THE POTENTIAL ROLE OF THE INHIBIN AND ACTIVIN FAMILY OF PROTEINS IN THE REPRODUCTIVE DYSFUNCTION OF TWO DOMESTICATED AVIAN SPECIES AND TWO CAPTIVE MAMMALIAN SPECIES

by

Jacquelyn Beth Hoffman

(Under the Direction of Adam J. Davis)

ABSTRACT

The transforming growth factor-β (TGF-β) superfamily of hormones includes inhibin, activin, and their receptors. Inhibin acts as a negative feedback regulator of pituitary FSH secretion while activin is a positive regulator of pituitary FSH secretion in addition to having a variety of ovarian autocrine and paracrine functions. By studying the expression of these hormones and their receptors, we may elucidate the mechanisms that govern follicular selection, maturation, and ovulation in mammalian and avian species. Additionally, reproductive pathophysiologies may be analyzed to understand the influences of captivity, domestication, and genetic selection, on the TGFβ superfamily of hormones.

Inhibin RIA detection studies were performed on plasma samples from anovulatory and ovulatory female elephants (*Elephas maximus, Loxodanta africana*) during the luteal and non-luteal phases of their ovulatory cycles. Cyclical patterns of inhibin were only detected in ovulatory Asian and African elephants. Levels of inhibin detected in anovulatory elephants were not significantly higher than ovulatory elephants and thus were not indicative of granulosa cell tumors.

Ovarian expression of mRNA for the inhibin/activin subunits was examined in two different genetic lines of turkey hens selected for egg production or rapid growth rate. Significant differences in subunit expression patterns and levels were detected between these two lines of hens. These differences in expression may potentially account for the differences in follicular hierarchy structure and egg production rates also observed in these birds.

mRNA expression studies of activin type IA and type IB receptors were examined in theca and granulosa tissue from the ovaries of the broiler breeder hen. Both receptor types were detected in theca and granulosa tissues, with type IA being expressed at significantly greater levels in both tissues. Both type IA and type IB activin receptors were expressed at significantly greater amounts in the theca tissue of hierarchical and non-hierarchical follicles as compared to the granulosa tissue. Granulosa cell culture experiments indicate that gonadotropins depress the mRNA expression of both type I receptors in hierarchical follicles. When combined with previous activin type IIA receptor expression data, these results suggest that activin may elicit its main effect in the theca tissue of the broiler breeder hen ovary.

INDEX WORDS: Activin, Inhibin, Granulosa, Ovulation, Broiler Breeder Hens, Turkey Hens, Elephants

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B.S.A., The University of Georgia, 2002

A Dissertation Submitted to the Graduate Faculty of the The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2006

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August 2006

DEDICATION

With tremendous gratitude, respect, and admiration, I dedicate this dissertation to my parents, Eileen and Larry, and to my family (human, canine, feline, and avian species alike), friends, loved ones, and mentors whose love and support has enabled me to pursue my education while preparing me to educate others in the future.

ACKNOWLEDGEMENTS

It is difficult to express thanks to someone whose actions have spoken volumes, whose mentoring has been unwaivering, and whose level of academic excellence and dedication to his students has provided me with a paradigm to strive to achieve. With an unexplainable level of appreciation, I acknowledge Dr. Adam Davis for instilling in me the desire to impact others' lives to the degree that he does on a daily basis. I would also like to thank Liz Freeman for her constant friendship, support, and excellent laboratory guidance and assistance. To Drew, my "partner in crime", thanks for always being there for me with constant support and encouragement…mwaa! Thanks to the National Science Foundation for sponsoring my graduate fellowship in the Science Behind Our Food grades K-12 grant and providing me with the opportunity to gain significant teaching experience. Finally, I would like to thank the faculty of the Department of Poultry Science and The University of Georgia for providing me with a wonderful education that I hope is only the beginning of my academic endeavors.

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CHAPTER 1

THE ROLE OF INHIBIN AND ACTIVIN IN REGULATING FOLLICLE-STIMULATING HORMONE PRODUCTION IN THE FEMALE REPRODUCTIVE AXIS

1.1 THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

The maintenance of normal reproductive function is dependent upon the synergistic interplay that exists between hypothalamic, pituitary, and gonadal factors. Neurons in the hypothalamus produce and secrete neurohormones which enter the primary capillary network and are carried to the secondary capillary network located in the anterior pituitary gland. This hypothalamohypophysial portal system thus acts as a conduit between the hypothalamus and the anterior pituitary gland. Neurohormones secreted by the hypothalamus may act on cells of the anterior pituitary gland through a stimulatory or inhibitory mechanism. Releasing hormones increase the secretion of anterior pituitary hormones while inhibiting hormones act in a contrary manner. The stimulatory and inhibitory responses elicited from the anterior pituitary gland by the neurohormones secreted from the hypothalamus are elicited in a highly specific fashion. Each releasing hormone stimulates and each inhibiting hormone inhibits the production and secretion of a specific hormone or hormones from the anterior pituitary gland. Releasing hormones cause anterior pituitary cells to secrete hormones that enter the secondary capillary network which eventually merges with the general circulation which then carries pituitary hormones to their target tissues. Thus, the hypothalamus is able to utilize neurohormones as chemical signals to regulate the secretory activity of the anterior pituitary through the use of the hypothalamohypophysial portal system.

Although several major hormones are released from hypothalamic neurons, the gonadotropin-releasing hormone (GnRH) is directly responsible for stimulating the anterior pituitary to produce the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH are hormones that are classified as gonadotropins because of their ability to promote growth and function of the gonads, which include the ovaries and testes.

LH and FSH secreted into the bloodstream bind to membrane-bound receptors located on gonadal tissue where they increase the intracellular synthesis of cAMP through G protein cascades. This activity results in the maturation of gametes, sperm cells in the testes and oocytes in the ovaries. Additionally, LH and FSH control the production of reproductive hormones such as estrogen and progesterone in the ovaries and testosterone in the testes.

1.2 OVERVIEW OF INHIBIN

Although the roles played by gonadal steroids, pituitary gonadotropins, and hypothalamic GnRH have been investigated for many years, research interests have shifted over time to examine the activity of nonsteroidal protein hormones of gonadal origin. Specifically, the polypeptide factors inhibin and activin have become of interest due to their selective regulation of pituitary FSH secretion (Halvorson and DeCherney, 1996).

The gonadal hormone inhibin was described more than 60 years ago by McCullagh as a water-soluble factor with the ability to suppress hypertrophy of the pituitary following castration in the rat (McCullagh, 1932). Approximately 50 years later, isolation and characterization of inhibin revealed the glycoprotein hormone to be a disulfide-linked heterodimer consisting of an α chain (relative molecular mass M_r = 18 kd) and one of two homologous β chains designated β_A and β_B (relative molecular mass M_r = 14 kd). Both the α and β-subunit proteins initially exist as larger pro-proteins that must undergo proteolytic cleavage in order to yield mature forms. In fact, the α-subunit may be detected in circulation in one of three forms: its monomeric pro-form, the fragment resulting from the cleavage of the pro-form, or bound to some form of the β-subunit.

Inhibin is most biologically potent when the two mature forms of the α and β-subunits combine (Vale et al., 1988). Depending upon the combination of the specific β subunit with the α chain, inhibin A (α-β_A) or inhibin B (α-β_B) is formed. Both isoforms of inhibin act to suppress pituitary FSH synthesis and secretion (Ying 1988, De Kretser and Robertson 1989, Vale *et al*. 1990).

The isolation and characterization of inhibin was met with significant challenges including the development of a bioassay for FSH that was sensitive enough to detect FSH levels in the blood (Steelman *et al*., 1953). Thus, the development of a RIA for gonadotropins (Midgley 1966, Midgley 1967) with the sensitivity to detect FSH fluctuations in the blood provided the evidence needed to demonstrate that water-soluble, steroid-free extracts of testes (Lee *et al*. 1976, Setchell *et al*. 1974) or follicular fluid (de Jong *et al*. 1976, Hopkinson *et al*. 1977, Schwarts *et al*. 1977) selectively suppress peripheral radioimmunoassayable FSH levels in experimental animals.

1.3 INHIBIN PRODUCTION AND ACTIVITY

Inhibin is primarily produced in the granulosa cell layer of ovarian follicles in mammalian females (Erickson and Hsueh, 1978; Meunier *et al*., 1988a) and production has been shown to vary with the maturation of the follicles (Woodruff *et al*., 1988). While in males, the sertoli cells are the predominate source of inhibin (Illingworth et al., 1996). Although inhibin was first isolated from gonadal sources, the α, β_A, and β_B mRNAs are expressed in a wide variety of extragonadal tissues including brain, spinal cord, pituitary, adrenal, spleen, placenta, bone marrow, and kidney (Bilezikgian and Vale, 1992; Meunier *et al*., 1988b).

