilbestrol (DES) (Kim et al., 2009a), or phytoestrogens (Jefferson et al., 2006). Additionally, transgenic modifications that lead to over expression of ovarian signaling factors, such as the inhibin α subunit (Inha), also can produce this pathology in rodents (McMullen et al., 2001). Therefore, MOFs can have multiple etiologies, but these factors may converge on a common signaling network regulating follicle assembly (Mayo et al., 2007).

Until a decade ago, research largely viewed increased frequencies of MOFs as a mammalian pathology resulting from improper estrogenic exposure. However, in 1994 our group reported MOFs at a very high frequency in female alligators exposed during embryonic development to environmental contaminants. Lake Apopka, Florida, is contaminated with various pesticides and anthropogenic nutrients (Guillette et al., 1999a; Heinz et al., 1991a; Rauschenberger et al., 2007), resulting in reproductive impairments to resident alligators

(Fujisaki et al., 2007; Milnes and Guillette, 2008; Woodward et al., 1993). Female hatchling alligators from Lake Apopka, after being administered a luteinizing hormone (LH) challenge, displayed MOFs (often 3-4 oocytes per follicle) and elevated plasma E_2 concentrations, compared to a low (0 - 3% of the follicles in an ovary) frequency observed in reference females of a similar age within the same study (Guillette et al., 1994b). Recently, studies of estrogenic exposures using another crocodilian species (*Caiman latirostris*) support our findings of altered gonadal morphology and endocrine physiology. Bisphenol A (BPA) is an industrial chemical shown to have estrogenic and anti-androgenic properties (Richter et al., 2007). Across vertebrates, BPA has the potential to induce a wide range of impacts on gonadal functions (Crain et al., 2007). *In ovo* exposure of developing caiman to E_2 or BPA resulted in male to female sex reversal (Stoker et al., 2003). Therefore, as previously observed in alligators (Crain et al., 1997), embryonic crocodilian gonads are responsive to exogenous steroidal signaling through both endogenous ligands and endocrine disrupting contaminant exposures. Further, laboratory raised female caiman exposed *in ovo* to E_2 or BPA, during the beginning of sex differentiation, displayed elevated circulating E_2 concentrations and higher proportions of advanced follicles as hatchlings. In animals incubated at female-producing temperatures and sex-reversed females incubated at male-producing temperatures, E_2 increased MOFs frequencies in juvenile (3- and 12-months post hatching) animals (Stoker et al., 2008). Further, BPA increased MOF frequency in sex-reversed females compared to control sex-reversed animals. These findings support a hypothesis that prenatal exposure to endocrine disrupting contaminants can result in long-lasting impacts on reproductive endpoints.

At hatching, alligator ovaries do not possess follicles. Follicle assembly occurs slowly over many months post-hatching (Forbes, 1940; Moore et al., 2008) and the mechanisms

regulating this process are being investigated. In mice, follicle assembly occurs over the first five days post-hatching and some of the signaling mechanisms that regulate follicle assembly are becoming clear. Transforming growth factor β superfamily signaling plays a vital role in follicle assembly and establishment of the ovarian follicle pool (Drummond, 2005; Trombly et al., 2009a). Activins are transforming growth factor ligands that act as ovarian paracrine signals and regulate a variety of endpoints, including growth and cellular differentiation (Onagbesan et al., 2009; Pangas et al., 2007). Activin signaling plays a role in regulating follicle assembly in neonatal mice. Augmentation of activin levels in neonatal mice increases germ and granulosa cell proliferation and primordial follicle numbers in juvenile animals (Bristol-Gould et al., 2006). Transforming growth factor β superfamily ligands are formed from large precursor proteins that are processed and assembled into mature dimers. Activin ligands are homo- or hetero-dimers of two β subunits (Inhba and Inhbb). 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). Production of inhibins or follistatin (Fst) 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). Follistatin is a TGF-β ligand antagonist that binds and neutralizes activins (Nakamura et al., 1992; Welt et al., 2002).

During germ cell nest breakdown and subsequent follicle assembly, an activin-dominated signaling milieu could be critical (Mayo et al., 2007; Trombly et al., 2009a). Activins participate in signaling cross talk with steroid hormones. Estrogens suppress activin gene expression (Kipp et al., 2007b) while, in turn, activins induce the expression of estrogen receptors (Kipp et al., 2007c). In rodents, it is hypothesized that high levels of maternal steroids impede follicle

assembly and after birth, steroid levels fall and potentiate ovarian follicle assembly (Chen et al., 2007; Kezele and Skinner, 2003; Pepling, 2006), possibly via TGF-β signaling pathways (Mayo et al., 2007).

In contrast, embryonic alligators do not maintain a direct maternal endocrine connection during embryonic development. However, alligator eggs are invested with a substantial, maternally derived yolk that supplies nutrient through out *in ovo* and post-hatching development. Environmental contaminants, including pesticides, are passed from mother to yolk (Rauschenberger et al., 2007). We have observed that hatchling alligators hatched from eggs removed from wild nests prior to sex determination and incubated under identical conditions with eggs from reference populations exhibited an increased frequency of MOFs. Therefore, factors that lead to an increased frequency of MOFs are not exogenous factors experienced during development in the nest, such as temperature or humidity, but more likely pass maternally to the embryo via the egg. We hypothesize that in female alligators from Lake Apopka, the maternal contribution of endocrine disrupting contaminants with estrogenic activity to the egg yolk subsequently alters inhibin/activin signaling in female offspring and, therefore, predisposes increases frequency of MOF formation.

Here we examine both basal and FSH-stimulated levels of circulating estradiol and testosterone and gonadal mRNA expression levels of *Inha*, *Inhba*, *Fst*, aromatase (*Cyp19a1*), and follicle stimulating hormone receptor (*Fshr*). Comparatively, ovaries of both embryonic and hatchling chicken are responsive to exogenous FSH, both *in vitro* (Pedernera et al., 1999) and *in vivo* (Gonzalez-Moran, 1998; Mendez-Herrera et al., 1998; Sanchez-Bringas et al., 2006), resulting in elevated circulating estradiol and ovarian cell proliferation. Further, in cultured chicken granulosa, activin signaling is necessary to maintain morphological differentiation

(Schmierer et al., 2003), whereas FSH increases expression of *Inha*, *Fst*, and *Inhba* mRNA (Davis et al., 2001; Safi et al., 2003).

Importantly, differences in the levels of these factors between contaminant exposed and reference animals could differentially increase in an ovary experimentally challenged with a gonadotropin treatment, as occurred just prior to necropsy in the cohort described in our previous work. We hypothesize that early exposure to abnormal estrogenic signaling alters both basal and stimulated levels of circulating steroid hormones and transcript levels of activin inhibin signaling factors.

Materials and Methods

We collected American alligator (*Alligator mississippiensis*, Daudin, 1801) eggs from nests at Lake Woodruff National Wildlife Refuge and Lake Apopka 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 at the University of Florida. Two of the eight clutches collected from Lake Apopka were entirely non-viable, whereas all Lake Woodruff clutches (n=17) contained viable eggs. We used seven Lake Woodruff and six Lake Apopka clutches of eggs for this study (the other clutches were used in other experiments). A subset of viable eggs from these clutches were 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 an IACUC approved protocol. Hatching animals were web tagged with numbered Monel tags and measured for body masses (BM), snout-vent lengths (SVL), and total lengths (TL). Alligators were co-housed in a temperature-controlled animal room in tanks (~20 neonates / 0.7 m^3), and experienced a 16:8 photoperiod with heat lamps for basking and daily water changes. Ambient room temperatures ranged from 27°C to 31°C. We

supplied no food during the experimental period since alligators subsist off the internalized yolk sac during this time (first two weeks post hatching).

Hatching order systematically assigned animals to one of four experimental groups: necropsy at one, two, or five days after hatching or to a grow-out experiment not addressed in this manuscript. Those animals examined either two or five days after hatching were part of similar FSH challenge studies of differing durations. Because reptile FSH preparations are not commercially available, we treated with ovine FSH (Sigma-Aldrich #F8174-1VL). Previous experimentation has shown robust hormonal and/or ovarian responsiveness to treatment in alligators (Edwards et al., 2004; Lance and Vliet, 1987) and reptiles (Jones et al., 1975; Jones and Swain, 2000), and FSH directly modulates activin signaling in a variety of species (Davis et al., 2001; Knight, 1996; Kumar et al., 1997). Challenge study animals received either a sham needle insertion or IM injections of 0.8% sterile saline vehicle (isotonic to alligator blood), low dose (10 ng/gram BM FSH), or high dose (50 ng/gram BM FSH) to the base of the tail on a daily basis. We administered all treatments in an injection volume of 90 µl between 11:00 and 12:00 h. Animals examined two days after hatching received one treatment on the day after hatching, whereas those examined 5 days after hatching received one treatment per day for the four consecutive post-hatching days.

Necropsies commenced at 12:00 h of appointed days. Immediately prior to euthanasia, 1 ml of blood was collected from the supravertebral blood vessel which was then followed by a lethal dose (0.5 mg/g BM) of sodium pentobarbital (Sigma) . Blood collected in a heparinized Vacutainer (BD Diagnostics) was kept on ice until centrifugation at 1,500 g for 20 min at 4^{o}C. Drawn off plasma was stored at -80^{o}C until radioimmunoassay (RIA). One ovary (from

alternating sides) was frozen in liquid nitrogen and stored at -80°C until RNA extraction. Standard paraffin histology of the contra-lateral gonad confirmed sex.

Our standard RNA isolation and reverse transcription (RT) procedures have been previously reported in detail (Milnes et al., 2008). Quantitative real-time PCR (Q-PCR) has been used to measured mRNA expression in American alligator tissues (Gunderson et al., 2006; Katsu et al., 2004; Kohno et al., 2008). Table 1 reports primer sequence information, annealing temperatures, and accession numbers. The MyiQ single color detection system (BioRad, Hercules, CA) performed Q-PCR following manufactures protocol using iQ SYBR Green Supermix (BioRad) in triplicate reaction volumes of 15 µl with 2 µl of RT product and specific primer pairs. Reactions were performed with relative standard curves of serially diluted cDNA. 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 $P < 0.05$. Unpaired Student's t-tests compared BM, SVL, and TL measurement of all hatching alligators and the subset employed in the experiments detailed in this manuscript by lake of origin. Circulating steroid or gonadal mRNA expression levels were not different between sham and vehicle treated animals (lowest observed p-value by independent t-tests; *Fst* mRNA $p = 0.17$). We combined these groups into a single control treatment group for further statistical analysis. Two-way ANOVA followed by least square means Tukey-Kramer post-tests, when appropriate, compared steroid hormone concentrations and mRNA expression levels between control groups of differing ages (ANOVA factors: age and lake of origin) and between control and FSH

treatment levels (ANOVA factors: treatment and lake of origin) for animals in the two-day old and five-day old FSH challenge studies. Statistical analysis between control groups of differing ages and FSH challenge experiments shared control animals of respective of ages and lake of origin. We further investigated significant treatment effects revealed by two-way ANOVA with subsequent one-way ANOVA by individual lake or origin and Dunnett's post-tests, if appropriate, to quantify lake of origin differences in FSH responsiveness compared to controls within age groups.

Results

Upon collection, egg viability was 81% (237/291) from Lake Woodruff and 68% (181/267) from Lake Apopka. Of the subset of these eggs co-incubated, 86% from Lake Woodruff (125/145) and 59% from Lake Apopka (99/167) hatched. Of these, 54 Lake Woodruff and 37 Lake Apopka alligators were allocated to the hatchling experimental groups (Figs. 1-3 present sample sizes).

Within all co-incubated eggs, Lake Woodruff alligators had greater body mass ($P < 0.001$; mean (g) ± SE: Woodruff 61.9 ± 0.5, Apopka 56.7 ± 0.6), but similar total length ($P = 0.25$; mean (cm) ± SE: Woodruff 24.5 ± 0.1, Apopka 24.7 ± 0.1) and snout vent length ($P = 0.21$; mean (cm) ± SE: Woodruff 11.7 ± 0.04, Apopka 11.8 ± 0.8) to Lake Apopka alligators. Within the subgroup of hatchling allocated to the experiments reported here, body measurements also differed by lake of origin. Lake Woodruff alligators had greater BM ($P < 0.001$; Mean (g) ± SE: Woodruff 62.0 ± 0.7, Apopka 55.2 ± 1.2) and TL ($P = 0.04$; Mean (cm) ± SE: Woodruff 24.5 ± 0.1, Apopka 24.1 ± 0.2), but similar SVL ($P = 0.08$; Mean (cm) ± SE: Woodruff 11.7 ± 0.6, Apopka 11.6 ± 0.9) to Lake Apopka alligators.

Both gross anatomy at necropsy and gonadal histology observations confirmed all hatchling animals were female. The ovarian cortex of all females contained oogonial clusters

and meiotic germ cells showing varying degrees of physical interaction with somatic cells. However, ovaries from these neonates, whether 1, 2 or 5 days old, from either lake, did not possess complete follicles. Ovarian expression levels of *Rpl8* mRNA were not different between control groups or between FSH challenge study groups (P = 0.78, 0.92, and 0.41, respectively).

Comparing control group females from both lakes at one, two, or five days after hatching, Lake Apopka alligators had greater plasma concentrations of E_2 (Fig. 1A; P < 0.001) and T (Fig. 1B; P < 0.001) compared to Lake Woodruff females. Gonadal *Inha* mRNA expression showed a lake by age effect (Fig. 1C; P = 0.009), with Lake Apopka expression levels greater than Lake Woodruff one day after hatch, but less than Lake Woodruff expression levels five days after hatching. Lake Woodruff alligators expressed greater gonadal mRNA levels of *Inhba* (Fig. 1D; P = 0.017) and *Fst* (Fig. 1E; P = 0.026) compared to those from Lake Apopka. Expression of *Cyp19a1* (Fig. 1F; P = 0.33 or *Fshr* mRNA (data not shown; P = 0.25) did not significantly vary by age or lake of origin.