With the exception of its negative feedback activity on anterior pituitary FSH synthesis and secretion, few other biological roles have been discovered for inhibin to date. In 1987, Hsueh *et al*. reported that inhibin could increase luteinizing hormone (LH)-stimulated steroidogenesis in cultured, immature leydig cells. A potentially significant role of the free inhibin α-subunit is that of gonadal tumor suppression. Matzuk *et al*. (1992a) observed that mice with the α-inhibin gene knocked out developed gonadal tumors at an excessively high rate. Similarly, two strains of hens differing in incidences of ovarian cancer were shown to differ in plasma total immunoreactive inhibin α-subunit protein concentrations. The strain with the higher incidence of cancer was shown to have lower total immunoreactive inhibin α-subunit concentrations (Olson *et al*., 1996).

From an applied animal production standpoint, many studies have shown that active immunization against the inhibin α-subunit alone neutralizes inhibin activity. For example, such immunizations have resulted in increased ovulation rates in ewes due to increased circulating FSH concentrations (Findlay *et al*., 1989), accelerated puberty and enhanced hen-day egg production in Japanese quail (Moreau *et al*., 1998), increased testicular weight in cockerels (Lovell *et al*., 2000) and increased numbers of preovulatory follicles in turkey hens (Ahn *et a*l., 2001). Furthermore, the lower egg production observed in broiler hens is associated with them having higher follicular mRNA expression of the inhibin α-subunit than laying hens (Safi *et al*.,1998; Slappey and Davis, 2003). In addition, Wang and Johnson (1993) reported that granulosa cell expression of the mRNA for the inhibin *α*-subunit was increased in F1 and F4 follicles in laying hens laying at a low rate (three to seven egg sequence) as compared to hens laying at a high rate (21or greater egg sequence).

1.4 DEVELOPMENT OF THE INHIBIN RADIOIMMUNOASSAY

After isolating and characterizing inhibin, monoclonal and polyclonal antibodies against synthetic peptides or native inhibin and its subunits were generated. From these antibodies, several radioimmunoassays were developed and were used in the measurement of inhibin levels in a variety of different species. McLachlan *et al*. (1986) generated rabbit antiserum raised against pure 31 kDa bovine inhibin thus producing one of the most widely used inhibin RIAs known as No. 1989. This RIA was termed the "Monash" radioimmunoassay for inhibin because of its development at Monash University in Australia. The antiserum developed at Monash University was directed against the inhibin α-subunit (McLachlan *et al*., 1986; McLachlan *et al*., 1987; Schneyer *et al*., 1990) and thus the immunoreactivity detected in the peripheral circulation by this RIA could be due to free α-subunit circulating as a monomer or dimeric inhibin (Sugino *et al*., 1989).

1.5 ADVENT OF TWO-SITE DIMERIC ELISAs

Although the "Monash" radioimmunoassay for inhibin (Robertson *et al*., 1989) provided a significant tool for clinical and physiological studies (Burger, 1993), the assay could not discriminate between dimeric bioactive inhibin forms and various forms of the free alpha subunit which exist in large quanitites in body fluids (Schneyer *et al*., 1990). Publications referred to the material measured by the Monash assay as "immunoreactive" inhibin in order to acknowledge the possibility that the assay might not accurately measure bioactive inhibin levels.
Numerous groups subsequently prepared monoclonal and polyclonal antibodies with the intent to develop two-site immunoassays which could specifically measure the dimeric forms of inhibin.

While the Genenetech group was successful in developing several assays using antibodies raised to recombinant inhibins (Baly et al., 1993), the affinity of the antibodies made was limited possibly because of inhibin's poor immunogenicity. In order to compensate for inhibin's poor immunogenicity, assays were developed that used antibodies raised to synthetic peptide portions of the inhibin α- and β- subunits. Poncelot *et al*. (1994) produced two-site assays for a variety of inhibin forms including free alpha forms, inhibin-A and B.

The application of these two-site assays to clinical material became quite common when the Groome laboratory of Oxford Brookes University in the United Kingdom developed a sensitive inhibin enzyme immunoassay by using synthetic peptide immunizations and focusing on the preparation of monoclonal antibodies with alkaline phosphatase as the label in order to make the reagents available for long term use (Groome, 1996). Currently, the Inhibin-A ELISA, Inhibin-B ELISA, Pro-Alpha C ELISA, Activin-A ELISA, Activin-AB ELISA assays which were all developed by Groome's laboratory, are commercially available to research groups through Diagnostic System Laboratories Inc. These assays have since been used for a variety of clinical and physiological studies including the detection of a circulating marker for granulosa cell tumors of the ovary (Cooke *et al*., 1995; Burger *et al*., 1996). Additionally, due to the specificity of these two-site ELISAs, specific forms of inhibin may be linked to different functions.

1.6 OVERVIEW OF ACTIVIN

The isolation and purification of inhibin resulted in the identification of an additional protein hormone, activin. Activin is formed by the dimerization of the inhibin β subunits yielding the homodimers activin A (β_A-β_A) and activin B (β_B-β_B), as well as the heterodimer activin AB (β_A–β_B) (Ying 1988, De Kretser and Robertson 1989, Vale *et al*. 1990). Activin A, activin B, and activin AB stimulate FSH synthesis and secretion from the anterior pituitary.

Thus, the opposing activities of activin and inhibin on FSH production in the anterior pituitary along with the variety of combinatorial assemblies of the α, β_A, and β_B subunits exemplify the complex synergistic regulation of the hypothalamic-pituitary-gonadal axis.

Unlike its antagonist inhibin, activin has been found to be associated with a variety of functions within the body. Such functions include neuron survival (Schubert *et al*., 1990), bone growth (Ogawa *et al*., 1992), injury response (Lai *et al*., 1996), and erythropoiesis (Murata *et al*., 1988) to name a few. Recently, much attention has been given to the paracrine and autocrine functions of activin in reproductive tissues. In male reproduction, activin has been shown to be a potent stimulator of spermatogonial proliferation in mammalian testes (Mather *et al*., 1990). Additionally, activin acts with FSH to stimulate sertoli cell proliferation (Boitani *et al*., 1995) and it may modulate steroidogenesis in leydig cells (Mauduit et al., 1991).

In the ovary, activin-A has been reported to stimulate inhibin-A secretion and to increase the mRNA expression for the inhibin α and β_A-subunits (LaPolt *et al*., 1989). Additionally, in mammalian species activin-A has been reported to stimulate the expression of the mRNA for the LH (Sugino *et al*., 1988a) and FSH receptors (Xiao *et al*., 1992; Nakamura *et al*., 1995). Activin has even been reported to influence progesterone production (Xiao *et al*., 1992; Fraser *et al*., 1995; Li *et al*., 1995; Miro *et al*., 1995; Wrathall and Knight, 1995; Shukovski *et al*., 1996; Ford and Howard, 1997). Recent work in chickens suggests that activin A may have an important regulatory role in the responsiveness of granulosa cells to gonadotropins while also modulating follicle development by attenuating cell proliferation (Johnson *et al*., 2006).

Specifically, the addition of 50 ng/mL of activin A to granulosa cells isolated from either the F1, F3+F4, or small yellow follicles (SYF) and cultured in M199 resulted in a significant increase in FSH receptors in the granulosa cells from all the follicle groups while a significant increase in LH receptors was detected in the F1 and F3+F4 follicles (Johnson *et al*., 2006). Additionally, activin A significantly reduced granulosa cell proliferation from all three follicle sizes (Johnson *et al*., 2006) supporting the potential for activin A to serve an important regulatory role in modulating follicular development.

1.7 INHIBIN/ACTIVIN SUBUNIT CHARACTERIZATION

The cloning of the inhibin/activin subunit cDNAs and genes generated significant insights into the inhibin/activin family of hormones. Specifically, the β-subunit was found to be highly conserved between a variety of species including: humans, cows, rats, and sheep.
In fact, mature β_A protein is 100% conserved in the aforementioned species while mature β_B protein differs by only one amino acid. This high degree of conservation across a multitude of species suggests the functional significance and evolutionary drive to maintain the structural integrity of the activin family (Vale *et al*., 1990).

In addition to discovering the high degree of conservation that exists among the inhibin/activin β_A and β_B subunits across species, the inhibin/activin proteins and the individual β-subunits were found to be structurally similar to the growth and differentiation factor, transforming growth factor-beta (TGF-β) (Mason *et al*., 1985). The TGF-β superfamily consists of a variety of growth and differentiation factors including mullerian inhibiting substance (MIS), bone morphogenic proteins (BMPs), and the amphibian protein VG-1 involved in embryogenesis (Dye *et al*. 1992, DePaolo *et al*. 1991, Mason *et al*. 1986, Stewart *et al*. 1986).

At the genomic level, the inhibin/activin α and β-subunits were discovered to be products of separate genes with different transcription elements controlling the expression of the independent subunit genes (Mason *et al*. 1989, Stewart *et al*. 1986). The presence of unique transcription elements for each individual subunit may be responsible for the complex control of individual inhibin/activin isoform synthesis in a tissue-, hormone-, or species-specific manner (Woodruff and Mather, 1995). In addition, activin's ability to direct assembly of the inhibin and activin protein dimers or auto-induce transcription may be a mechanism for the control of functional diversity (Woodruff and Mather, 1995).

1.8 OVERVIEW OF FOLLISTATIN: A BINDING PROTEIN OF INHIBIN AND ACTIVIN

In addition to the discovery of inhibin and activin in the mid-1980s, another member of the FSH-modulating family, follistatin, was identified in bovine and porcine follicular fluid due to its FSH-suppressing activity (Robertson *et al.* 1987, Ueno *et al*. 1987). Follistatin is a single chain glycosylated protein that exists in a variety of different isoforms that are generated by alternate splicing and differing degrees of glycosylation. The amino acid sequences of the rat, human, sheep and pig follistatin are more than 97% homologous (Shimasaki *et al*., 1989; Tisdall *et al*., 1992). Follistatin is primarily produced by the granulosa cells of the ovary (Findlay *et al.* 1992, DePaolo 1991) and FSH and activin regulate its production (Findlay *et al*., 1993).

Follistatin is a soluble-binding protein that binds both inhibin and activin through the common β-subunit (Nakamura *et al*. 1990, Shimonaka *et al*. 1990). However, follistatin binds activin with a greater affinity than inhibin (Shimonaka *et al*., 1991; Krummen *et al*., 1993). Almost all of the biological actions of activin are neutralized when it is bound by follistatin (Mather *et al*., 1993).

Changes in the bioactivity of inhibin once bound to follistatin have not been characterized. Gene knockouts of follistatin in mice result in impeded growth, decreased diaphragm and intercostals muscle mass, palate defects, skeletal defects, taut skin, abnormal whiskers and teeth, and inadequate breathing which inevitably results in death within a few hours of birth (Matzuk *et al*., 1995b). In comparison to activin and inhibin knockout mice, the effects of follistatin gene knockout were more extensive suggesting a role for follistatin that is unrelated to activin and/or inhibin (Matzuk *et al.*, 1995b).

1.9 OVERVIEW OF α_2-MACROGLOBULIN: A BINDING PROTEIN OF INHIBIN AND ACTIVIN

In addition to follistatin, a second soluble-binding protein for inhibin and activin was identified as α_2-macroglobulin (Krummen *et al*., 1993). α_2-macroglobulin is a broad-spectrum protease inhibitor that is known for its ability to bind a variety of growth factors, including TGF-β (Krummen *et al*., 1993). α_2-macroglobulin binds both inhibin and activin in a low-affinity, high-capacity manner in contrast to follistatin which binds both ligands in a high-affinity, low-capacity manner. Binding of activin to α_2-macroglobulin has not been observed to affect activin function (Mather *et al*., 1993). Production and regulation of α_2-macroglobulin occurs in a hormonally dependent fashion in the gonads, as well as in a variety of other tissues, and thus may participate in the bioavailability of inhibin and activin for local actions (Gaddy-Kurten 1989, Nakatani *et al*. 1991).

1.10 ACTIVIN RECEPTORS: CELLULAR SIGNALING BY THE SERINE KINASE RECEPTOR FAMILY

Different receptors have been identified as having the ability to bind activin and/or related TGF-β family members (Massagué 1990, Mathews 1991). Initial progress toward understanding the activin signaling mechanism was achieved with the elucidation of the structural characteristics of activin receptors. By utilizing an expression cloning approach based on the binding of radiolabeled activin A in mammalian cell tissue cultures, a receptor complex was isolated, subsequently cloned and called ActRII (Mathews and Vale, 1991). Sequencing analysis of ActRII, revealed it consisted of a single membrane-spanning domain, a small extracellular ligand-binding domain, and an intracellular protein kinase domain. Subsequent analyses of type II receptor sequences in a variety of species have revealed a high degree of evolutionary conservation in accordance with the conservation seen in the β-subunits of activin. Conservation of activin type II receptor sequences is so great that only two amino acid differences exist between the mouse and human sequences (Matzuk and Bradley, 1992).

By using ActRII cDNA as a hybridization probe or the ActRII cDNA sequence as a means to direct construction of degenerate oligonucleotides for polymerase chain reaction (PCR), a closely related type II receptor ActRIIB was subsequently detected in several animal species (Attisano *et al*. 1992, Mathews *et al*. 1992, Hemmati-Brivanlou *et al*. 1992, Legerski *et al*. 1992, Nishimatsu *et al*. 1992). Four different isoforms of ActRIIB are detected in the mouse and they result from alternate splicing which is due to the inclusion or omission of two short sequences, either in the extracellular domain or in the intracellular domain N-terminal to the kinase moiety (Attisano *et al*., 1992).

These isoforms were found to differ in their ligand-binding affinity for activin A with those molecules having the full extracellular domain sequence exhibiting a higher affinity towards activin A (Attisano *et al*., 1992). The detection and identification of the ActII and ActIIB receptors as serine-threonine kinases was a unique discovery, at the time, as most of the known receptor kinases were tyrosine-specific (Ullrich and Schlessinger, 1990).

After detecting and sequencing the ActRIIs, numerous additional receptor serine kinases were cloned by using PCR based methodology with oligonucleotides based on the ActRII sequences (Ebner *et al*., 1993). At first, these molecules were classified as orphan receptors because they did not bind activin, inhibin, TGF-β, or bone morphogenetic proteins (BMPs). Further analyses revealed these molecules to be activin type I receptors (ActRI) which complemented type II receptors to generate activin's intracellular signal. ActRI, also known as SKR1, Tsk7L R1, ALK2, or ActX1R, was the first type I receptor clone to be characterized and was cloned from humans (Attisano *et al*., 1993). ActRIB, the second type I receptor identified and cloned in humans is also known as ALK4 (Ten Dijke *et al*., 1993; Ten Dijke *et al*., 1994).

The cooperative manner in which the activin receptors interact to bind activin and elicit activin's downstream intracellular signal is now fairly well established. In general, activin binds to a type II receptor (ActRII or ActRIIB) which then recruits and transphosphorylates a type I receptor (ActRI or ActRIB) which leads to the phosphorylation of intracellular downstream Smads (Kaivo-oja *et a*l., 2006). Depending upon the specific combination of type I and type II receptors, different Smad proteins are activated through phosphorylation by the type I receptor. Proteins belonging to the Smad family were first identified in the fruit fly Drosophila melanogaster by Sekelsky *et al.* in the mid 1990s.

They detected an intracellular protein named Mad that mediated the signaling of decapentaplegic (dpp), a member of the TGF-β superfamily that corresponds to mammalian bone morphogenetic protein 2 or 4 (BMP-2/4) (Sekelsky *et al*., 1995). Eight different Smads have since been detected and have been identified as evolutionarily conserved proteins that act as mediators of transcriptional activation for members of the TGF-β superfamily (Kaivo-oja *et a*l., 2006). Smads are classified as receptor activated (R-) Smads (Smad 1, -2, -3, -5, -8), common-partner (Co-) Smads (Smad 4), or inhibitory (I-) Smads (Smad 6 and -7) (Kaivo-oja *et a*l., 2006). Depending upon the combination of type I and type II receptors, different R-Smads are activated through phosphorylation by the type I receptor following ligand binding. R-Smad 2 and -3 are phosphorylated by activated TGF-β/activin type I receptors, while Smad 1, -5, and -8 act downstream of BMP type I receptors. Activated R-Smads then form oligomeric complexes with Co-Smad 4 and are translocated into the nucleus where they regulate target gene expression. In contrast to R-Smads, inhibitory Smads block TGF-β superfamily signaling by binding to the type I receptor (Smad 7) or by competing with the activated R-Smad for binding to Co-Smad 4 (Smad 6). Smad 7 can competitively inhibit both the TGF-β/activin and BMP pathway R-Smads while Smad 6 acts solely as an inhibitor of BMP signaling (Shi *et al*., 2003; Moustaka *et al*., 2001; Attisano *et al*., 2002).