Within two-day-old, FSH challenged animals, plasma E_2 and T concentrations were greater in Lake Apopka alligators compared to Lake Woodruff (Fig. 2A,B; P < 0.001 for each). Additionally, FSH treatment resulted in a significant elevation of plasma E_2 concentrations irrespective of lake (Fig. 2A; P = 0.008). Analysis of treatment effects by individual lake of origin showed circulating E_2 concentrations for Lake Apopka animals responded to both low and high FSH doses (P = 0.027 and 0.015, respectively), whereas Lake Woodruff animals responded only to high dose FSH (P = 0.49 and 0.007, respectively). Gonadal expression of *Inha* mRNA showed a lake by treatment effect (Fig. 2C; P = 0.002), with Lake Apopka alligators showing a greater expression level response to the high FSH dose than Lake Woodruff alligators. Analysis of treatment effects by individual lake of origin showed the both Lake Woodruff and Apopka

animals responded to both low and high dosages with elevated *Inha* levels (Woodruff: low P =0.006, high P < 0.001; Apopka: low P = 0.003, high P < 0.001). Significant differences in *Inhba*, *Fst*, *Cyp19a1*, and *Fshr* mRNA expression levels were not observed (Figs. 2D-F and data not shown).

Within five-day old, FSH challenge study animals, plasma E_2 and T concentrations were greater in Lake Apopka alligators compared to Lake Woodruff (Fig. 3A,B; P < 0.005 and 0.001, respectively). Additionally, FSH treatment resulted in a significant elevation of plasma E_2 concentration (Fig. 3A; P < 0.001). Analysis of treatment effects by individual lake of origin showed circulating E_2 concentrations of both Lake Apopka and Woodruff animal responded only at high doses of FSH (P < 0.001 and 0.005, respectively). Both lake of origin and treatment effects were observed in mRNA expression levels of *Inha* (Fig. 3C; lake of origin P < 0.03, treatment P < 0.001), *Fst* (Fig. 3E; lake of origin P = 0.003, treatment P =0.03), *Cyp19a1* (Fig. 3F; lake of origin P = 0.001, treatment P = 0.004), and *Fshr* (data not shown; lake of origin P = 0.006, treatment P = 0.002). Lake of origin effects showed that Lake Woodruff animals expressed greater levels of gonadal *Inha*, *Fst*, *Cyp19a1*, and *Fshr* mRNA than Lake Apopka animals. Analysis of treatment effects by individual lake of origin showed that both Lake Woodruff and Apopka animals responded to both low and high dosages of FSH with elevated *Inha* levels (Woodruff: low P =0.033, high P < 0.001; Apopka: low P = 0.044, high P < 0.001). In contrast, only Lake Woodruff animals responded to the FSH challenges with elevated mRNA levels: *Fst* (Woodruff: low P = 0.09, high P = 0.022; Apopka: low P = 0.63, high P = 0.39), *Cyp19a1* (Woodruff: low P = 0.033, high P < 0.001; Apopka: low P = 0.42, high P = 0.42), and *Fshr* (Woodruff: low P = 0.47, high P = 0.006; Apopka: low P = 0.42, high P = 0.17).

Discussion

Recent research in rodents has demonstrated that an interaction between activin and estrogen signaling participates in the breakdown of germ cell nest and the assembly of ovarian follicles. Here, we investigated the basal and gonadotropin stimulated levels of circulating hormones and gonadal mRNA expression of activin signaling factors in hatchling alligators from a contaminated and a reference environment. We observed evidence of elevated circulating steroids (E_2 and T) in alligators from the contaminated environment and conversely, greater mRNA expression levels of activin signaling factors (*Inhba* and *Fst*) in alligators from the reference environment. Additionally, Inha mRNA expression levels in ovaries from Lake Apopka females changed substantially during the post hatching period from greater than that seen in ovaries from Lake Woodruff females one day after hatching to lesser than those observed at five days after hatching. Hatchling alligators have not yet formed ovarian follicles; therefore, these endocrine differences are likely to directly affect subsequent ovarian development.

We observed these endocrine differences in animals that exhibited differing egg viability rates at the nest (Woodruff- 81%, Apopka-68%) and post-incubation hatch rates (Woodruff-86%, Apopka-59%). This hatching success dimorphism continues observations of decreased egg viability and hatching success in Lake Apopka eggs observed for almost three decades (Fujisaki et al., 2007; Milnes and Guillette, 2008).

While the role of FSH in ovarian nest breakdown and follicle assembly is unknown in alligators, we observed clear differences in response to a gonadotrophin challenge. While alligators from both lakes showed responses to a single high dose FSH injection, only Lake Apopka animals showed a significant E_2 increase to the low dose treatment and Lake Apopka animals *Inha* mRNA expression levels responded more robustly than Lake Woodruff alligators to the high dose treatment. In five-day old challenged alligators, we observed responses in

circulating E_2, and ovarian *Inha*, *Fst*, *Cyp19a1*, and *Fshr* mRNA expression. Animals from both lakes responded with elevated plasma E_2 concentrations to high dose treatments and increased *Inha* mRNA expression to both treatment levels. However, only Lake Woodruff animals significantly responded to FSH with elevated *Fst*, *Cyp19a1*, and Fshr mRNA expressions. Therefore, *Fst* mRNA expression in Lake Woodruff animals is both greater at basal levels and is more inducible by FSH challenge than in Lake Apopka alligators. In mice, Wnt4 is a signaling factor that suppresses male gonadal tissue differentiation during ovary development and regulates Fst expression (Yao et al., 2004b). Since Fst expression also is critical for female germ cells survival, this Wnt4-Fst signaling cascade is both anti-testis and pro-ovary. Therefore, lower basal and stimulated *Fst* expression levels could be a robust sign of impaired reproductive health of Lake Apopka alligators.

Aromatase (translated from *Cyp19a1* mRNA) is an enzyme that converts androgens to estrogens. While we observed elevated circulating E_2 concentrations in untreated Lake Apopka alligators and in FSH stimulated alligators from both lakes, *Cyp19a1* mRNA expression levels were not different between non-stimulated alligators from either lakes and the FSH challenge elevated expression levels only in five-day old Lake Woodruff alligators. Circulating steroid hormone concentrations in an organism are the result of an integration of synthesis, plasma storage, hepatic biotransformation and clearance. Changes to these parameters can alter measured concentrations of any steroid. We observed elevated basal testosterone concentrations in Lake Apopka animals, therefore supplying an elevated level of available substrate for aromatization. Additionally, our laboratory group has demonstrated that contaminant exposures can change expression levels of enzymes involved in hepatic steroid degradation (Gunderson et al., 2001). Finally, measured mRNA levels do not always predict rates of protein translation,

length of mRNA stability, or length of enzymatic activity of the transcribed product. Therefore, numerous physiological processes can dissociate Cyp19a1 mRNA levels from circulating E_2 concentrations.

Using reported means, standard errors, and samples sizes from our 1994 study, we calculated that the LH challenge significantly increased circulating concentrations of E_2 in Lake Apopka alligators ($P = 0.47$), but not in Lake Woodruff animals ($P = 0.74$). Comparing this previous work to data presented here, in both studies, basal circulating E_2 levels were greater in Lake Apopka than in Lake Woodruff alligators. Further, Lake Apopka animals showed greater E_2 responsiveness following treatment with either LH (Guillette et al., 1994a) or FSH (current study). In ovo exposure of caiman embryos to exogenous E_2 or BPA resulted in increased circulating E_2 concentrations in 10 day of hatchlings, although this difference was no longer observed at three- to twelve-months post-hatching (Stoker et al., 2008). Taken together, these three studies strengthen the hypothesis that embryonic exposure to endocrine active substances can affect the endocrine milieu of hatching crocodilians, and likely other vertebrate species as well.

Ten-day old caiman display ovarian follicles (Stoker et al., 2003) with maturation levels not observed in alligator ovaries until months after hatching (Moore et al., 2008). The basis for this species-specific difference in developmental timing is unknown, however broad-snouted caiman are smaller as adults compared to American alligators (adult total lengths ~ 2.0-3.5 compared to 3.0-4.5 m) and are reproductive at smaller sizes, but lay larger eggs than alligators (Thorbjarnarson, 1996). In addition to increased circulating E_2 concentrations, E_2 and BPA treatments increased follicular maturation in hatchling caiman and elevated MOF frequencies in three to twelve month juveniles (Stoker et al., 2008). This period encompasses the six-month

age when we observed MOFs in American alligators (Guillette et al., 1994a). Altered hatchling follicle dynamics in caiman presented an inverted-U dose response curve with effects observed only at lower E_2 and BPA doses. Therefore, in crocodilians substantial ovarian morphological development occurs in the months after hatching and the processes underlying this maturation may not respond in a linear manner to varying degrees of exogenous influence.

While numerous studies have demonstrated increase MOF frequencies in estrogenically treated rodents (Iguchi et al., 1990; Iguchi and Takasugi, 1986; Kim et al., 2009a; Nakamura et al., 2008), only recently has research investigated the effects of similar treatments on activin signaling pathways. Treatments with E_2 or DES of neonatal mice resulted in increased MOF frequencies and altered activin signaling 19 d post natally (Kipp et al., 2007b). Alterations of activin signaling were observed as decreased *Inha*, *Inhba*, and *Inhbb* mRNA expressions and decreased Inhba and activin A ovarian protein levels. Treatments did not alter *Fst* mRNA levels. We observed consistently decreased *Inhba* and a rapid decreasing in *Inha* mRNA expression levels in contaminant-exposed alligators over the observed period. In contrast, *Fst* mRNA levels decreased in contaminant-exposed alligators compared to no alteration observed in estrogenically treated mice. In contrast to the increased maturation of follicles observed in caiman treated in ovo with E_2 (Stoker et al., 2008), neonatal E_2 and DES treatment of mice resulted in fewer small antral follicles at day 19, as compared to controls. Therefore, while these exposure experiments share some similarity in molecular and physiological effects, differing, species-specific impacts can also be observed.

In the mouse ovary, estrogen and activin signaling proteins co-localize in granulosa cells. While the location of synthesis of these factors is unknown in the pre-follicular crocodilian ovary, we can compare spatial expression patterns in other vertebrates with morphologically

similar ovaries (Moore et al., 2008) to formulate a hypothesis. Steroidogenic cells differentiate and migrate from the nephrogenous mesenchyme in chicken and mouse ovaries (Sekido and Lovell-Badge, 2007). In embryonic, pre-follicular chicken left ovaries, aromatase protein localizes to the medullary cords, not to the overlying cortex (Govoroun et al., 2004). Similarly, embryonic turtle ovaries express aromatase around the regressing medullary cords and at the cortex/medulla boundary after sex differentiation (Ramsey et al., 2007). In chicken ovary, two weeks after hatching, medullary steroidogenic cells migrate toward the cortex and incorporate into the thecal layers of developing follicles (Narbaitz and DeRobertis, 1968; Pedernera et al., 1988b). In juvenile (five-week old) chickens, aromatase is only detected in the thecal cells of developing follicles, granulosa cells do not express aromatase (Govoroun et al., 2004; Oreal et al., 2002). Avian granulosa cells of the pre-hierarchical follicles exclusively express most activin signaling factors (Onagbesan et al., 2009). In light of the ovarian cortex possessing follicular cells that express activin, inhibin, and follistatin and an ovarian medulla containing the majority of steroidogenic cells before migration and differentiation into theca around small follicles, we hypothesize that in the pre-follicular alligator ovary, activin and estrogen signaling segregate spatially between cortex and medulla, respectively.

Embryonic chicken ovarian medullary cells bind FSH (Woods et al., 1991) and this gonadotropin induces steroidogenesis (Gomez et al., 2001). At hatching, chickens treated with FSH in ovo respond with increased plasma E_2 concentrations, thickening of the ovarian cortex and medullary cords (Gonzalez-Moran and Mancilla, 1998), increased number of oogonia, and impeded meiosis observed as diminished numbers of oocytes (Gonzalez-Moran, 1998). We support a hypothesis proposed by these researchers that an FSH challenge does not directly augment germ or somatic cell numbers, direct somatic differentiation, or change entry into

meiosis in the ovarian cortex, but rather, this produces a transient effect due to altered medullary steroidogenesis. We expand this hypothesis to state that an FSH challenge alters cortical activin signaling by way of elevating plasma and tissue E_2 concentrations that interact with activin signaling dynamics. Furthermore, as suggested in this study, exposure to environmental contaminants can alter this response and interaction.

Amniote follicle formation is a function of initial germ cell proliferation followed by precipitous loss concomitant with complex interactions between the oocytes and somatic cells (Pepling and Spradling, 2001). Oocyte survival depends on follicular formation, those that fail to be surrounded by granulosa cells forming normal follicles undergo apoptosis (Pru and Tilly, 2001). The majority of the morphological processes described here are conserved among vertebrates (Matova and Cooley, 2001; Pepling et al., 1999) and appear to share many of the molecular mechanisms underpinning gonadal maturation. As with the 'canary in the coal mine', we propose that if MOFs can be induced in wildlife by contaminant exposure with estrogenic activity, putatively through alteration in activin signaling, similar responses could also be observed in natural populations of mammals, including humans (Crain et al., 2008). Understanding and documenting these conserved mechanisms among vertebrates will strengthen causal and mechanistic relationships, allowing a better understanding of this phenomenon across vertebrates and allowing us to reduce or prevent these abnormalities in future populations.