1.11 INHIBIN RECEPTOR MODELS

The receptor system and molecular mechanisms by which inhibin acts on its target cells are poorly understood. Two main models concerning the mechanism governing inhibin activity have been developed. The first model is based on the premise that activin-stimulated FSH synthesis and release by the pituitary gonadotrope is antagonized by gonadally derived inhibins.

The two isoforms of inhibin, inhibin A and B, bind to the activin type II receptors, though at a lower affinity than the activins, but do not stimulate intracellular signaling. Theoretically, therefore, inhibins could prevent activin signaling through competitive binding if they are present at higher concentrations than the activins (Dyson and Gurdon, 1998) but, in reality, inhibins have been shown to antagonize activin signaling when the two ligand types are present at equimolar concentrations (Carroll *et al*., 1989; Rivier and Vale, 1991; Weiss *et al*., 1993).

Such observations led to the second model used to explain the action of inhibin which is based on the hypothesis that inhibin binding proteins or co-receptors exist that act to increase the affinity of the inhibins for the activin receptors or propagate inhibin-specific intracellular signals (Bilezikigan, 1991; Lebrun and Vale, 1997). Specifically, two candidate inhibin co-receptors, betaglycan and InhBP/p120, were isolated, and proposed due to their ability to interact with activin receptors to augment inhibin antagonism of activin action (Chong, 2000; Lewis *et al*., 2000; Chapman and Woodruff, 2001). Betaglycan is a transmembrane protein with a large ectodomain but no obvious signaling domain. Betaglycan was originally characterized as a TGF-β type III receptor, which is necessary for TGF-β2 signaling (Lopez-Casillas et al., 1993). InhBP/p120 was purified from bovine pituitary membrane extracts using an inhibin A affinity column (Chong et al., 2000). Like betaglycan, InhBP/p120 is predicted to contain a large extracellular domain, a single transmembrane domain and a short, kinase deficient intracellular tail. It is highly expressed in inhibin target tissues, namely the anterior pituitary, and also interacts with components of the activin receptor complex (Bernard and Woodruff, 2001; Chapman and Woodruff, 2001).

Although originally purified based on inhibin affinity chromatography, Chapman *et al*. (2002) and Bernard *et al.* (2002) subsequently found that InhBP/p120 did not bind inhibin A or B when expressed alone or in combination with activin receptors. These findings indicate that a re-examination of the role of this protein in inhibin biology is required. Additionally, Chapman *et al*. (2002) reported that the inhibin isoforms bind betaglycan with high affinity. The most recent model of inhibin action proposes that inhibin binds betaglycan with high affinity and then forms a highly stable but non-intracellular signaling complex with an activin type II receptor (Lewis *et al*., 2000). Formation of the inhibin betaglycan-activin type II receptor complex provides a mechanism for inhibin antagonism of activin signaling since the ActRII molecule in this complex is no longer available for the formation of an activin receptor complex (Chapman *et al*., 2002). The stability of the inhibin betaglycan-activin type II receptor complex allows inhibin to antagonize activin actions even when it is present in lower concentrations than activins (Chapman *et al*., 2002).

Although there has not been much success in detecting an inhibin receptor that generates an intracellular response upon inhibin binding, there is evidence of inhibin specific actions that would require the presence of an independent inhibin receptor to transduce an inhibin signal (Murata et al., 1996; Prendergast et al., 2004). In addition, inhibin binding proteins have been identified that do not correspond to either betaglycan or InhBP/p120 (Draper *et al*., 1998). Thus there is ongoing research to isolate and characterize an inhibin-specific receptor (Phillips and Woodruff, 2004).

1.12 SUMMARY

Maintaining normal reproductive function relies upon the synergistic interactions between hypothalamic, pituitary, and gonadal factors. Through the hypothalamohypophysial portal system gonadotropin-releasing hormone (GnRH) stimulates the anterior pituitary to produce the gonadotropins, LH and FSH. Inhibin and activin, which are produced abundantly by the gonads, regulate FSH secretion from the anterior pituitary gland.

Inhibin suppresses FSH secretion while activin stimulates FSH secretion. Inhibin's ability to suppress FSH production and secretion is based on its ability to antagonize activin function. This antagonism is based on the fact that inhibin can interfere with the formation of the activin receptor complex. Activin binds to an activin type II receptor (ActRII or ActRIIB) which then recruits an activin type I receptor (ActRI or ActRIB) to form a receptor complex that generates an intracellular message. Inhibin binds to a cell surface protein called betaglycan, and once betaglycan is bound by inhibin it complexes with an activin type II receptor which makes it unavailable for the formation of an active activin receptor complex. Interestingly, the inhibin betaglycan receptor complex does not elicit an intracellular signal. Finally, activin's ability to stimulate FSH production can also be prevented by the soluble binding protein follistatin which can bind activin making it unavailable to bind to an activin type II receptor.

CHAPTER 2

INHIBIN'S POTENTIAL ROLE IN THE REPRODUCTIVE DYSFUNCTION OF TWO CAPTIVE MAMMALIAN SPECIES

2.1 NON-SUSTAINING CAPTIVE POPULATIONS OF AFRICAN AND ASIAN ELEPHANTS

Currently the number of captive elephants in North American zoos, wildlife preserves, and parks is dwindling. This small population of captive elephants is precious as these exotic animals serve as ambassadors for their species; educating youth and adults alike about the importance of wildlife conservation. Although the majority of our population will never observe these creatures in their natural habitat, they can gain a great appreciation for the magnificence of these creatures by observing them in zoos or parks.

Asian and African captive elephants in North America are at risk of 'captive extinction' due to the limitations placed on importing elephants from the wild into captivity as well as the deficiencies in captive breeding programs. Recent surveys showed that less than 20% of Asian and 10% of African captive elephants have produced offspring (Asian Elephant Studbook, 2000, 2001). Due to the difficulties in developing self-sustaining captive populations of African and Asian elephants and the rapid rate at which the captive population is aging, there is a need to increase our limited knowledge of elephant reproductive biology. Only then will we have the tools to establish self-sustaining populations of captive elephants.

2.2 "FLATLINERS"

In an effort to increase reproductive rates, the Elephant Taxon Advisory Group/Species Survival Plan instituted a reproductive endocrine monitoring program for captive elephants to learn more about their reproductive biology and to help identify viable breeding candidates. One of the findings of this effort is that many female elephants of reproductive age are not cycling normally. Termed "flatliners", these females exhibit ovarian inactivity, as evidenced by consistently stable, baseline serum progestogen concentrations (Brown, 2000).

A recent survey reported that up to 14% of Asian and 29% of African elephants in North America are not cycling normally (Brown et al., 2004a). The cause of the acyclicity is unknown, but may be related to a variety of reproductive problems such as reproductive tract pathologies, stress, hypothalamic-pituitary disruptions, hormone receptor dysfunctions, and neoplasias (Knobil and Neill, 1998). The probability that the etiology of acyclicity is the same for all females is unlikely, however, there may be commonalities among acyclic elephants. Unfortunately, no effective treatment for reproductive inactivity in elephants has been discovered (Brown, 2000). Attempts to administer exogenous GnRH and/or human chorionic gonadotropin, both effective treatments for reproductive dysfunctions in other mammalian species, including ovarian cysts, have not proven to be effective in the elephant (Brown *et al*., 1999a). Ultimately, it is important to elucidate what factors are related to reproductive dysfunction in elephants so that mitigating actions can be taken to prevent 'captive extinction.'

2.3 OVERVIEW OF THE FEMALE ELEPHANT'S REPRODUCTIVE BIOLOGY

In order to understand what factors are related to reproductive dysfunctions in female elephants, it is necessary to first understand the fundamentals of female elephant reproductive biology and endocrinology. Differences in reproductive development exist between captive female elephants and their wild counterparts. For example, captive female elephants appear to reach puberty several years earlier than those in the wild. In captivity, sexual maturity and the first pubertal luteal phase increase in progestogens has been observed in females between the ages of 7 and 8 years in comparison to wild elephants who reach sexual maturity at approximately thirteen years of age (Brown *et al*., 2000). Similarly, reproductive senescence occurs in captive elephants earlier than in the wild with studbook records indicating that few female elephants (cows) produce a calf after 30 years of age.