Table 6-1. 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		Katsu et al. 2004
Inhibin α (Inha)	ACAATCCACTTGTCCCAGCC CAACTGCCACCGCGC	70.0	68	DQ010151
Activin βA (Inhba)	ACCCACAGGTTACCGTGCTAA GCCAGAGGTGCCCGCTATA	63.8	67	DQ101152
Follistatin (Fst)	CGAGTGTGCCCTCCTCAAA TGCCCTGATACTGGACTTCAAGT	66.5	65	DQ010156
Aromatase (Cyp19a1)	CAGCCAGTTGTGGACTTGATCA TTGTCCCCTTTTTCACAGGATAG	62.0	79	AY029233
Follicle Stimulating Hormone Receptor (Fshr)	GAAATTACCAAACGAGGTTTTTCAA GGGCAGGAAACTGATTCTTGTC	60.0	81	DQ010157

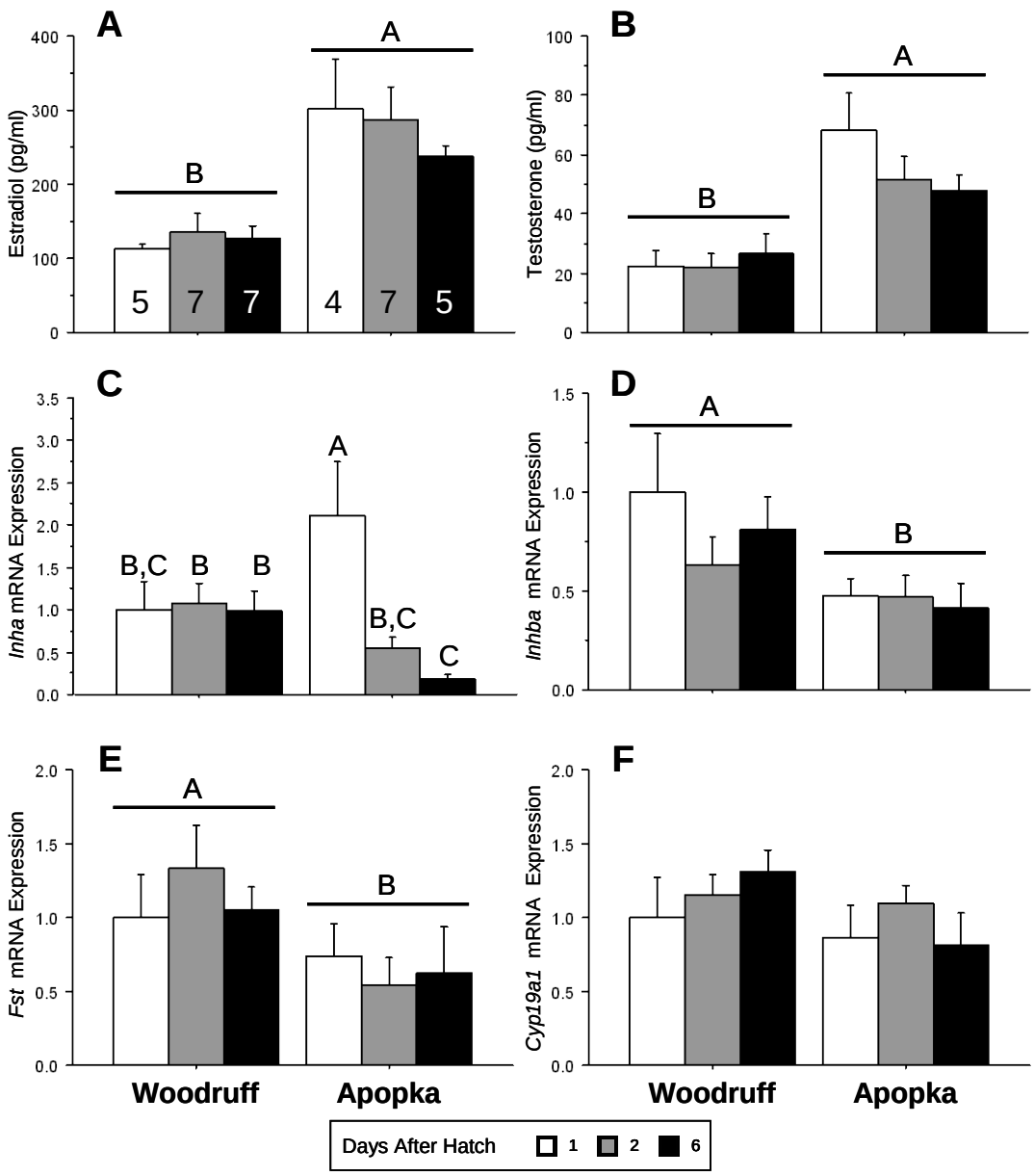

Figure 6-1. Basal circulating steroids concentrations and ovarian mRNA expression levels, Mean (±SEM), in hatchling alligators: estradiol and sample sizes (A), testosterone (B), *Inha* (C), *Inhba* (D), *Fst* (E) and *Cyp19a1* (F) in Lake Woodruff and Lake Apopka alligators. Days after hatching: white bars = one, gray bars = two, black bars = five. All sample means are normalized using ribosomal protein L8 (*Rpl8*) expression and standardized for each endpoint to Lake Woodruff female, day 1 expression = one. Horizontal line above bars indicates statistical significance by lake of origin ($P < 0.05$). Different letters above the bars indicate age by lake of origin statistical significances.

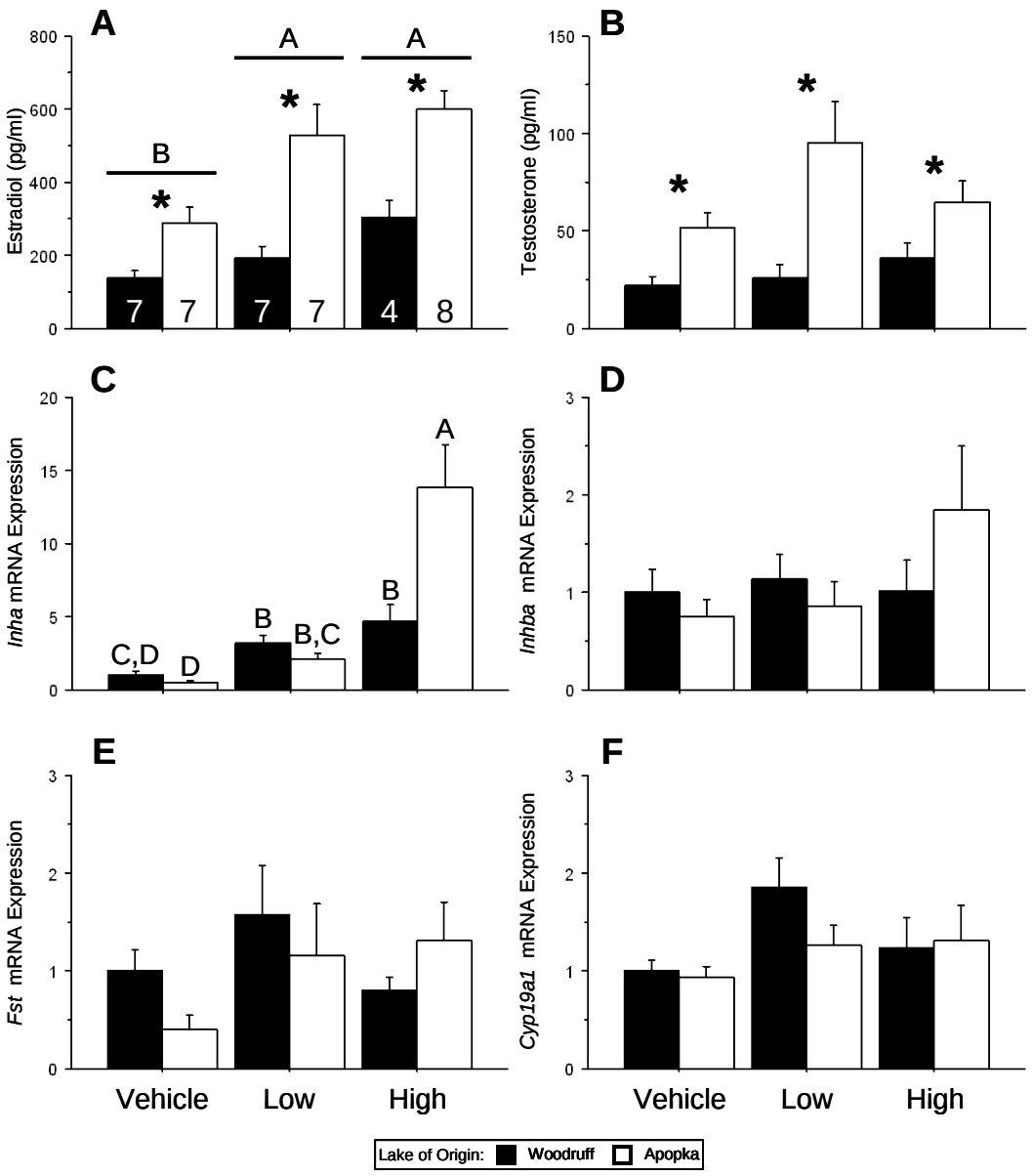

Figure 6-2. Circulating steroid concentrations and ovarian mRNA expression levels in two-day old FSH challenged alligators. Bars report means (±SEM): estradiol and sample sizes (A), testosterone (B), *Inha* (C), *Inhba* (D), *Fst* (E) and *Cyp19a1* (F) in Lake Woodruff (black bars) and Lake Apopka (white bars) alligators. All sample means are normalized using ribosomal protein L8 (*Rpl8*) expression and standardized for each endpoint to Lake Woodruff female, vehicle treated expression = one. Horizontal line above bars indicates statistical significance by treatment ($P < 0.05$). Asterisks above bars indicate significant difference by lake of origin. Different letters above the bars indicate age by lake of origin statistical significances.

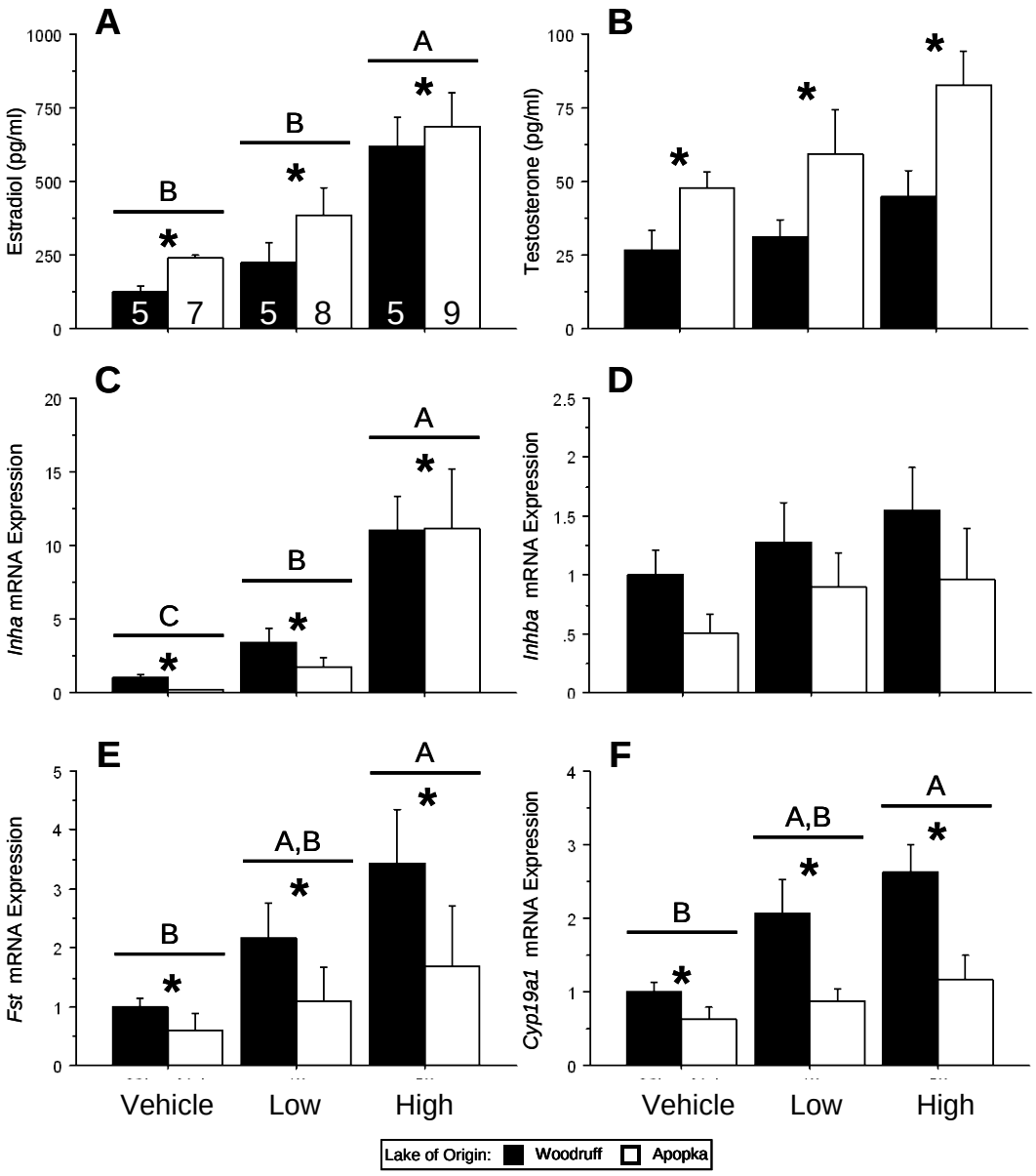

Figure 6- 3. Circulating steroid concentrations and ovarian mRNA expression levels in five-day old FSH challenged alligators. Bars report means (±SEM): estradiol and sample sizes (A), testosterone (B), *Inha* (C), *Inhba* (D), *Fst* (E) and *Cyp19a1* (F) in Lake Woodruff (black bars) and Lake Apopka (white bars) alligators. All sample means are normalized using ribosomal protein L8 (*Rpl8*) expression and standardized for each endpoint to Lake Woodruff female, vehicle treated expression = one. Horizontal line above bars indicates statistical significance by treatment ($P < 0.05$). Asterisks above bars indicate significant difference by lake of origin. Different letters above the bars indicate age by lake of origin statistical significances.

CHAPTER 7
CONCLUSIONS MAKE FOR THE BEST NEW BEGINNINGS

Perspective

The foundation for my doctoral research was laid in 1994 with the publication of a manuscript with a peculiar photograph showing malformed American alligator oocytes (Guillette et al., 1994a). While decreased egg viability, increased hatching mortality, and altered circulating plasma steroid levels were also reported in this paper, this image may have created the greatest impact in the scientific and environmental communities. Images are often highly evocative and motivating. Also, this description of a pathology, - the multioocytic or polyovular follicle - known to be observed in mice and human females exposed pre- or perinatally to estrogens, was the initial report of this pathology in a wildlife species. As of April 2008, this publication was cited 557 times.

While the observed morphology stood on its own merit as not normal, the causative factors underlying this peculiar morphology were unclear. Within the growing world of endocrine disruption research, the usual suspect of altered steroid hormone signaling was an easy mark, but could not tell the whole story. When I undertook this research, entry into a new biological frontier for alligator reproductive research was required. I will argue that this body of work will also take endocrine disruption research into a novel realm and, in part, away from only investigating alteration in classic steroid hormone signaling dynamics.

I began this research at a time when there was substantial expansion in the understanding of how paracrine signaling factors regulate ovarian follicle assembly. This required continual synthesis and implementation as these new findings were published throughout the period of my dissertation research. An additional challenge was to transfer a current body of research performed on laboratory rodents to a wildlife species. This entailed knowing the limitation of

my study species, developing new experimental tools, and maximizing the results obtained from each yearly egg collections.

Seminal research by Profs. Teresa Woodruff and Kelly Mayo has demonstrated the role of Transforming Growth Factor β superfamily ligands in the paracrine regulation of normal ovarian follicle assembly (Bristol-Gould et al., 2006; Kipp et al., 2007b; Kipp et al., 2007c; Mayo et al., 2007; McMullen et al., 2001). Specifically, they expanded our understanding of the role activin signaling plays in follicle assembly and, further, the complexity of activin/estrogen signaling cross talk during this dynamic process. Therefore, to investigate the endocrine underpinnings of how environmental contamination can result in ovarian follicle malformation, I needed to expand three areas of reproductive knowledge in the American alligator. First, characterize both the timing and morphological development of follicle assembly in alligator ovaries. Second, characterize normal expression levels of paracrine signaling factors that could modulate follicle assembly during this developmental period. Lastly, investigate if differences exist in these signaling patterns between alligator ovaries exposed to environmental contaminants and those from a reference population. Through achieving these goals, a characterization of the etiology of multioocytic follicle (MOF) formation can be put forward. However, the path is often more valuable than the destination. The MOF morphology is an overt sign of altered biological processes. It may be that alterations in these underlying signaling processes pose a greater impact on ovarian reproductive fitness as a whole than the altered morphology.

Results and Advancements

To establish normal expression levels of a suite of estrogen and activin signaling factors, we first investigated gonads of ovaries and testes of 13-month-old alligator gonads in Chapters 2 and 3. By studying gonads of both sexes, these studies revealed sexually dimorphic mRNA expression patterns. These patterns established expectations for mRNA expression patterns in

hatchling ovaries. Androgen and estrogen receptor mRNA expression levels were not profoundly different between ovary and testis**.** Greater sexually dimorphism mRNA expressions were observed with steroidogenic factors such as the enzymes aromatase (Cyp19a1) and 17 alpha-hydroxylase (Cyp17a1). This finding supports a hypothesis that both androgen and estrogen signaling are important to both ovary and testis. Activin/estrogen cross talk dynamics are mediated partially through modulation of estrogen receptor expression (Kipp et al., 2007c). Similar expression levels of estrogen receptor in both ovary and testis supports a hypothesis that this cross talk functions in both testis and ovary. Further, we observed evidence that environmental contaminant exposure could affect steroid hormone receptor mRNA expression levels. Some of these environmentally induced differences in mRNA expression when investigated by cluster analysis showed complex expression patterns. This complexity is putatively the result of gene by environment interactions, a direct result of studying genetically heterogeneous alligator populations

In contrast to steroid hormone mRNA receptor expression levels, alligator gonads expressed many TGFβ signaling factors in robust, sexually dimorphic patterns. These mRNA expression patterns are similar to those reported in other vertebrates, including mammals. In testes, we observed greater βB subunit (*Inhbb*) and α subunit (*Inha*) levels, whereas ovaries expressed greater mRNA levels of follistatin (*Fst*) and growth differention factor-9 (Gdf9). Again, incubation temperature did not markedly change these dimorphic expression milieus which are critical for ovarian and testicular health (Loveland et al., 2007; Trombly et al., 2009a) In contrast, exposure to environmental contaminants again showed an ability to modify these expression patterns. In alligators exposed to a complex mixture of environmental contaminants, such is found in alligator eggs obtained from Lake Apopka females, we observed a loss of sexual

dimorphic expression of *Inhbb* in males from Lake Apopka and *Fst* and *Gdf9* in females from Lake Apopka when compared to same sex and age alligators from a reference population. As with steroid hormone receptor and steroidogenic factor expression, a gene by environment modulation of individual susceptibility was shown through cluster analysis.

In Chapter 4, we demonstrated that under laboratory conditions, ovary and testis undergo pronounced morphological changes during the first five months post hatching. In gonads of both sexes, we observed the formation and proliferation of steroidogenic tissues, thecal cell layers in ovarian follicles and Leydig cell clusters in the interstitial tissue of the testes. Further, we observed meiotic progression of germ cells. In ovaries, follicles assembled around diplotene oocytes whereas in testes, spermatogonia initiated meiosis. It seems clear that compared to wild animals, optimum rearing conditions under laboratory conditions accelerates gonadal development. Extracellular matrix structures that developed during this period could directly impact physiological and endocrine changes through simple physical effects such as tissue compartmentalization or through direct signaling activity within gonadal tissues.

We demonstrated that morphological development of alligator gonads relates to changes in expression level of signaling factors in Chapter 5. At one week after hatching, we observed medullary remodeling in association with elevated activin signaling factor and aromatase mRNA expressions. A putative quiescent period followed during which many transcript levels showed a nadir. At three and five months following hatching and concomitant with follicle assembly ovaries showed increased expression levels of activin signaling factors, aromatase, *Gdf9* and the mitotic marker *Pcna*. In testes, expansion of interstitial tissues and differentiation of cell therein at three and five months after hatching could be related to a concomitant decreases in mRNA expression of activin signaling factors. Additionally, a rise in Gdf9 mRNA expression level at

five months after hatching aligns with increased spermatogenic activity. Here we have made initial progress on a deeper understanding of the molecular factors regulating alligator gonadal differentiation.

In Chapter 6, we observed that during the first five days post hatching, contaminant exposure changes both plasma steroid concentrations and gonadal gene expression patterns in alligator ovaries. Elevated plasma steroid hormone concentrations in contaminant-exposed animals could impair subsequent follicle assembly. Contaminant exposure was also associated with lower basal levels are TGFB signaling factors that have been determined to be vital for ovarian health in other species. In light of elevated steroid hormone concentrations, these lower *Inhba* and *Fst* mRNA expression levels could further compromise follicle assembly in contaminant-exposed alligators. In addition to differing basal levels, animals from contaminated environments showed differing responses to FSH challenges, for example greater responsiveness of estradiol levels at day 2 and diminished *Fst* and *Cyp19a1* mRNA level responsiveness at 5 days after hatching.

Addressing the questions first posed in the research, we were able to characterize both the timing and morphological development of follicle assembly in alligator ovaries and contrast this morphological development to that observed in testes during the identical period. Post hatching alligator ovarian development is both extensive and prolonged. These characteristics make this species well suited for ovary development studies. We characterized normal expression levels of paracrine signaling factors that could modulate follicle assembly during this developmental period. Many of our observations are in agreement with those reported in mammalian and avian ovaries. Again, the prolonged period in which these TGFβ directed morphological developments occur could aid in future investigation of these processes. We identified differences in TGFβ

signaling patterns between alligator ovaries exposed to environmental contaminants and those from a reference population.

Synthesis and Advancement

Throughout this current experimentation, we did not induce MOFs in alligator ovaries. However, during the same research period another laboratory group induced MOFs with in ovo exposure to estrogenic compounds in another crocodilian species (Stoker et al., 2008). These experimental data support our hypothesis that abnormal estrogenic signaling during ovarian differentiation leads to altered follicle assembly and suggests that all crocodilians, potentially all vertebrates, could show similar susceptibilities. Currently, an estrogenic exposure study similar to the one inducing MOFs in caiman is underway in our laboratory. While the results of this work unknown at this time, an induction of the MOF morphology will not draw this experimental line of inquiry to a conclusion. That is, the induction of MOFs is only a morphological sign of an underlying malady in physiology as well; the morphology is only one of the real issues. If we induce MOFs, showing an associated difference in steroid hormone or TGFβ signaling directly addresses causality of this pathology.

Post hatchling alligator gonads divide into two distinct compartments, a germ cell containing cortex overlying medullary tissues. Localizing cell specific mRNA expression patterns of the signaling factors measured in my work is vital to advancing our understanding of signaling kinetics and tissue specificity. I predict profound differences in mRNA expression between cortical and medullary compartments. Understanding the interactions of these regions is vital to understanding alligator – archosarian -ovarian development. Research has begun to clarify these differences in morphologically similar chicken ovaries. This body of research could supply a road map to guide further research using in situ hybridization and laser capture microdissection techniques.

Lastly, the results of this dissertation lead to a hypothesis that estrogen/activin signaling cross talk modulates alligator ovarian follicle assembly. We are currently performing experiments that expose alligators to exogenous estrogens. The complement to this study is to treat alligators during critical developmental windows with recombinant activins. Activins have been demonstrated to increase follicle pools in neonatal rodents (Bristol-Gould et al., 2006). If estrogens have the ability to impair alligator follicle assembly, then activins many augment this process. Finally, I also demonstrated that contaminant exposure could alter steroid hormone mRNA expression levels. If estrogen/activin signaling cross talk is modulated through receptor expression, then further investigations could uncover differences in expression that could affect follicle assembly. All of these finding have furthered our understanding what is apparently a conserved process of follicle assembly across vertebrates (Pepling et al., 1999).

LIST OF REFERENCES

Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM. 2000. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. Endocrinology 141(5):1795-1803.

Abrass CK, Peterson CV, Raugi GJ. 1988. Phenotypic-expression of collagen types in mesangial matrix of diabetic and nondiabetic rats. Diabetes 37(12):1695-1702.

Adler SG, Feld S, Striker L, Striker G, LaPage J, Esposito C, Aboulhosn J, Barba L, Cha DR, Nast CC. 2000. Glomerular type IV collagen in patients with diabetic nephropathy with and without additional glomerular disease. Kidney Int 57(5):2084-2092.

Aire TA, Ozegbe PC. 2007. The testicular capsule and peritubular tissue of birds: Morphometry, histology, ultrastructure and immunohistochemistry. J Anat 210(6):731-740.

Akingbemi BT. 2005. Estrogen regulation of testicular function. Reprod Biol Endocrinol 3(51).

Amer FI, Shahin M. 1975. The post-hatching development of the gonads in the fowl, *Gallus domesticus*. Ann Zool (Wars) 11:1-24.

Andersson AM, Juul A, Petersen JH, Muller J, Groome NP, Skakkebaek NE. 1997. Serum inhibin B in healthy pubertal and adolescent boys: Relation to age, stage of puberty, and follicle-stimulating hormone, luteinizing hormone, testosterone, and estradiol levels. J Clin Endocrinol Metab 82(12):3976-3981.

Antenos M, Zhu J, Jetly NM, Woodruff TK. 2008. An activin/furin regulatory loop modulates the processing and secretion of inhibin alpha- and beta(B)-subunit dimers in pituitary gonadotrope cells. J Biol Chem 283(48):33059-33068.

Asem EK, Feng SL, Stingley-Salazar SR, Turek JJ, Peter AT, Robinson JP. 2000a. Basal lamina of avian ovarian follicle: Influence on morphology of granulosa cells in-vitro. Comp Biochem Physiol C 125(2):189-201.

Asem EK, Stingley-Salazar SR, Robinson JP, Turek JJ. 2000b. Effect of basal lamina on progesterone production by chicken granulosa cells in vitro - influence of follicular development. Comp Biochem Physiol C 125(2):233-244.

Barakat B, O'Connor AE, Gold E, de Kretser DM, Loveland KL. 2008. Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. Reproduction 136(3):345-359.

Belaid B, Richard-Mercier N, Pieau C, Dorizzi M. 2001. Sex reversal and aromatase in the european pond turtle: Treatment with letrozole after the thermosensitive period for sex determination. J Exp Zool 290(5):490-497.

Bergmann M, Schindelmeiser J. 1987. Development of the blood-testis barrier in the domestic-fowl (*Gallus domesticus*). Int J Androl 10(2):481-488.

Bergmann M, Schindelmeiser J, Greven H. 1984. The blood-testis barrier in vertebrates having different testicular organization. Cell Tissue Res 238(1):145-150.

Berkholtz CB, Lai BE, Woodruff TK, Shea LD. 2006a. Distribution of extracellular matrix proteins type I collagen, type IV collagen, fibronectin, and laminin in mouse folliculogenesis. Histochem Cell Biol 126(5):583-592.

Berkholtz CB, Shea LD, Woodruff TK. 2006b. Extracellular matrix functions in follicle maturation. Seminars In Reproductive Medicine 24(4):262-269.

Bersinger NA, Odegard RA. 2007. Second-trimester serum levels of placenta growth factor (PLGF) and inhibin A are increased in smokers. Implications for pre-eclampsia risk assessment. Immuno-Analyse & Biologie Specialisee 22(1):19-23.

Billiar RB, Zachos NC, Burch MG, Albrecht ED, Pepe GJ. 2003. Up-regulation of alpha-inhibin expression in the fetal ovary of estrogen-suppressed baboons is associated with impaired fetal ovarian folliculogenesis. Biol Reprod 68(6):1989-1996.

Bozkurt HH, Aktas A, Ulkay MB, Firat UB. 2007. Sertoli cell proliferation during the post hatching period in domestic fowl. J Vet Sci 8(3):219-222.

Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Cook RW, Kipp JL, Shea LD, Mayo KE, Woodruff TK. 2006. Postnatal regulation of germ cells by activin: The establishment of the initial follicle pool. Dev Biol 298(1):132-148.

Britt KL, Saunders PK, McPherson SJ, Misso ML, Simpson ER, Findlay JK. 2004. Estrogen actions on follicle formation and early follicle development. Biol Reprod 71(5):1712-1723.

Bruggeman V, Van As P, Decuypere E. 2002. Developmental endocrinology of the reproductive axis in the chicken embryo. Comp Biochem Physiol A Physiol 131(4):839-846.

Burger J, Fossi C, McClellan-Green P, Orlando EF. 2007. Methodologies, bioindicators, and biomarkers for assessing gender-related differences in wildlife exposed to environmental chemicals. Environ Res 104(1):135-152.

Burns KH, Yan CN, Kumar TR, Matzuk MM. 2001. Analysis of ovarian gene expression in follicle-stimulating hormone beta knockout mice. Endocrinology 142(7):2742-2751.

Buzzard JJ, Farnworth PG, de Kretser DM, O'Connor AE, Wreford NG, Morrison JR. 2003. Proliferative phase Sertoli cells display a developmentally regulated response to activin in vitro. Endocrinology 144(2):474-483.

Buzzard JJ, Loveland KL, O'Bryan MK, O'Connor AE, Bakker M, Hayashi T, Wreford NG, Morrison JR, de Kretser DM. 2004. Changes in circulating and testicular levels of inhibin A and B and activin A during postnatal development in the rat. Endocrinology 145(7):3532-3541.

Callebaut M. 1968a. [3h]uridine incorporation during previtellogenesis and early vitellogenesis in oocytes of chick (*Gallus gallus*). J Embryol Exp Morphol 20:169-174.