Free-ranging females, in contrast, can produce calves well into their 50s with less difficulty (Brown, 2000). Potentially, differences between captive and free-ranging elephants can be attributed to altered endocrine function, ovarian/uterine activity, or a multitude of reproductive tract pathologies, such as uterine and ovarian cysts (Hildebrandt et al., 2000a).

Upon sexual maturation, the female elephant exhibits an estrous cycle that has been characterized as consisting of an 8- to 12- week luteal phase and a 4- to 6-week non-luteal phase, allowing a female to cycle approximately four times a year. Cyclicity is characterized by measuring serum or plasma progestogens, specifically 5α-reduced pregnane which is the major circulating progestogen in elephants (Heistermannet al., 1997; Hodges et al., 1997; Schwarzenberger et al., 1997; Hodges, 1998).

Unlike other mammals where ovulation is induced by a single, pre-ovulatory LH surge at the end of the follicular phase of the estrous cycle, the elephant is unique in that two precisely timed LH surges occur during the non-luteal or follicular phase (Knobil and Neill, 1998). The first surge occurs between 12 and 21 days after progestogens decline to baseline and the second surge occurs approximately 3 weeks (19-22 days) after the first LH surge (Kapustin et al., 1996; Brown et al., 1999b). Although the surges are similar quantitatively and qualitatively, only the second surge induces ovulation. Thus, the first surge is typically described as the anovulatory LH (anLH) surge while the second surge is called the ovulatory LH (ovLH) surge. While the ovLH surge is known to induce ovulation and corpus luteum (CL) formation, the function of the anLH is not clear (Brown, 2000).

During the luteal phase, elevated progestogens inhibit follicular development and LH release. As progestogen levels decline towards baseline, follicular activity resumes.
As the non-luteal phase begins, FSH levels become elevated recruiting follicles and initiating two successive waves of follicular development which culminate in the distinct anLH and ovLH surges. The first follicular wave consists of multiple follicles that do not reach Graafian size or ovulate, but actually regress after the anLH surge. Although the anLH surge is triggered by elevated estrogens, the follicles in the first wave are not steroidogenically competent . It has been proposed that their incompetency may be due to inadequate gonadotropin receptor numbers (Brown, 2000). Ultimately, these follicles luteinize in response to the anLH surge and form accessory corpora lutea (CLs) that become steroidogenically active, secreting progestogens, a few days before the ovLH surge (Brown, 2000).

The second follicular wave occurs during the next three weeks and results in the formation of one large dominant follicle that is ovulated approximately 24 hours after the ovLH surge. After the postovulatory CL forms, a shift in steroidogenic activity from the accessory CLs to the newly formed, post-ovulatory follicle occurs. Progestogens continue to increase as the CL matures and approximately one week after CL formation FSH levels begin to rise gradually (Brown, 2000).

Unlike other mammals, FSH concentrations peak at the end of the luteal phase and decrease progressively during the non-luteal phase (Brown *et al*., 1991; Brown *et al*., 1999b). In other species, FSH concentrations are typically elevated at the same time as the pre-ovulatory LH surge and exhibit a secondary FSH rise after ovulation (Knobil and Neill, 1998).

Two hypotheses have been made regarding the function of the unique pattern of FSH secretion in the elephant: 1) it may serve to recruit functional follicles during the last few weeks of the non-luteal phase leading up to ovulation, as occurs in the horse (Ginther, 1992); or 2) it may act to sustain waves of follicular development throughout the inter-luteal period as suggested by ultrasound findings (Hermes et al., 2000).

2.4 PREGNANCY, PARTURITION, AND THE POSTPARTUM PERIOD

Elephants do not exhibit seasonal breeding and generally produce one calf every 3 to 4 years after a gestation period of 20-22 months (Poole and Moss, 1989). The gestation period is diagnosed on the basis of elevated progestogens beyond the normal luteal phase (after about week 12). Approximately 2-5 days before birth (range, 1-10 days), a decrease in progestogens occurs (Brown and Lehnhardt, 1995; Carden et al., 1998; Doyle et al., 1999). After giving birth, the postpartum anestrous period begins and its length is primarily dependent on lactational status. On average, the elephant's lactational anestrus lasts about 46 weeks and is characterized by low progestogen concentrations (Olsen et al., 1994). Problems with retained placenta, reduced milk production, death of a calf, or premature weaning, however, can reduce the postpartum period to as short as 8 weeks (Olsen et al., 1994; Brown and Lehnhardt, 1995).

2.5 INHIBIN AS A BIOMARKER FOR OVARIAN CANCER

Ovarian cancer is classified as the most lethal human gynecological malignancy due to diagnostic difficulties that typically only permit detection of the cancers at a late clinical stage (Holschneider and Berek, 2000). Ovarian tumors are divided into histological categories according to the cell type that the tumors arise from which include: germ cells, stroma, or surface epithelium.

Initially, interest in inhibin as an ovarian cancer tumor marker evolved as a result of studies examining inhibin α-subunit knockout mice (Matzuk *et al*., 1992) and later increased due to observations that serum inhibin levels were elevated in women with granulosa cell tumors (GCTs) (Lappohn *et al*., 1989). GCTs are hormonally active tumors derived from sex-cord stromal tissue and can be diagnostically detected through measurements of inhibin, testosterone, and progesterone. Studies in mares indicate that inhibin is elevated in approximately 90% of mares with GCTs (Christman et al., 1999). Elevated inhibin concentrations along with ovarian inactivity can thus be indicative of ovarian tumors in species such as humans as well as horses (Burger et al., 1996; Christman et al., 1999).

In humans, variation in the inhibin form detected in serum exists between specific types of tumors and may even vary within a class of tumors. For example, in some GCT's, dimeric inhibins, especially inhibin B, are the primary inhibin forms detected in serum, while in mucinous carcinomas the free inhibin α-subunit is primarily detected (Robertson et al., 1999). The variation in inhibin forms detected in humans is often a reflection of the origin of the cancer since inhibin A is primarily produced by mature follicles and copora lutea while inhibin B is formed by developing antral follicles (Tong *et al*., 2003). After menopause or a surgical oophorectomy, circulating levels of inhibin A and B, and free α-subunit decline to nondetectable concentrations thus providing a useful low-level baseline for development of a sensitive inhibin assay (Robertson *et al*., 1999). Such assays may still be used to detect tumors in post-menopausal women due to elevated inhibin levels detected in serum (Robertson *et al*., 1999). It is important to measure all α-subunit-containing forms (inhibins A and B, and the free α-subunit) in order to detect all cancer types (Robertson *et al*., 1999).

2.6 DETECTION OF INHIBIN α-PEPTIDE IN ASIAN FEMALE ELEPHANTS

While Knobil and Neill (1998) confirmed that elephants exhibit the same inverse relationship between FSH and inhibin as exists in other species, there is still a paucity of information on inhibin's role in cyclic and acyclic captive female elephants. Previously, serum concentrations of inhibin were quantified throughout the estrous cycle of nine cycling Asian female elephants using antibodies formed against a synthetic portion of the porcine inhibin-α-peptide. Serum concentrations of immunoreactive inhibin α-subunit were found to be negatively correlated with FSH during the non-luteal phase as would be expected due to inhibin's negative feedback regulation of FSH (Brown et al., 1999). However, the lack of a dimeric antisera assay, at that time, could not definitively confirm that the molecule detected was, in fact, biologically active inhibin and not just circulating inhibin α-subunit.

Previous studies have also found significant differences ($P<0.05$) in mean FSH levels between cycling and non-cycling Asian and African elephants, where maximum FSH concentrations in non-cycling females were consistently similar to baseline levels observed in cycling females and failed to increase and initiate follicular development (Brown et al., 2004). The depressed secretion of pituitary FSH in acyclic elephants may indicate the presence of elevated secretory concentrations of inhibin which in turn could be indicative of granulosa cell tumors.

2.7 ENDOCRINOLOGY OF BULL ELEPHANTS

In comparison to the scant amount of knowledge available regarding the reproductive biology of female elephants, even more limited information has been gained regarding the reproductive endocrinology of bull elephants.

Research on bull elephants has primarily focused on investigating musth, a period of heightened aggressive and sexual behavior that lasts for periods of a few weeks to several months and is characterized by increased temporal gland drainage, urine dribbling, and androgen secretion (Schmidt, 1993; Mikota et al., 1994; Niemuller et al., 1998). Factors such as age, nutrition, and social status determine when a bull exhibits musth (Jainudeen et al., 1972; Cooper et al., 1990; Lincoln and Ratnasooriya, 1996).

Analogous to the early sexual maturation seen in captive female elephants, bulls in captivity can begin musth as early as 10-15 years of age while their wild counterparts seldom exhibit signs of musth before the age of 25. During periods of non-musth, concentrations of circulating androgens, including testosterone, dihydrotestosterone, and androstenedione, are low (<2 ng/mL), with occasional spikes up to 10 ng/mL (Jainudeen et al., 1972; Rasmussen et al., 1984, 1990; Hall-Martin and van der Walt, 1984; Cooper et al., 1990; Niemuller and Liptrap, 1991; Brown et al., 1993). In contrast, concentrations of testosterone during musth increase significantly, averaging 10-20 ng/mL in pre- and post-musth and sometimes exceeding 50 ng/mL during peak musth (Brown, 2000). Musth is not seasonal (Poole, 1994) and is not a prerequisite for breeding. Often times, the overaggressiveness accompanying musth can reduce breeding interest in captive bulls (Schmidt, 1993). Little is known about the reproductive endocrinology surrounding musth or the successful breeding of captive bull elephants. In fact, no studies have been conducted, to our knowledge, on inhibin production in bulls.

2.8 SUMMARY

The risk of 'captive extinction' of Asian and African elephants may become a reality without the development of self-sustaining captive populations of African and Asian elephants.

While progress has been made in understanding the estrous cycle of the female elephant, it is still necessary to acquire a greater understanding of elephant reproductive biology. Additionally, the establishment of endocrine monitoring programs and the development of effective treatments for reproductive pathologies is critical to the success of these captive populations. One of the greatest challenges facing the development of a successful captive breeding program is determining the cause(s) of acyclicity observed in a large number of African and Asian captive female elephants. Elucidation of the female elephant's double LH surge along with an understanding of follicular recruitment and maturation have only begun to unravel this puzzle. Knowledge of bull reproductive physiology is even more limited leaving much to be discovered.

CHAPTER 3

INVOLVEMENT OF THE INHIBIN AND ACTIVIN FAMILY OF PROTEINS IN REGULATING OVARIAN DEVELOPMENT IN AVIAN SPECIES

3.1 FOLLICULAR DEVELOPMENT AND OVARY STRUCTURE IN THE HEN

The structure of the hen's ovary provides an excellent paradigm for studying the roles of inhibin and activin in modulating follicular growth and development. In avian species, as in mammalian species, follicular development is a highly regulated process. However, avian species differ from mammalian species due to their consecutive ovulations which occur, depending on the species, about every 24 or 48 hours apart. Ovulation is easily predicted in domesticated poultry because it occurs approximately 20-40 minutes after oviposition (egg laying).

Structurally, the ovary of the hen consists of pools of follicles at different developmental stages which comprise the follicular hierarchy. The ovary contains 5 to 8 large yellow follicles that are arranged in a hierarchy on the basis of size which inherently represents the sequence in which the follicles will be ovulated. Each of these large, preovulatory hierarchical follicles consists of yellow yolk and has a diameter ranging from about 12-40 mm, based upon their stage of maturation. The largest of the preovulatory follicles is designated as the F1 follicle and will be ovulated within the next 24 hours. Subsequent to the F1 follicle, the F2 follicle is the next largest in size and the remaining preovulatory hierarchical follicles are named in a similar fashion. After the F1 follicle is ovulated, the F2 follicle continues to mature and is now designated as the F1 follicle. Similarly, the succeeding follicles in the hierarchy each advance one place and an additional follicle is recruited into the hierarchy from a pool of small, yellow, non-hierarchical follicles.

The non-hierarchical follicles consist of a pool of small yellow follicles (SYF) that are approximately 5-12 mm in diameter and a pool of smaller white follicles that are less than 5 mm in diameter.

The white follicles consist of white yolk and are further classified by size into two groups: the large white follicles (LWF, 2-5 mm) and the small white follicles (SWF, <2 mm). As was previously mentioned, recruitment of follicles into the hierarchy occurs following each consecutive ovulation. One follicle is recruited from the pool of SYF and thus enters the group of preovulatory hierarchical follicles that are destined for ovulation while several LWF advance to become SYF. Follicular recruitment into the hierarchy is, however, a highly selective process. In fact, it has been estimated that for every 20 ovarian follicles in the hen's ovary that grow to a size of 6-8 mm in diameter only one will be selected into the preovulatory hierarchy (Gilbert et al., 1983). The remaining follicles will undergo follicular atresia (Gilbert et al., 1983).

3.2 HISTOLOGICAL STRUCTURE OF THE HEN'S FOLLICLE

In addition to the highly regulated and visually apparent avian follicular hierarchy, the histological structure of the hen's ovarian follicle also makes it an ideal model for study. The tissue layers of the ovarian follicle from the exterior of the follicle to the interior of the follicle include: the theca layer, a basement membrane, a granulosa layer, an inner perivitelline layer, and the plasma membrane of the oocyte that surrounds the yolk. The theca layer contains the vasculature of the follicle and can be further divided into two layers, the theca interna and externa. In the white-yolk containing non-hierarchical follicles the theca layer is less developed while the granulosa cells form several layers (Etches, 1990). In contrast, yellow-yolk containing hierarchical follicles have a monolayer of granulosa cells that can be easily dissected away and separated from the theca cell layers.

The ability to manually separate the granulosa and theca tissues in the hierarchical follicles as well as to enzymatically separate these tissues in the non-hierarchical follicles allows for the conduction of expression studies that are tissue specific in avian species. Such studies are not as feasible in mammals where the theca and granulosa cells of the follicle are more intermixed and not easily separated.

3.3 DETECTION AND CHARACTERIZATION OF AVIAN INHIBIN

The bioactivity of inhibin in the chicken ovary was first reported in 1988 in two separate studies which established that granulosa cell conditioned medium exhibited selective suppression of FSH secretion when incubated with rat (Akashiba *et al*., 1988) or sheep (Tsonis *et al*., 1988) anterior pituitary cells. Akashiba *et al*. (1988) further analyzed the differential production of inhibin by examining the FSH suppressing abilities of granulosa cells from various sized hierarchical follicles. This study determined that a greater amount of biologically active inhibin was produced by the F1 follicle as compared to the F3 follicle. Tsonis *et al*. (1988) examined both granulosa and theca cells for inhibin activity and found that theca cell conditioned medium suppressed FSH secretion from ovine pituitary cells to a lesser degree than granulosa conditioned medium. These studies established the differential production of inhibin in follicles at different developmental stages and the differential production of inhibin by different ovarian cell types.

Advancements in understanding avian inhibin biology continued when Vanmontfort *et al*. (1992) utilized the Monash assay, a mammalian radioimmunoassay system, (Robertson *et al*., 1988; validated for the chicken) to detect immunoreactive inhibin α-subunit in the laying hen.

By inducing molt and thus causing ovarian regression, Vanmontfort's group was able to detect a decline in immunoreactive inhibin α-subunit that paralleled the decline detected in plasma progesterone levels which suggested that the large follicles of the ovary were the primary sources of inhibin α-subunit in the hen (Vanmontfort *et al*., 1992).

Other studies conducted by Johnson *et al*., (1993b) confirmed that the ovary, and more specifically, the largest follicles, are the principal source of secreted immunoreactive inhibin α-subunit in the hen. Hens injected with equine chorionic gonadotropin (eCG) to increase the number of large follicles present on the ovary had significantly increased plasma concentrations of immunoreactive inhibin α-subunit by the sixth day of treatment when compared to untreated controls (Johnson *et al*., 1993b). In addition, removing the 3-4 largest follicles from the hen's ovary significantly reduced plasma immunoreactive inhibin α-subunit levels by 50% 6 h after their removal when compared to sham-operated animals (Johnson *et al*., 1993b).

3.4 MOLECULAR CLONING OF THE AVIAN INHIBIN/ACTIVIN SUBUNIT

In avian species, follicular fluid is absent and thus cannot be utilized as an enriched source of secreted inhibin. Scientists were therefore prompted to use a molecular approach to characterize inhibin in the hen (Johnson, 1997). The high degree of conservation (>80%) exhibited by the cDNA sequence for the α-subunit of inhibin among mammalian species (Ying, 1987) led to the use of a cDNA for the porcine inhibin α-subunit as a probe to detect chicken inhibin α-subunit expression in granulosa cells. By using the porcine inhibin α-subunit as a probe, an mRNA transcript of 1.7 kb was detected in RNA extracted from chicken granulosa cells (Johnson and Wang, 1993).