Callebaut M. 1968b. Extracorporal development of quail oocytes. Experientia 24(12):1242-1243.

Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. 1998. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 204(2):373-384.

Cardeilhac PT. Reproductive physiology of the American alligator. Proceeding of the 1st Annual Alligator Production Conference; 1981; University of Florida, Gainesville.

Carreau S, Delalande C, Silandre D, Bourguiba S, Lambard S. 2006. Aromatase and estrogen receptors in male reproduction. Mol Cell Endocrinol 246(1-2):65-68.

Chen HY, Boontheung P, Loo RRO, Xie YM, Loo JA, Rao JY, Collins MD. 2008. Proteomic analysis to characterize differential mouse strain sensitivity to cadmium-induced forelimb teratogenesis. Birth Defects Res A 82(4):187-199.

Chen Y, Jefferson WN, Newbold RR, Padilla-Banks E, Pepling ME. 2007. Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. Endocrinology 148(8):3580-3590.

Chia R, Achilli F, Festing MFW, Fisher EMC. 2005. The origins and uses of mouse outbred stocks. Nat Genet 37(11):1181-1186.

Coe TS, Hamilton PB, Griffiths AM, Hodgson DJ, Wahab MA, Tyler CR. 2009. Genetic variation in strains of zebrafish (*Danio rerio*) and the implications for ecotoxicology studies. Ecotoxicology 18(1):144-150.

Coulson RA, Hernandez T. 1983. Natural history. Alligator metabolism: Studies on chemical-reactions in vivo. Elmsford, New York: Pergamon Press Inc. p 1-14.

Crain DA, Eriksen M, Iguchi T, Jobling S, Laufer H, LeBlanc GA, Guillette LJ. 2007. An ecological assessment of bisphenol-A: Evidence from comparative biology. Reprod Toxicol 24(2):225-239.

Crain DA, Guillette LJ, Rooney AA, Pickford DB. 1997. Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. Environ Health Perspect 105(5):528-533.

Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho SM, Hunt P, Iguchi T, Juul A, McLachlan JA, Schwartz J, Skakkebaek N, Soto AM, Swan S, Walker C, Woodruff TK, Woodruff TJ, Giudice LC, Guillette LJ. 2008. Female reproductive disorders: The roles of endocrine-disrupting compounds and developmental timing. Fertil Steril 90(4):911-940.

da Silva SJM, Bayne RAL, Cambray N, Hartley PS, McNeilly AS, Anderson RA. 2004. Expression of activin subunits and receptors in the developing human ovary: Activin A promotes germ cell survival and proliferation before primordial follicle formation. Dev Biol 266(2):334-345.

Dai J, Xie CS, Vincent R, Churg A. 2003. Air pollution particles produce airway wall remodeling in rat tracheal explants. Am J Respir Cell Mol Biol 29(3):352-358.

Davis AJ, Brooks CF, Johnson PA. 2001. Follicle-stimulating hormone regulation of inhibin alpha- and beta(B)-subunit and follistatin messenger ribonucleic acid in cultured avian granulosa cells. Biol Reprod 64(1):100-106.

Davis LM, Glenn TC, Strickland DC, Guillette LJ, Elsey RM, Rhodes WE, Dessauer HC, Sawyer RH. 2002. Microsatellite DNA analyses support an east-west phylogeographic split of American alligator populations. J Exp Zool 294(4):352-372.

de Kretser DM, Buzzard JJ, Okuma Y, O'Connor AE, Hayashi T, Lin SY, Morrison JR, Loveland KL, Hedger MP. 2004. The role of activin, follistatin and inhibin in testicular physiology. Mol Cell Endocrinol 225(1-2):57-64.

Delbes G, Levacher C, Habert R. 2006. Estrogen effects on fetal and neonatal testicular development. Reproduction 132(4):527-538.

Diaz ES, Pellizzari E, Casanova M, Cigorraga SB, Denduchis B. 2005. Type IV collagen induces down-regulation of steroidogenic response to gonadotropins in adult rat Leydig cells involving mitogen-activated protein kinase. Mol Reprod Dev 72(2):208-215.

Dong JW, Albertini DF, Nishimori K, Kumar TR, Lu NF, Matzuk MM. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383(6600):531-535.

Dornas RAP, Oliveira AG, Dias MO, Mahecha GAB, Oliveira CA. 2008. Comparative expression of androgen receptor in the testis and epididymal region of roosters (*Gallus domesticus*) and drakes (*Anas platyrhynchos*). Gen Comp Endocrinol 155(3):773-779.

Drozdowsky BS, Whittaker SG. 1999. Workplace hazards to reproduction and development. Olympia, WA: Safety and Health Assessment and Research for Prevention, Washington State Department of Labor and Industries.

Drummond AE. 2005. TGF beta signalling in the development of ovarian function. Cell Tissue Res 322(1):107-115.

Drummond AE, Findlay JK, Ireland JJ. 2004. Animal models of inhibin action. Seminars In Reproductive Medicine 22(3):243-252.

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. 2000. Effect of single and compound knockouts of estrogen receptors alpha (ER alpha) and beta (ER beta) on mouse reproductive phenotypes. Development 127(19):4277-4291.

Dykema JC, Mayo KE. 1994. 2 messenger ribonucleic-acids encoding the common beta(B)-chain of inhibin and activin have distinct 5'-initiation sites and are differentially regulated in rat granulosa-cells. Endocrinology 135(2):702-711.

Edwards TM, Gunderson MP, Milnes MR, Guillette LJ. 2004. Gonadotropin-induced testosterone response in peripubertal male alligators. General and Comparative Endocrinology 135(3):372-380.

Edwards TM, Moore BC, Guillette LJ. 2006a. Reproductive dysgenesis in wildlife: A comparative view. Int J Androl 29(1):109-120.

Elf PK. 2003. Yolk steroid hormones and sex determination in reptiles with TSD. General And Comparative Endocrinology 132(3):349-355.

Elvin JA, Yan CN, Wang P, Nishimori K, Matzuk MM. 1999. Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 13(6):1018-1034.

Emmen JMA, Couse JF, Elmore SA, Yates MM, Kissling GE, Korach KS. 2005. In vitro growth and ovulation of follicles from ovaries of estrogen receptor (ER)alpha and ER beta null mice indicate a role for ER beta in follicular maturation. Endocrinology 146(6):2817-2826.

Ethier JF, Findlay JK. 2001. Roles of activin and its signal transduction mechanisms in reproductive tissues. Reproduction 121(5):667-675.

Feng ZM, Bardin CW, Chen CLC. 1989. Characterization and regulation of testicular inhibin beta-subunit messenger-RNA. Mol Endocrinol 3(6):939-948.

Ferguson MW. 1985. Reproductive biology and embryology of the crocodilians. In: Gleason PJ, editor. Biol reptil. New York, N.Y.: John Wiley and Sons. p 329-491.

Ferguson MWJ, Joanen T. 1983. Temperature-dependent sex determination in *Alligator mississippiensis*. J Zool 200(JUN):143-177.

Ferriman EL, Sehmi IK, Jones R, Cuckle HS. 1999. The effect of smoking in pregnancy on maternal serum inhibin A levels. Prenat Diagn 19(4):372-374.

Findlay JK, Drummond AE, Dyson M, Baillie A, Robertson DM, Ethier JF. 2001. Production and actions of inhibin and activin during folliculogenesis in the rat. Mol Cell Endocrinol 180(1-2):139-144.

Fitzpatrick SL, Sindoni DM, Shughrue PJ, Lane MV, Merchenthaler IJ, Frail DE. 1998. Expression of growth differentiation factor-9 messenger ribonucleic acid in ovarian and nonovarian rodent and human tissues. Endocrinology 139(5):2571-2578.

Forbes TR. 1937. Studies on the reproductive system of the alligator I. The effects of prolonged injections of pituitary whole gland extract in the immature alligator. Anat Rec 70(1):113-137.

Forbes TR. 1938. Studies on the reproductive system of the alligator II the effecfs of prolonged injections of oestrone in the immature alligator. J Exp Zool 78(3):335-367.

Forbes TR. 1940. Studies of the reproductive system of the alligator IV. Observations of the development of the gonad, the adrenal cortex, and the Mullerian duct. Contrib Embryol 174:131-154.

Forbes TR. 1956. The development of the reproductive system of A lizard, *Anolis carolinensis*. Am J Anat 98(1):139-157.

Fowler PA, Dora NJ, McFerran H, Amezaga MR, Miller DW, Lea RG, Cash P, McNeilly AS, Evans NP, Cotinot C, Sharpe RM, Rhind SM. 2008. In utero exposure to low doses of environmental pollutants disrupts fetal ovarian development in sheep. Mol Hum Reprod 14(5):269-280.

Fujisaki I, Rice KG, Woodward AR, Percival HF. 2007. Possible generational effects of habitat degradation on alligator reproduction. J Wildl Manage 71(7):2284-2289.

Gelly JL, Richoux JP, Leheup BP, Grignon G. 1989. Immunolocalization of type-IV collagen and laminin during rat gonadal morphogenesis and postnatal-development of the testis and epididymis. Histochemistry 93(1):31-37.

Genissel C, Levallet J, Carreau S. 2001. Regulation of cytochrome p450 aromatase gene expression in adult rat Leydig cells: Comparison with estradiol production. J Endocrinol 168(1):95-105.

Gomez Y, Velazquez PN, Peralta-Delgado I, Mendez MC, Vilchis F, Juarez-Oropeza MA, Pedernera E. 2001. Follicle-stimulating hormone regulates steroidogenic enzymes in cultured cells of the chick embryo ovary. Gen Comp Endocrinol 121(3):305-315.

Gondos B, Zamboni L. 1969. Ovarian development - functional importance of germ cell interconnections. Fertil Steril 20(1):176-&.

Gonzalez-Moran G. 1997. A stereological study of the different cell populations in chicken testes treated with follicle-stimulating hormone during embryonic development. Anat Histol Embryol 26(4):311-317.

Gonzalez-Moran G. 1998. Effect of follicle-stimulating hormone on different cell sub-populations in the ovary of newly hatched chicks treated during embryonic development. Br Poult Sci 39(1):128-132.

Gonzalez-Moran G, Gonzalez del Pliego M, Pedernera E. 1985. Morphological-changes in the ovary of newly hatched chickens treated with chorionic-gonadotropin during embryonic-development. Gen Comp Endocrinol 59(1):162-167.

Gonzalez-Moran G, Mancilla C. 1998. Histomorphometric analysis in the ovary of newly hatched chicks treated with follicle-stimulating hormone during embryonic development. Eur J Morphol 36(1):11-18.

Gonzalezmoran G, Gonzalezdelpliego M, Pedernera E. 1985. Morphological-changes in the ovary of newly hatched chickens treated with chorionic-gonadotropin during embryonic-development. General And Comparative Endocrinology 59(1):162-167.

Gould ML, Hurst PR, Nicholson HD. 2007. The effects of oestrogen receptors alpha and beta on testicular cell number and steroidogenesis in mice. Reproduction 134(2):271-279.

Govoroun MS, Pannetier M, Pailhoux E, Cocquet J, Brillard JP, Couty I, Batellier F, Cotinot C. 2004. Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev Dyn 231(4):859-870.

Gribbins KM, Elsey RM, Gist DH. 2006. Cytological evaluation of the germ cell development strategy within the testis of the American alligator, *Alligator mississippiensis*. Acta Zoologica 87(1):59-69.

Guillette LJ, Brock JW, Rooney AA, Woodward AR. 1999a. Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. Arch Environ Contam Toxicol 36(4):447-455.

Guillette LJ, Crain DA, Gunderson MP, Kools SAE, Milnes MR, Orlando EF, Rooney AA, Woodward AR. 2000a. Alligators and endocrine disrupting contaminants: A current perspective. Am Zool 40(3):438-452.

Guillette LJ, Crain DA, Gunderson MP, Kools SAE, Milnes MR, Orlando EF, Rooney AA, Woodward AR. 2000b. Alligators and endocrine disrupting contaminants: A current perspective. Am Zool 40(3):438-452.

Guillette LJ, Crain DA, Rooney AA, Pickford DB. 1995. Organization versus activation - the role of endocrine-disrupting contaminants (EDCs) during embryonic-development in wildlife. Environ Health Perspect 103:157-164.

Guillette LJ, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. 1994. Developmental abnormalities of the gonad and abnormal sex-hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect 102(8):680-688.

Guillette LJ, Gunderson MP. 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122(6):857-864.

Guillette LJ, Pickford DB, Crain DA, Rooney AA, Percival HF. 1996. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen Comp Endocrinol 101(1):32-42.

Guillette LJ, Woodward AR, Crain DA, Pickford DB, Rooney AA, Percival HF. 1999b. Plasma steroid concentrations and male phallus size in juvenile alligators from seven Florida lakes. Gen Comp Endocrinol 116(3):356-372.

Gunderson MP, Kohno S, Blumberg B, Iguchi T, Guillette LJ. 2006. Up-regulation of the alligator CYP3A77 gene by toxaphene and dexamethasone and its short term effect on plasma testosterone concentrations. Aquat Toxicol 78(3):272-283.

Gunderson MP, LeBlanc GA, Guillette LJ. 2001. Alterations in sexually dimorphic biotransformation of testosterone in juvenile American alligators (*Alligator mississippiensis*) from contaminated lakes. Environ Health Perspect 109(12):1257-1264.

Guo QX, Kumar TR, Woodruff T, Hadsell LA, DeMayo FJ, Matzuk MM. 1998. Overexpression of mouse follistatin causes reproductive defects in transgenic mice. Mol Endocrinol 12(1):96-106.

Guraya SS. 1962. Structure and function of so-called yolk-nucleus in oogenesis of birds. Q J Microsc Sci 103(4):411-&.

Hand DJ, Heard NA. 2005. Finding groups in gene expression data. J Biomed Biotechnol(2):215-225.

Harper JM. 2008. Wild-derived mouse stocks: An underappreciated tool for aging research. Age 30(2-3):135-145.

Harvey SJ, Zheng KQ, Sado Y, Naito I, Ninomiya Y, Jacobs RM, Hudson BG, Thorner PS. 1998. Role of distinct type IV collagen networks in glomerular development and function. Kidney Int 54(6):1857-1866.

Heinz GH, Percival HF, Jennings ML. 1991a. Contaminants in American alligator eggs from Lake Apopka, Lake Griffin, and Lake Okeechobee, Florida. Environ Monit Assess 16(3):277-285.

Herbert JD, Coulson TD, Coulson RA. 2002. Growth rates of Chinese and American alligators. Comp Biochem Physiol A 131(4):909-916.

Hotchkiss AK, Rider CV, Blystone CR, Wilson VS, Hartig PC, Ankley GT, Foster PM, Gray CL, Gray LE. 2008. Fifteen years after "wingspread" - environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go. Toxicol Sci 105(2):235-259.

Hudson QJ, Smith CA, Sinclair AH. 2005. Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary. Dev Dyn 233(3):1052-1055.

Huet C, Monget P, Pisselet C, Monniaux D. 1997. Changes in extracellular matrix components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. Biol Reprod 56(4):1025-1034.

Huet C, Pisselet C, Mandon-Pepin B, Monget P, Monniaux D. 2001. Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: Relationships between cell shape and function. J Endocrinol 169(2):347-360.

Humason GL. 1979. Animal tissue techniques. San Francisco: W.H. Freeman.

Hunt PA, Hassold TJ. 2008. Human female meiosis: What makes a good egg go bad? Trends Genet 24(2):86-93.

Iguchi T. 1992. Cellular effects of early exposure to sex hormones and antihormones. Int Rev Cytol 139:1-57.

Iguchi T, Fukazawa Y, Uesugi Y, Takasugi N. 1990. Polyovular follicles in mouse ovaries exposed neonatally to diethylstilbestrol in vivo and in vitro. Biol Reprod 43:478-484.

Iguchi T, Katsu Y. 2008. Commonality in signaling of endocrine disruption from snail to human. Bioscience 58(11):1061-1067.

Iguchi T, Ohta Y, Fukazawa Y, Takasugi N. 1987. Strain differences in the induction of polyovular follicles by neonatal treatment with diethylstilbestrol in mice. Med Sci Res 15(21-22):1407-1408.

Iguchi T, Takasugi N. 1986. Polyovular follicles in the ovary of immature mice exposed prenatally to diethylstilbestrol. Anat Embryol (Berl) 175(1):53-55.

Iguchi T, Takasugi N, Bern HA, Mills KT. 1986. Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. Teratology 34(1):29-35.

Iguchi T, Todoroki R, Takasugi N, Petrow V. 1988. The effects of an aromatase inhibitor and A 5-alpha-reductase inhibitor upon the occurrence of polyovular follicles, persistent anovulation, and permanent vaginal stratification in mice treated neonatally with testosterone. Biol Reprod 39(3):689-697.

Irving-Rodgers HF, Rodgers RJ. 2006. Extracellular matrix of the developing ovarian follicle. Semin Reprod Med 24(4):195-203.

Itman C, Mendis S, Barakat B, Loveland KL. 2006. All in the family: TGF-beta family action in testis development. Reproduction 132(2):233-246.

Jefferson W, Newbold R, Padilla-Banks E, Pepling M. 2006. Neonatal genistein treatment alters ovarian differentiation in the mouse: Inhibition of oocyte nest breakdown and increased oocyte survival. Biol Reprod 74(1):161-168.

Jefferson WN, Couse JF, Padilla-Banks E, Korach KS, Newbold RR. 2002. Neonatal exposure to genistein induces estrogen receptor (er) alpha expression, and multi-oocyte follicles in the maturing mouse ovary: Evidence for er beta and nonestrogenic actions. Biol Reprod 66:189-190.

Joanen T, McNease L. 1980. Reproductive biology of the American alligator in southwest Louisiana. In: Murphy JB, Collins JT, editors. Reproductive biology and disease of captive reptiles. Lawrence, Kansas. p 153-159.

Joanen T, McNease LL. 1989. Ecology and physiology of nesting and early development of the American alligator. Am Zool 29(3):987-998.

Jobling S, Tyler CR. 2003. Endocrine disruption in wild freshwater fish. Pure Appl Chem 75(11-12):2219-2234.

Johnson PA, Dickens MJ, Kent TR, Giles JR. 2005. Expression and function of growth differentiation factor-9 in an oviparous species, *Gallus domesticus*. Biol Reprod 72(5):1095-1100.

Jones RE, Tokarz RR, Lagreek FT. 1975. Endocrine control of clutch size in reptiles.5. FSH-induced follicular formation and growth in immature ovaries of *Anolis-carolinensis*. Gen Comp Endocr 26(3):354-367.

Jones SM, Swain R. 2000. Effects of exogenous FSH on follicular recruitment in a viviparous lizard niveoscincus metallicus (scincidae). Comp Biochem Phys A 127(4):487-493.

Jorgez CJ, Klysik M, Jamin SP, Behringer RR, Matzuk MM. 2004. Granulosa cell-specific inactivation of follistatin causes female fertility defects. Mol Endocrinol 18(4):953-967.

Katoh H, Ogino Y, Yamada G. 2006. Cloning and expression analysis of androgen receptor gene in chicken embryogenesis. FEBS Lett 580(6):1607-1615.

Katsu Y, Bermudez DS, Braun EL, Helbing C, Miyagawa S, Gunderson MP, Kohno S, Bryan TA, Guillette LJ, Iguchi T. 2004. Molecular cloning of the estrogen and progesterone receptors of the American alligator. Gen Comp Endocrinol 136(1):122-133.

Kezele P, Skinner MK. 2003. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: Endocrine model of follicle assembly. Endocrinology 144(8):3329-3337.

Kezele PR, Ague JM, Nilsson E, Skinner MK. 2005. Alterations in the ovarian transcriptome during primordial follicle assembly and development. Biol Reprod 72(1):241-255.

Kim H, Hayashi S, Chambon P, Watanabe H, Iguchi T, Sato T. 2009a. Effects of diethylstilbestrol on ovarian follicle development in neonatal mice. Reprod Toxicol 27(1):55-62.

Kim H, Hayasi S, Chambon P, Watanabe H, Iguchi T, Sato T. 2009b. Effects of diethylstilbestrol on ovarain follicle development in neonatal mice. Reprod Toxicol 27:55-62.

Kipp JJL, Kilen SM, Woodruff TK, Mayo KE. 2007. Activin regulates estrogen receptor gene expression in the mouse ovary. J Biol Chem 282(50):36755-36765.

Kipp JL, Kilen SM, Bristol-Gould S, Woodruff TK, Mayo KE. 2007b. Neonatal exposure to estrogens suppresses activin expression and signaling in the mouse ovary. Endocrinology 148(5):1968-1976.

Kirigaya A, Sato T, Iguchi T, Hayashi S. 2004. Involvement of MIS and ER beta in induction of polyovular follicles (pf) in mouse ovary treated with des neonatally. Zool Sci 21(12):1343-1343.

Knight PG. 1996. Roles of inhibins, activins, and follistatin in the female reproductive system. Front Neuroendocrinol 17(4):476-509.

Koba N, Mori M, Ha Y, Mizushima S, Tsukada A, Saito N, Ono T, Shimada K. 2008a. Effects of aromatase inhibitor (fadrozole)-induced sex-reversal on gonadal differentiation and mRNA expression of p450arom, AMH and ER alpha in embryos and growth in posthatching quail. Poult Sci 45(2):116-124.

Koba N, Ohfuji T, Ha Y, Mizushima S, Tsukada A, Saito N, Shimada K. 2008b. Expression of p450arom, AMH and ER alpha mRNA in gonads of turkey, duck and goose within one week of age. Poult Sci 45(3):220-226.

Koba N, Ohfuji T, Ha Y, Mizushima S, Tsukada A, Saito N, Shimada K. 2008c. Profiles of mRNA expression of FOXL2, p450arom, DMRT 1, AMH, p450(c17), SF1, ER alpha and AR, in relation to gonadal sex differentiation in duck embryo. J Poult Sci 45(2):132-138.

Kohno S, Bermudez DS, Katsu Y, Iguchi T, Guillette LJ. 2008. Gene expression patterns in juvenile American alligators (*Alligator mississippiensis*) exposed to environmental contaminants. Aquat Toxicol 88(2):95-101.

Krishna TSR, Kong XP, Gary S, Burgers PM, Kuriyan J. 1994. Crystal-structure of the eukaryotic DNA-polymerase processivity factor PCNA. Cell 79(7):1233-1243.

Kumar TR, Varani S, Wreford NG, Telfer NM, de Kretser DM, Matzuk MM. 2001. Male reproductive phenotypes in double mutant mice lacking both FSH beta and activin receptor IIA. Endocrinology 142(8):3512-3518.

Kumar TR, Wang Y, Lu NF, Matzuk MM. 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15(2):201-204.

Kumaran JDS, Turner CW. 1949. The normal development of the testes in the white plymouth rock. Poult Sci 28(4):511-520.

Lahlou N, Roger M. 2004. Inhibin B in pubertal development and pubertal disorders. Semin Reprod Med 22(3):165-175.

Lance VA. 1989. Reproductive-cycle of the American alligator. Am Zool 29(3):999-1018.

Lance VA. 2003. Alligator physiology and life history: The importance of temperature. Exp Gerontol 38(7):801-805.

Lance VA, Vliet KA. 1987. Effect of mammalian gonadotropins on testosterone secretion in male alligators. Journal of Experimental Zoology 241(1):91-94.

Lee VH, Britt JH, Dunbar BS. 1996. Localization of laminin proteins during early follicular development in pig and rabbit ovaries. J Reprod Fertil 108(1):115-122.

Lenie S, Smitz J. 2008. Estrogen receptor subtypes localization shifts in cultured mouse ovarian follicles. Histochem Cell Biol 129(6):827-840.

Lewis KA, Gray PC, Blount AL, MacConnell LA, Wiater E, Bilezikjian LM, Vale W. 2000. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature 404(6776):411-414.

Licht P, Pearson AK. 1969. Effects of mammalian gonadotropins (FSH and LH) on testes of lizard *anolis carolinensis*. Gen Comp Endocrinol 13(3):367-&.

Liu JQ, Kuulasmaa T, Kosma VM, Butzow R, Vanttinen T, Hyden-Granskog C, Voutilainen R. 2003. Expression of betaglycan, an inhibin coreceptor, in normal human ovaries and ovarian sex cord-stromal tumors and its regulation in cultured human granulosa-luteal cells. J Clin Endocrinol Metab 88(10):5002-5008.

Loffler KA, Zarkower D, Koopman P. 2003. Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology 144(7):3237-3243.

Loveland KL, Dias V, Meachem S, Meyts ERD. 2007. The transforming growth factor-beta superfamily in early spermatogenesis: Potential relevance to testicular dysgenesis. Int J Androl 30(4):377-384.

Lovell TM, Gladwell RT, Cunningham FJ, Groome NP, Knight PG. 1998. Differential changes in inhibin A, activin A, and total alpha-subunit levels in granulosa and thecal layers of developing preovulatory follicles in the chicken. Endocrinology 139(3):1164-1171.

Lovell TM, Knight PG, Groome NP, Gladwell RT. 2000. Measurement of dimeric inhibins and effects of active immunization against inhibin alpha-subunit on plasma hormones and testis morphology in the developing cockerel. Biol Reprod 63(1):213-221.

Ma XP, Dong YL, Matzuk MM, Kumar TR. 2004. Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. Proc Natl Acad Sci U S A 101(49):17294-17299.

Maekawa M, Kamimura K, Nagano T. 1996. Peritubular myoid cells in the testis: Their structure and function. Arch Histol Cytol 59(1):1-13.

Maheshwari A, Fowler PA. 2008. Primordial follicular assembly in humans - revisited. Zygote 16(4):285-296.

Main KM, Toppari J, Suomi AM, Kaleva M, Chellakooty M, Schmidt IM, Virtanen HE, Boisen KA, Kai CM, Damgaard IN, Skakkebaek NE. 2006. Larger testes and higher inhibin B levels in Finnish than in Danish newborn boys. J Clin Endocrinol Metab 91(7):2732-2737.

Makanji Y, Walton KL, Wilce MC, Chan KL, Robertson DM, Harrison CA. 2008. Suppression of inhibin A biological activity by alterations in the binding site for betaglycan. J Biol Chem 283(24):16743-16751.

Martens JWM, deWinter JP, Timmerman MA, McLuskey A, VanSchaik RHN, Themmen APN, deJong FH. 1997. Inhibin interferes with activin signaling at the level of the activin receptor complex in Chinese hamster ovary cells. Endocrinology 138(7):2928-2936.

Masson GR. 1995. Environmental influences on reproductive potential, clutch viability and embryonic mortality of the American alligator in Florida [Ph.D. Dissertation]: University of Florida.

Matova N, Cooley L. 2001. Comparative aspects of animal oogenesis. Dev Biol 231(2):291-320.

Matsumoto T, Shiina H, Kawano H, Sato T, Kato S. 2008. Androgen receptor functions in male and female physiology. J Steroid Biochem Mol Biol 109(3-5):236-241.

Mayo K, Jameson L, Woodruff TK. 2007. Eggs in the nest. Endocrinology 148(8):3577-3579.

Mazatid S, Guyot R, Guig UJB, Coudoue N, Le Magueresse-Battistoni B, Margre S. 2005. Basal membrane remodeling during follicle histogenesis in the rat ovary: Contribution of proteinase of the MMP and PA families. Dev Biol 277(2):403-416.

McMullen ML, Cho BN, Yates CJ, Mayo KE. 2001. Gonadal pathologies in transgenic mice expressing the rat inhibin alpha-subunit. Endocrinology 142(11):5005-5014.

McNatty KP, Fidler AE, Juengel JL, Quirke LD, Smith PR, Heath DA, Lundy T, O'Connell A. 2000. Growth and paracrine factors regulating follicular formation and cellular function. Mol Cell Endocrinol 163(1-2):11-20.

Meachem SJ, Nieschlag E, Simoni M. 2001. Inhibin B in male reproduction: Pathophysiology and clinical relevance. Eur J Endocrinol 145(5):561-571.

Meehan T, Schlatt S, O'Bryan MK, de Kretser DM, Loveland KL. 2000. Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. Dev Biol 220(2):225-237.

Mendez-Herrera MC, Tamez L, Candido A, Reyes-Esparza JA, Pedernera E. 1998. Follicle stimulating hormone increases somatic and germ cell number in the ovary during chick embryo development. Gen Comp Endocrinol 111(2):207-215.

Menke DB, Page DC. 2002. Sexually dimorphic gene expression in the developing mouse gonad. Gene Expr Patterns 2(3):359-367.

Michel U, Mcmaster JW, Findlay JK. 1992. Regulation of steady-state follistatin messenger-rna levels in rat granulosa-cells invitro. J Mol Endocrinol 9(2):147-156.