Additional use of the porcine α-subunit cDNA probe to screen a chicken granulosa cell cDNA library led to the isolation of a clone (1585 bp) that contained the complete open-reading frame for chicken inhibin α-subunit and exhibited 59% homology to the porcine α-subunit open reading frame (Wang and Johnson, 1993b). Further examination of the sequence for the chicken inhibin α-subunit indicated that the chicken inhibin α-subunit, like mammalian species, is synthesized as a precursor protein and subsequently processed into its mature form. The number and position of cysteine residues as well as the location of glycosylation sites in the mature chicken inhibin α-subunit resembled those found in mammalian sequences (Johnson, 1997).

Another mammalian cDNA probe (rat) was used to detect the mRNA for the inhibin/activin β_A-subunit in chicken granulosa cells (Chen and Johnson, 1996b). First, through Northern blot analysis, an mRNA transcript of 8.4 kb was detected. Next, this probe was used to isolate a cDNA clone encoding for the full length coding sequence for the inhibin/activin β_A-subunit from a chicken granulosa cell cDNA library. This clone was 1,540 bp in length, 85% identical in nucleotide sequence, and 98% similar in deduced amino acid sequence to the mature region of the rat β_A-subunit cDNA clone (Chen and Johnson, 1996b).

Lastly, a partial PCR-generated cDNA clone of the avian β_B-subunit was reported by Mitrani *et al*. (1990) which led to the screening of a chicken granulosa cell cDNA library and the determination of the complete coding sequence for the chicken β_B-subunit (Hecht *et al*., 2000). Like the β_A-subunit, the mature region of the chicken β_B-subunit was found to be approximately 80% identical in nucleotide sequence and 95% identical in deduced amino acid sequence to a variety of reported mammalian sequences (Hecht *et al*., 2000).

3.5 EXPRESSION OF THE INHIBIN/ACTIVIN SUBUNITS IN THE HEN'S FOLLICLES

Using Northern analysis, Davis and Johnson (1998) examined the mRNA expression of the inhibin/activin subunits in the granulosa layers of the large hierarchical follicles and small non-hierarchical follicles of the domestic laying hen. They found the mRNA expression of the inhibin α-subunit to be highest in the F5 follicle with significant declines in expression for larger or smaller follicles. They did not detect any mRNA for the inhibin α-subunit in the large white follicles. The inhibin/activin β_A-subunit mRNA was solely expressed in the hiearchical follicles with expression in the F1 follicle being twice that of the other follicles. Finally, expression of the mRNA for the inhibin/activin β_B-subunit was only detected in the F6 - F8 follicles and small yellow follicles (SYF) (Davis and Johnson, 1998).

The data from the mRNA expression studies suggest that inhibin-A is predominantly produced by the F1 follicle since it has the highest level of expression of the β_A-subunit in addition to having abundant α-subunit mRNA expression. Lovell *et al*., (1998) confirmed this hypothesis by using a specific two-site ELISA to examine the inhibin-A and activin-A content of the granulosa and theca layers of the four largest follicles. They reported that the content of inhibin-A increased approximately 40 fold from the F3 granulosa cells to the F1 granulosa cells. Additionally, they determined that while inhibin-A production was primarily limited to the granulosa cells, activin-A was present in both granulosa and theca cells. The level of activin-A, however, was 2-3 times greater in the theca cells than the granulosa cells (Lovell *et al*., 1998). More recently, Johnson *et al*. (2005) reported that the SYF secrete the largest amount of inhibin B.

3.6 MOLECULAR CLONING OF FOLLISTATIN IN AVIAN SPECIES

Follistatin was first cloned in an avian species by Connolly *et al.* (1995) from chick embryos. In addition to cloning follistatin, Connolly's group reported the predicted amino acid sequence of follistatin and examined its expression pattern in early developing chick embryos. Connolly *et al*. (1995) did not report the cDNA sequence for chicken follilstatin. The cDNA coding sequence as well as the predicted amino acid sequence for chick follistatin was later cloned and reported by Graham and Lumsden (1996). By using PCR analysis and Northern blot analysis respectively, Davis and Johnson (1998) reported the tissue mRNA distribution of follistatin and examined the expression of the mRNA for follistatin in granulosa layers of differently sized preovulatory follicles. They detected follistatin in the brain, pituitary, thyroid, muscle, heart, intestine, lung, pancreas, liver, spleen, kidney, adrenal, shell gland, magnum, isthmus, and infundibulum by PCR. By Northern analysis, mRNA for follistatin was detected in samples obtained from granulosa cells of only the F5, F6-F8, SY, and LW follicles and by far the greatest amount of follistatin was detected in the SYF samples (Davis and Johnson, 1998). Thus follistatin mRNA expression in the hen preovulatory follicles paralleled the mRNA expression of the inhibin/activin β_B-subunit.

3.7 MOLECULAR CLONING AND EXPRESSION OF THE ACTIVIN TYPE II AND TYPE I RECEPTORS AND BETAGLYCAN IN AVIAN SPECIES

ActRII and ActRIIB cDNA clones were initially both isolated from chick embryo cDNA libraries (Nohno *et al*., 1992; Stern *et al*., 1995). Nohno *et al*. (1992) and Stern *et al*. (1995) concluded both ActRII and ActRIIB, were identical in their ability to bind activin A based on binding assays. Slappey and Davis (2003) examined the expression pattern of the mRNA for the activin type II receptors during follicular development in broiler breeder hens.

By Northern analysis of total RNA isolated from individual granulosa and theca layers of the F1 through F5 follicles, a pool of the F6 and F7 follicles, the small yellow follicles, and from the combined granulosa and theca layers of the large white follicles of six birds, two ActRII mRNA transcripts of 6.5 and 3.7 kb were detected in all of the granulosa and theca samples. Interestingly, they detected a difference in expression of these two transcripts between granulosa and theca cells. In granulosa cells, differential expression of the 6.5 kb ActRII transcript was observed based on follicle size while the 3.7 kb ActRII transcript expression was constant across the follicular hierarchy. However, in theca cells, the expression of the larger transcript was constant while expression of the smaller transcript varied with follicle size. The significance of the differential expression of the two transcripts is unclear but has been reported in mammalian species as well (de Winter *et al*., 1992; Ito *et al*., 1993; Wu *et al*., 1994). ActRIIB mRNA expression was not detectable in any of the samples by Northern analysis (Slappey and Davis, 2003). However, Lovell *et al*. (2005) and (2006) characterized the expression of the mRNA for the activin type II receptors by real-time quantitative PCR in preovulatory and prehierarchical follicles of the laying hen ovary as well as in the hen's anterior pituitary gland. Significantly greater amounts of mRNA for the activin type IIB receptor were detected in theca tissue compared to granulosa tissue regardless of follicular maturation (Lovell *et al*., 2006).

Lai *et al*. (2000) cloned and investigated the expression and function of activin type I (ActRI) receptors in avian embryology. Subsequently, Lovell *et al*. (2005) and (2006) reported its detection in preovulatory and prehierarchical follicles of the laying hen ovary and the hen's anterior pituitary gland. Like the activin type II receptors, real-time quantitative PCR revealed that significantly greater amounts of mRNA for the ActRI receptor was detected in theca tissue as compared to granulosa tissue in all follicles (Lovell *et al*., 2006).

To date, a sequence for chicken ActRIB has not been submitted to Genbank, and there is no expression data for this receptor in avian species.

Sweeney and Johnson (2005) cloned chicken betaglycan and reported it was more abundantly expressed in the theca layer compared with the granulosa layer for all follicle sizes. They interpreted this to exemplify a potential paracrine role of inhibin in the hen since inhibin is produced predominately by the granulosa cells. Additionally, they observed abundant colocalization of betaglycan and FSH in the anterior pituitary of the hen which is consistent with known inhibin effects (Sweeney and Johnson, 2005).

3.8 REGULATION OF THE INHIBIN/ACTIVIN FAMILY

Understanding the regulation of the inhibin/activin family members in the hen ovary is paramount to elucidating the role of this family of proteins in follicular development. Several in vitro studies have been conducted to examine the regulatory roles of LH and FSH on the inhibin/activin family. LH or FSH added to granulosa cell cultures from the hierarchy follicles of domestic laying hens induces the expression of inhibin α-subunit mRNA and protein (Vanmontfort et al., 1992; Davis et al., 1999). FSH has also been reported to significantly stimulate the secretion of immunoreactive inhibin α-subunit in F4+F5, SY, and LW follicles (Davis *et al*., 2001). Most likely, this stimulation resulted from the enhanced expression of the mRNA for the inhibin α-subunit in the granulosa cells from the F4 + F5 follicles and SYF and the induction of this message to detectable levels in the LWF. In contrast, although estradiol had a positive effect on the expression of the mRNA for the inhibin α-subunit in F4 + F5 and SYF granulosa cell cultures, immunoreactive inhibin α-subunit was not increased (Davis *et al*., 2000).