Milnes MR, Allen D, Bryan TA, Sedacca CD, Guillette LJ. 2004a. Developmental effects of embryonic exposure to toxaphene in the American alligator (*Alligator mississippiensis*). Comp Biochem Phys C 138(1):81-87.

Milnes MR, Allen D, Bryan TA, Sedacca CD, Guillette LJ. 2004b. Developmental effects of embryonic exposure to toxaphene in the American alligator (*Alligator mississippiensis*). Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 138(1):81-87.

Milnes MR, Bermudez DS, Bryan TA, Gunderson MP, Guillette LJ. 2005. Altered neonatal development and endocrine function in *Alligator mississippiensis* associated with a contaminated environment. Biol Reprod 73(5):1004-1010.

Milnes MR, Bryan TA, Katsu Y, Kohno S, Moore BC, Iguchi T, Guillette LJ. 2008. Increased posthatching mortality and loss of sexually dimorphic gene expression in alligators (*Alligator mississippiensis*) from a contaminated environment. Biol Reprod 78(5):932-938.

Milnes MR, Guillette LJ. 2008. Alligator tales: New lessons about environmental contaminants from a sentinel species. Bioscience 58(11):1027-1036.

Milnes MR, Woodward AR, Rooney AA, Guillette LJ. 2002. Plasma steroid concentrations in relation to size and age in juvenile alligators from two Florida lakes. Comp Biochem Physiol A Comp Physiol 131(4):923-930.

Modi WS, Crews D. 2005. Sex chromosomes and sex determination in reptiles - commentary. Curr Opin Genet Dev 15(6):660-665.

Moore BC, Hyndman KA. 200X. Morphology and histochemistry of juvenile American alligator nephrons. Anatomical Record (accepted).

Moore BC, Milnes MR, Kohno S, Katsu Y, Iguchi T, Guillette LJ. Accepted. Influences of sex, incubation temperature, and environmental quality on gonadal estrogen and androgen steroid receptor mRNA expression in juvenile American alligators (*Alligator mississippiensis*). Biol Reprod.

Moore BC, Uribe-Aranzabal MC, Boggs ASP, Guillette LJ. 2008. Developmental morphology of the neonatal alligator (*Alligator mississippiensis*) ovary. J Morphol 269(3):302-312.

Mousa MA, Mousa SA. 2003. Immunohistochemical localization of inhibin and activin-like proteins in the brain, pituitary gland, and the ovary of thin-lipped grey mullet, *Liza ramada* (Risso). Gen Comp Endocrinol 132(3):434-443.

Muretto P, Chilosi M, Rabitti C, Tommasoni S, Colato C. 2001. Biovularity and "coalescence of primary follicles" in ovaries with mature teratomas. Int J Surg Pathol 9(2):121-125.

Nakamura T, Hasegawa Y, Sugino K, Kogawa K, Titani K, Sugino H. 1992. Follistatin inhibits activin-induced differentiation of rat follicular granulosa-cells invitro. Biochimica Et Biophysica Acta 1135(1):103-109.

Nakamura T, Katsu Y, Watanabe H, Iguchi T. 2008. Estrogen receptor subtypes selectively mediate female mouse reproductive abnormalities induced by neonatal exposure to estrogenic chemicals. Toxicology 253(1-3):117-124.

Narbaitz R, Adler R. 1966. Submicroscopical observations on differentiation of chick gonads. J Embryol Exp Morphol 16:41-&.

Narbaitz R, Derobert EM. 1968. Postnatal evolution of steroidogenic cells in chick ovary. Histochemie 15(3):187-&.

Narbaitz R, DeRobertis EM. 1968. Postnatal evolution of steroidogenic cells in chick ovary. Histochemie 15(3):187-&.

Ni YD, Zhou YC, Lu LZ, Grossmann R, Zhao RQ. 2007. Developmental changes of FSH-r, LH-r, ER-beta and GnRH-I expression in the ovary of prepubertal ducks (*Anas platyrhynchos*). Anim Reprod Sci 100(3-4):318-328.

Nicholls PK, Harrison CA, Gilchrist RB, Farnworth PG, Stanton PG. In Press. Growth differntiation factor 9 (GDF9) is A germ-cell regulator of Sertoli cell function. Endocrinology.

Nitta H, Mason JI, Bahr JM. 1993. Localization of 3-beta-hydroxysteroid dehydrogenase in the chicken ovarian follicle shifts from the theca layer to granulosa layer with follicular maturation. Biol Reprod 48(1):110-116.

Nitta H, Osawa Y, Bahr JM. 1991a. Immunolocalization of steroidogenic cells in small follicles of the chicken ovary - anatomical arrangement and location of steroidogenic cells change during follicular development. Domest Anim Endocrinol 8(4):587-594.

Nitta H, Osawa Y, Bahr JM. 1991b. Multiple steroidogenic cell-populations in the thecal layer of preovulatory follicles of the chicken ovary. Endocrinology 129(4):2033-2040.

O'Donnell L, Robertson KM, Jones ME, Simpson ER. 2001. Estrogen and spermatogenesis. Endocr Rev 22(3):289-318.

Oktay K, Schenken RS, Nelson JF. 1995. Proliferating cell nuclear antigen marks the initiation of follicular-growth in the rat. Biol Reprod 53(2):295-301.

Onagbesan O, Bruggeman V, Decuypere E. 2009. Intra-ovarian growth factors regulating ovarian function in avian species: A review. Anim Reprod Sci 111(2-4):121-140.

Onagbesan OM, Safi M, Decuypere E, Bruggeman V. 2004. Developmental changes in inhibin alpha and inhibin/activin beta A and beta B mRNA levels in the gonads during post-hatch prepubertal development of male and female chickens. Mol Reprod Dev 68(3):319-326.

Oreal E, Mazaud S, Picard JY, Magre S, Carre-Eusebe D. 2002. Different patterns of anti-Mullerian hormone expression, as related to DMRT1, SF-1, WT1, GATA-4, WNT-4, and LHX9 expression, in the chick differentiating gonads. Dev Dyn 225(3):221-232.

Organ CL, Janes DE. 2008. Evolution of sex chromosomes in sauropsida. Integr Comp Biol 48(4):512-519.

Orlando EF, Guillette LJ. 2001. A re-examination of variation associated with environmentally stressed organisms. Hum Reprod Update 7(3):265-272.

Oshima Y, Uno Y, Matsuda Y, Kobayashi T, Nakamura M. 2008. Molecular cloning and gene expression of Foxl2 in the frog *Rana rugosa*. Gen Comp Endocrinol 159(2-3):170-177.

Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G, Schlessinger D. 2005. Foxl2 is required for commitment to ovary differentiation. Hum Mol Genet 14(14):2053-2062.

Ottolenghi C, Pelosi E, Tran J, Colombino M, Douglass E, Nedorezov T, Cao A, Forabosco A, Schlessinger D. 2007a. Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. Hum Mol Genet 16(23):2795-2804.

Ottolenghi C, Uda M, Crisponi L, Omari S, Cao A, Forabosco A, Schlessinger D. 2007b. Determination and stability of sex. Bioessays 29(1):15-25.

Pangas SA, Jorgez CJ, Tran M, Agno J, Li XH, Brown CW, Kumar TR, Matzuk MM. 2007. Intraovarian activins are required for female fertility. Mol Endocrinol 21(10):2458-2471.

Pangas SA, Woodruff TK. 2000. Activin signal transduction pathways. Trends Endocrinol Metab 11(8):309-314.

Pannetier M, Fabre S, Batista F, Kocer A, Renault L, Jolivet G, Mandon-Pepin B, Cotinot C, Veltla R, Pailhoux E. 2006. FOXL2 activates p450 aromatase gene transcription: Towards a better characterization of the early steps of mammalian ovarian development. Journal Of Molecular Endocrinology 36(3):399-413.

Pedernera E, Gomez Y, Velazquez P, Juarezoropeza MA, Delpliego MG. 1988a. Identification of steroidogenic cell subpopulations in the ovary of the newly hatched chicken. Gen Comp Endocrinol 71(1):153-162.

Pedernera E, Gomez Y, Velazquez P, Juarezoropeza MA, Delpliego MG. 1988b. Identification of steroidogenic cell subpopulations in the ovary of the newly hatched chicken. General And Comparative Endocrinology 71(1):153-162.

Pedernera E, Solis L, Peralta I, Velazquez PN. 1999. Proliferative and steroidogenic effects of follicle-stimulating hormone during chick embryo gonadal development. Gen Comp Endocrinol 116(2):213-220.

Pelliniemi LJ, Frojdman K. 2001. Structural and regulatory macromolecules in sex differentiation of gonads. J Exp Zool 290(5):523-528.

Pepe GJ, Billiar RB, Albrecht ED. 2006. Regulation of baboon fetal ovarian folliculogenesis by estrogen. Mol Cell Endocrinol 247(1-2):41-46.

Pepling ME. 2006. From primordial germ cell to primordial follicle: Mammalian female germ cell development. Genesis 44(12):622-632.

Pepling ME, de Cuevas M, Spradling AC. 1999. Germline cysts: A conserved phase of germ cell development? Trends Cell Biol 9(7):257-262.

Pepling ME, Spradling AC. 2001. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Developmental Biology 234(2):339-351.

Petrino TR, Toussaint G, Lin YWP. 2007. Role of inhibin and activin in the modulation of gonadotropin- and steroid-induced oocyte maturation in the teleost *Fundulus heteroclitus*. Reprod Biol Endocrinol 5.

Pieau C, Dorizzi M. 2004. Oestrogens and temperature-dependent sex determination in reptiles: All is in the gonads. J Endocrinol 181(3):367-377.

Pru JK, Tilly JL. 2001. Programmed cell death in the ovary: Insights and future prospects using genetic technologies. Molecular Endocrinology 15(6):845-853.

Puchtler H, Waldrop FS. 1978. Silver impregnation methods for reticulum fibers and reticulin - reinvestigation of their origins and specifity. Histochemistry 57(3):177-187.

Raivio T, Dunkel L. 2002. Inhibins in childhood and puberty. Best Pract Res Clin Endoc Metab 16(1):43-52.

Ramaswam LS, Jacob D. 1965. Effect of testosterone propionate on urogenital organs of immature crocodile *Crocodylus palustris* lesson. Experientia 21(4):206.

Ramsey M, Crews D. 2007. Steroid signaling system responds differently to temperature and hormone manipulation in the red-eared slider turtle (*Trachemys scripta elegans*), a reptile with temperature-dependent sex determination. Sex Dev 1(3):181-196.

Ramsey M, Shoemaker C, Crews D. 2007. Gonadal expression of Sf1 and aromatase during sex determination in the red-eared slider turtle (Trachemys scripta), a reptile with temperature-dependent sex determination. Differentiation 75(10):978-991.

Rauschenberger RH, Wiebe JJ, Sepulveda MS, Scarborough JE, Gross TS. 2007. Parental exposure to pesticides and poor clutch viability in American alligators. Environ Sci Technol 41(15):5559-5563.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307(5715):1625-1630.

Rhen T, Metzger K, Schroeder A, Woodward R. 2007. Expression of putative sex-determining genes during the thermosensitive period of gonad development in the snapping turtle, Chelydra serpentina. Sex Dev 1(4):255-270.

Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR, vom Saal FSV. 2007. In vivo effects of bisphenol A in laboratory rodent studies. Reprod Toxicol 24(2):199-224.

Robertson KM, O'Donnell L, Jones MEE, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER. 1999. Impairment of spermatogenesis in mice lacking a functional aromatase (*Cyp 19*) gene. Proc Natl Acad Sci U S A 96(14):7986-7991.

Rodgers RJ, Irving-Rodgers HF, Russell DL. 2003. Extracellular matrix of the developing ovarian follicle. Reproduction 126(4):415-424.

Rodgers RJ, Rodgers HFI. 2002. Extracellular matrix of the bovine ovarian membrana granulosa. Mol Cell Endocrinol 191(1):57-64.

Rombauts L, Vanmontfort D, Decuypere E, Verhoeven G. 1995. Gonadotropin-regulated production of immunoactive inhibin and androgen by cultured testicular cells from chicken embryos. Gen Comp Endocrinol 99(2):152-160.

Rombauts L, Vanmontfort D, Decuypere E, Verhoeven G. 1996. Inhibin and activin have antagonistic paracrine effects on gonadal steroidogenesis during the development of the chicken embryo. Biol Reprod 54(6):1229-1237.

Rooney AA, Crain DA, Woodward AR, Guillette LJ. 2004. Seasonal variation in plasma sex steroid concentrations in juvenile American alligators. Gen Comp Endocrinol 135(1):25-34.

Rotstein DS, Schoeb TR, Davis LM, Glenn TC, Arnold BS, Gross TS. 2002. Detection by microsatellite analysis of early embryonic mortality in an alligator population in Florida. J Wildl Dis 38(1):160-165.

Roy SK, Albee L. 2000. Requirement for follicle-stimulating hormone action in the formation of primordial follicles during perinatal ovarian development in the hamster. Endocrinology 141(12):4449-4456.

Saalfeld DT, Webb KK, Conway WC, Calkins GE, Duguay JP. 2008. Growth and condition of American alligators (*Alligator mississippiensis*) in an inland wetland of east Texas. Southeast Nat 7(3):541-550.

Safi M, Onagbesan OM, Bruggeman V, Vleugels B, Volckaert G, Decuypere E. 2003. Regulation of inhibin alpha- and beta(A)-subunit messenger ribonucleic acid levels by gonadotropins and IGF-I in cultured chicken granulosa cells. Gen Comp Endocrinol 131(2):159-167.

Safi M, Onagbesan OM, Volckaert G, Vanmontfort D, Bruggeman V, Decuypere E. 2001. Developmental expression of activin/inhibin alpha- and beta(A)-subunit genes in the gonads of male and female chick embryos. Gen Comp Endocrinol 122(3):304-311.

Sanchez-Bringas G, Salazar O, Pedernera E, Mendez C. 2006. Follicle-stimulating hormone treatment reverses the effect of hypophysectomy on cell proliferation in the chicken embryo ovary. Gen Comp Endocrinol 149(2):134-140.

Sar M, Welsch F. 1999. Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. Endocrinology 140(2):963-971.

Schlatt S, Weinbauer GF. 1994. Immunohistochemical localization of proliferating cell nuclear antigen as a tool to study cell-proliferation in rodent and primate testes. Int J Androl 17(4):214-222.

Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, Treier M. 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development 131(4):933-942.

Schmierer B, Schuster MK, Shkumatava A, Kuchler K. 2003. Activin and follicle-stimulating hormone signaling are required for long-term culture of functionally differentiated primary granulosa cells from the chicken ovary. Biol Reprod 68(2):620-627.

Schomberg DW, Couse JF, Mukherjee A, Lubahn DB, Sar M, Mayo KE, Korach KS. 1999. Targeted disruption of the estrogen receptor-alpha gene in female mice: Characterization of ovarian responses and phenotype in the adult. Endocrinology 140(6):2733-2744.

Sekido R, Lovell-Badge R. 2007. Mechanisms of gonadal morphogenesis are not conserved between chick and mouse. Dev Biol 302(1):132-142.

Sharara FI, Seifer DB, Flaws JA. 1998. Environmental toxicants and female reproduction. Fertil Steril 70(4):613-622.

Shariati M, Noorafshan A, Mokhtari M, Askari HR. 2008. The effects of trifluralin on LH, FSH and testosterone hormone levels and testis histological changes in adult rats. Int J Fertil Steril 2(1):23-28.

Shayu D, Rao AJ. 2006. Expression of functional aromatase in the epididymis: Role of androgens and LH in modulation of expression and activity. Mol Cell Endocrinol 249(1-2):40-50.

Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, Sakari M, Takada I, Nakamura T, Metzger D, Chambon P, Kanno J, Yoshikawa H, Kato S. 2006. Premature ovarian failure in androgen receptor-deficient mice. Proc Natl Acad Sci U S A 103(1):224-229.

Sierens JE, Sneddon SF, Collins F, Millar MR, Saunders PTK. 2005. Estrogens in testis biology. Testicular cell dynamics and endocrine signaling. p 65-76.

Siu MKY, Cheng CY. 2004a. Dynamic cross-talk between cells and the extracellular matrix in the testis. Bioessays 26(9):978-992.

Siu MKY, Cheng CY. 2004b. Extracellular matrix: Recent advances on its role in junction dynamics in the seminiferous epithelium during spermatogenesis. Biol Reprod 71(2):375-391.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. 2001. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum Reprod 16(5):972-978.

Smith CA, Elf PK, Lang JW, Joss JMP. 1995. Aromatase enzyme-activity during gonadal sex-differentiation in alligator embryos. Differentiation 58(4):281-290.

Smith CA, Joss JMP. 1995. Immunohistochemical localization of laminin and cytokeratin in embryonic alligator gonads. Acta Zoologica 76(3):249-256.

Speake BK, Thompson MB. 1999. Comparative aspects of yolk lipid utilisation in birds and reptiles. Poultry Avian Biol Rev 10(4):181-211.

Spearow JL, Barkley M. 2001. Reassessment of models used to test xenobiotics for oestrogenic potency is overdue. Hum Reprod 16(5):1027-1029.

Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. 1999. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science 285(5431):1259-1261.

Steinmetz R, Mitchner NA, Grant A, Allen DL, Bigsby RM, Ben-Jonathan N. 1998. The xenoestrogen bisphenol A induces growth, differentiation, and C-fos gene expression in the female reproductive tract. Endocrinology 139(6):2741-2747.

Stoker C, Beldomenico PM, Bosquiazzo VL, Zayas MA, Rey F, Rodriguez H, Munoz-de-Toro M, Luque EH. 2008. Developmental exposure to endocrine disruptor chemicals alters follicular dynamics and steroid levels in caiman latirostris. Gen Comp Endocrinol 156(3):603-612.

Stoker C, Rey F, Rodriguez H, Ramos JG, Sirosky P, Larriera A, Luque EH, Munoz-de-Toro M. 2003. Sex reversal effects on *Caiman latirostris* exposed to environmentally relevant doses of the xenoestrogen bisphenol A. Gen Comp Endocrinol 133(3):287-296.

Suzuki A, Sugihara A, Uchida K, Sato T, Ohta Y, Katsu Y, Watanabe H, Iguchi T. 2002. Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reprod Toxicol 16(2):107-116.

Swan SH, Brazil C, Drobnis EZ, Liu F, Kruse RL, Hatch M, Redmon JB, Wang C, Overstreet JW. 2003. Geographic differences in semen quality of fertile us males. Environ Health Perspect 111(4):414-420.

Swan SH, Elkin EP, Fenster L. 1997. Have sperm densities declined? A reanalysis of global trend data. Environ Health Perspect 105(11):1228-1232.

Tena-Sempere M, Navarro J, Pinilla L, Gonzalez LC, Huhtaniemi I, Aguilar E. 2000. Neonatal exposure to estrogen differentially alters estrogen receptor alpha and beta mRNA expression in rat testis during postnatal development. J Endocrinol 165(2):345-357.

Thompson TB, Lerch TF, Cook RW, Woodruff TK, Jardetzky TS. 2005. The structure of the follistatin: Activin complex reveals antagonism of both type I and type II receptor binding. Dev Cell 9(4):535-543.

Thorbjarnarson JB. 1996. Reproductive characteristics of the order crocodylia. Herpetologica 52(1):8-24.

Trombly DJ, Woodruff TK, Mayo KE. 2009a. Roles for transforming growth factor beta superfamily proteins in early folliculogenesis. Semin Reprod Med 27(1):14-23.

Trombly DJ, Woodruff TK, Mayo KE. 2009b. Suppression of notch signaling in the neonatal mouse ovary decreases primordial follicle formation. Endocrinology 150(2):1014-1024.

Tuuri T, Eramaa M, VanSchaik RHN, Ritvos O. 1996. Differential regulation of inhibin/activin alpha- and beta(A)-subunit and follistatin mRNAs by cyclic amp and phorbol ester in cultured human granulosa-luteal cells. Mol Cell Endocrinol 121(1):1-10.

Tyler CR, Jobling S, Sumpter JP. 1998. Endocrine disruption in wildlife: A critical review of the evidence. Crit Rev Toxicol 28:319-361.

Uda M, Ottolenghi C, Deiana M, Kimber W, Forabosco A, Cao A, Schlessinger D, Pilia G. 2004. Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet 13(11):1171-1181.

Uhlenhaut NH, Treier M. 2006. Foxl2 - function in ovarian development. Mol Genet Metab 88(3):225-234.

Ukeshima A, Fujimoto T. 1991. A fine morphological-study of germ-cells in asymmetrically developing right and left ovaries of the chick. Anat Rec 230(3):378-386.

Unsicker K, Burnstock G. 1975. Myoid cells in peritubular tissue (lamina-propria) of reptilian testis. Cell And Tissue Research 163(4):545-560.

Uribe MCA, Guillette LJ. 2000. Oogenesis and ovarian histology of the American alligator *Alligator mississippiensis*. J Morphol 245(3):225-240.

Valleley EMA, Harrison CJ, Cook Y, Ferguson MWJ, Sharpe PT. 1994. The karyotype of *Alligator mississippiensis*, and chromosomal mapping of the zfy/x homolog, zfc. Chromosoma 103(7):502-507.

van Tienhoven A. 1983. Reproductive physiology of vertebrates. Ithaca: Cornell Univerity Press.

Vancza EM, Galdanes K, Gunnison A, Hatch G, Gordon T. 2009. Age, strain, and gender as factors for increased sensitivity of the mouse lung to inhaled ozone. Toxicol Sci 107(2):535-543.

Vizziano-Cantonnet D, Baron D, Mahe S, Cauty C, Fostier A, Guiguen Y. 2008. Estrogen treatment up-regulates female genes but does not suppress all early testicular markers during rainbow trout male-to-female gonadal transdifferentiation. J Mol Endocrinol 41(5-6):277-288.

Wachs DS, Coffler MS, Malcom PJ, Chang RJ. 2006. Comparison of follicle-stimulating-hormone-stimulated dimeric inhibin and estradiol responses as indicators of granulosa cell function in polycystic ovary syndrome and normal women. J Clin Endocrinol Metab 91(8):2920-2925.

Walters KA, Allan CM, Handelsman DJ. 2008. Androgen actions and the ovary. Biol Reprod 78(3):380-389.

Wang C, Roy SK. 2007. Development of primordial follicles in the hamster: Role of estradiol-17 beta. Endocrinology 148(4):1707-1716.

Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi KI, Nagahama Y. 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with AD4 binding protein/steroidogenic factor 1. Mol Endocrinol 21(3):712-725.

Wang JR, Roy SK. 2004. Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: Modulation by follicle-stimulating hormone. Biol Reprod 70(3):577-585.

Wang XM, Harris RE, Bayston LJ, Ashe HL. 2008. Type IV collagens regulate BMP signaling in *Drosophila*. Nature 455(7209):72-U49.

Wang Y, Ge W. 2004. Developmental profiles of activin beta A, beta B, and follistatin expression in the zebrafish ovary: Evidence for their differential roles during sexual maturation and ovulatory cycle. Biol Reprod 71(6):2056-2064.

Weakley BS. 1967. Light and electron microscopy of developing germ cells and follicle cells in ovary of golden hamster - 24 hours before birth to 8 days post partum. J Anat 101:435-&.

Weber MA, Groos S, Aumuller G, Konrad L. 2002. Post-natal development of the rat testis: Steroid hormone receptor distribution and extracellular matrix deposition. Andrologia 34(1):41-54.

Welt C, Sidis Y, Keutmann H, Schneyer A. 2002. Activins, inhibins, and follistatins: From endocrinology to signaling. A paradigm for the new millennium. Experimental Biology and Medicine 227(9):724-752.

Welt CK, Adams JM, Sluss PM, Hall JE. 1999. Inhibin A and inhibin B responses to gonadotropin withdrawal depends on stage of follicle development. J Clin Endocrinol Metab 84(6):2163-2169.

Welt CK, Schneyer AL. 2001. Differential regulation of inhibin B rand inhibin A by follicle-stimulating hormone and local growth factors in human granulosa cells from small antral follicles. J Clin Endocrinol Metab 86(1):330-336.

Weng Q, Wang HB, Medan MS, Jin WZ, Xia GL, Watanabe G, Taya K. 2006. Expression of inhibin/activin subunits in the ovaries of fetal and neonatal mice. Journal Of Reproduction And Development 52(5):607-616.

Woodruff TK. 1999. Editorial: Hope, hypothesis, and the inhibin receptor. Does specific inhibin binding suggest there is a specific inhibin receptor? Endocrinology 140(1):3-5.

Woods JE, Damianideskeenan M, Thommes RC. 1991. Fsh-binding and tsh-binding cells in the ovary of the developing chick-embryo. Gen Comp Endocrinol 82(3):487-494.

Woodward AR, Jennings ML, Percival HF, Moore CT. 1993. Low clutch viability of American alligators on Lake Apopka. Fl Sci 56:52-63.

Yan CN, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM. 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15(6):854-866.

Yao HHC. 2005. The pathway to femaleness: Current knowledge on embryonic development of the ovary. Mol Cell Endocrinol 230(1-2):87-93.

Yao HHC, Aardema J, Holthusen K. 2006. Sexually dimorphic regulation of inhibin beta B in establishing gonadal vasculature in mice. Biol Reprod 74(5):978-983.

Yao HHC, Capel B. 2005. Temperature, genes, and sex: A comparative view of sex determination in *Trachemys scripta* and *Mus musculus*. J Biochem (Tokyo) 138(1):5-12.

Yao HHC, DiNapoli L, Capel B. 2004a. Cellular mechanisms of sex determination in the red-eared slider turtle, *Trachemys scripta*. Mech Dev 121(11):1393-1401.

Yao HHC, Matzuk MM, Jorgez CJ, Menke DB, Page DC, Swain A, Capel B. 2004b. Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. Dev Dyn 230(2):210-215.

Yoshimura Y, Chang C, Okamoto T, Tamura T. 1993. Immunolocalization of androgen receptor in the small, preovulatory, and postovulatory follicles of laying hens. Gen Comp Endocrinol 91(1):81-89.

Zhao Y, Luck MR. 1995. Gene-expression and protein distribution of collagen, fibronectin and laminin in bovine follicles and corpora-lutea. J Reprod Fertil 104(1):115-123.

BIOGRAPHICAL SKETCH

Brandon C. Moore was born June,1970 to Richard and Maureen Moore. He spent his childhood playing every sport available, reading dorky science fiction literature, and looking for animals in ditches and woods. The home in which he grew up was full of love, laughter, and good food. Time to time he did strange things, like setting a late night alarm so he could wake and observe a meteor shower with his amateur telescope in the dead of a snowy winter night. Despite these eccentricities, he performed fairly well in school, survived a qualityCatholic education, earned All-State honors in football, and accepted a full-scholarship to Duke University in 1988.

At the Gothic Wonderland, he rambled about the academic campus trying to find his way while competing in college football. This period was dichotomous with toil and party, development and immaturity, drive and distractibility, and focus and frivolity. At the end of this collegiate odyssey, he had earned a B.S. in sociology and All-Conference academic accolades.

While the National Football League passed him over in the 1993 draft, he walked on with the New England Patriots, practiced every day like it was his last, and made the roster. Thanks to Coach Bill Parcells, Sensei Andre Tippett, and Johnny Parker. This fortunate happening resulted in an NFL career, ultimately ending with the Arizona Cardinals in 1996. Thus began three years in limbo.

After trying a variety of unsatisfying careers, a realization occurred that he was most happy looking for animals in ditches and other bucolic places. Therefore, the decision was made to return to the education arena. With much luck, he met Dr. Matthew Grober, who liked the drive and wonder he observed enough to allow Brandon to join his research team. For three wonderful summers, he researched sex change dynamics in a small marine fish using SCUBA at Catalina

Island, California. Kelp forests are the most beautiful place in the world. During this period, a dormant scientific soul was reborn.

In 2002, Brandon started a Ph.D. program with Dr. Louis J. Guillette at the University of Florida, Department of Zoology. Dr. Guillette proved the perfect mentor to direct his enthusiasm and drive. For the last seven years, Brandon has taken airboats into the wonderful lakes of central Florida on moonlit night, endeavored to understand the complexities of living organisms in context of their environment, and perfected laboratory skills to investigate the detailed workings of the American alligator reproductive tract. During this period, he married the most caring person he has ever met and, with her, brought into to the world a wonderful, caring, and curious child. He received his Ph.D. from the University of Florida in the spring of 2009. As this challenging and rewarding period ends, this family looks to new opportunities and adventures in the new frontier land of New Orleans, Louisiana.