Addition of estradiol to granulosa cell cultures from the F4 +F5 follicles, SYF, and LWF generally had a positive effect on the expression of the mRNA for follistatin and the inhibin/activin β_B-subunits (Davis *et al*., 2000).

More recently, the autocrine and paracrine roles of activin A and inhibin A have been examined in the hen's ovary in order to understand the manner in which they might act as a local regulator of follicular maturation. In general, the addition of activin A to granulosa cell cultures induces the mRNA expression of LH (Johnson *et al*., 2004; Johnson *et al*., 2006) and FSH (Davis et al., 2001; Johnson et al., 2004; Johnson et al., 2006) receptors. Activin A also inhibits granulosa cell proliferation in granulosa cell cultures from hierarchical follicles (Davis *et al*., 2001; Johnson *et al*., 2006) but not in granulosa cell cultures from prehierarchical follicles (Johnson *et al*., 2006). In contrast, the addition of inhibin A to cultured granulosa cells did not effect the expression of the LH or FSH receptors and had no effect on granulosa cell proliferation (Johnson *et al*., 2006).

3.9 SUMMARY

The use of the hen's ovary as a model for studying the roles of inhibin and activin in regulating follicular development and growth has provided much insight into the biological activities of these proteins. The abundance of granulosa and theca tissue surrounding the hen's large preovulatory follicles as well as the ability to easily separate these layers has allowed cell specific analyses of the inhibin and activin subunits once they were cloned in the chicken. By using Northern analysis and real-time quanitative PCR, the mRNA expression of these subunits has been characterized in the hierarchical as well as non-hierarchical follicles of the hen's ovary.

Additionally, the cloning of follistatin, activin type I and type II receptors, and betaglycan have further revealed the intricate manner in which inhibin and activin might function in the hen ovary. Finally, by examining the regulation of FSH and LH receptors as well as granulosa cell proliferation in the hen's ovary the autocrine and paracrine roles of activin A and inhibin A in regulating follicular maturation are becoming clearer.

CHAPTER 4

STATEMENT OF PURPOSE

While the TGFβ superfamily has been recognized as serving a variety of endocrine, autocrine, and paracrine roles in regulating follicular maturation, much is left to be discovered about this family of proteins. The purpose of these studies was to add to our understanding of the roles of inhibin and activin in the reproductive functions and dysfunctions observed in mammalian and avian species. Specifically, one purpose of this research is to examine the effects of genetic selection on the inhibin/activin subunits and follistatin by studying their expression in the preovulatory follicles of two lines of turkey hens selected for rapid growth or egg production. Additionally, this study will focus on characterizing the follicular hierarchies of these two genetic lines in order to correlate these differences with differences in follistatin and inhibin/activin subunit mRNA expression data. While both of the activin type II receptors and ActRIA have been cloned and their expression studied in the hen, ActRIB has not been cloned in the hen and thus remains the last piece of the puzzle needed to understand this intricate signaling cascade. Therefore, in order to gain a complete understanding of the activin receptor signaling cascade in the hen, the second goal of this research is to characterize the expression of ActRIA and ActRIB in the theca and granulosa tissues of the broiler breeder hen ovary and to examine their regulation by FSH, LH, estradiol, and testosterone

The last aim of this research is to determine if the large number of acyclic female elephants in captivity which are known to have suppressed serum FSH levels, also have elevated serum levels of inhibin which could be indicative of granulosa cell tumors. Additionally, inhibin will be measured in bull elephants in musth and non-musth in order to further understand their reproductive endocrine patterns.

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CHAPTER 5

FOLLICULAR DEVELOPMENT AND EXPRESSION OF THE MESSENGER RIBONUCLEIC ACID FOR THE INHIBIN/ACTIVIN SUBUNITS IN TWO GENETIC LINES OF TURKEY HENS THAT DIFFER IN TOTAL EGG PRODUCTION

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To be submitted to *Biology of Reproduction*

ABSTRACT

The characterization of the follicular hierarchy and the expression of mRNA for follistatin and the inhibin/activin subunits was investigated in the follicles of two lines of turkey hens selected for over 40 generations for either increased egg production (E-line) or increased body weight (F-line). The follicular hierarchies of six hens from both the E- and F-lines were characterized in middle (45 weeks of age) and late production (58 weeks of age). Relative follicular weights for individual hierarchical follicles (>12mm), pooled small yellow follicles (SYF 5-12mm), and large white follicles (LWF 2-5 mm) were calculated. Total RNA was extracted for Northern blot analysis from individual granulosa layers of the F1 thru F4 follicles, and from the combined granulosa and theca layers of small yellow follicles and large white follicles from an additional 6 hens from each genetic line. E-line hens displayed a more distinct follicular hierarchy than F-line hens at 45 and 58 weeks. Although total follicular weight relative to body size was greater at both 45 and 58 weeks of age for the E-line hens than the F-line hens, the total number of hierarchical follicles was greater in the F-line hens at both 45 and 58 weeks of age. Expression of follistatin and the inhibin β_B-subunit was highest in non-hierarchical follicles while the expression of the inhibin α- and β_A-subunits was highest in the hierarchical follicles. The inhibin α- and β_A -subunit mRNA expression pattern in the four largest follicles of the F-line hens was not similar to the E-line hens nor characteristic of laying hens that have a high rate of egg production. The lack of a clear size hierarchy and the unusual inhibin subunit mRNA expression in the largest hierarchical follicles in F-line hens may account for the multiple ovulations and poor egg production observed in these birds.

Key words: *turkey, follicle, inhibin, activin, follistatin, growth, egg-production*

INTRODUCTION

Inhibin and activin are two members of the transforming growth factor β (TGFβ) superfamily whose regulation of FSH release from the anterior pituitary has been well documented (Mather et *al*., 1997; Rombauts *et al*., 1996; Halvorson and DeCherney, 1996; Findlay 1993, 1994). Inhibin is a heterodimeric protein composed of an α- and β-subunit. Depending upon which of the two distinct but similar β-subunits (β_A and β_B) is combined with the α-subunit, inhibin exists as either inhibin-A (α-β_A) or inhibin-B (α-β_B). In contrast, activin may form as either a homodimer or heterodimer of the β-subunits, resulting in the homodimers activin-A (β_A-β_A) and activin-B (β_A-β_A) or the heterodimer activin-AB (β_A-β_B). Inhibin suppresses FSH release from the pituitary while activin stimulates pituitary secretion of FSH (Ying, 1988). Additionally, the inhibin α-subunit acts as a gonadal tumor suppressor (Matzuk *et al*., 1992) and activin has been associated with a multitude of diverse functions as reviewed by (Mather *et al*., 1992; Woodruff, 1998; Woodruff, 2002; Chapman *et al*., 2003). Activin and inhibin are bound by follistatin, a soluble-binding protein that binds both hormones through their common β-subunit (Nakamura *et al*. 1990). Follistatin binds activin with a greater affinity than inhibin (Shimonaka *et al*., 1991; Krummen *et al*., 1993) and neutralizes almost all of the biological actions of activin upon binding (Mather *et al*., 1993).

The laying hen ovary provides a unique model for studying follicular development due to the organization of its yolk-filled preovulatory follicles into a hierarchy according to size. The largest follicle which will be ovulated within 24-26 hours is designated the F1 follicle, and the second largest follicle, which will be ovulated 24-26 hours after the F1 follicle, is the F2 follicle, and so on.

The smallest hierarchical preovulatory follicle matures each day from a pool of small yellow follicles (SYF) that are 5-12 mm in diameter which in turn mature from a pool of large white follicles (LWF) that are less than 5 mm in diameter.

Both the mRNA and protein expression of the inhibin/activin subunits and follistatin are well characterized in the laying hen ovary (Davis and Johnson, 1998; Lovell *et al*., 1998; 2003; Johnson *et al*., 2005). In general, the granulosa cells of the largest follicles produce inhibin-A while the small follicles produce inhibin-B (Lovell *et al*., 1998; 2003; Johnson *et al*., 2005). Specifically, the F1 follicle is the primary source of inhibin-A (Lovell *et al*., 1998). Activin-A production is greater in the theca cells than the granulosa cells (Lovell *et al*., 1998). Activin-A production steadily increases in a follicle as it matures from the small prehierarchical stage through to the F4 stage where it declines rapidly as the follicle continues to mature (Lovell *et al*., 2003). Follistatin is produced by both theca and granulosa tissue and production by the granulosa tissue is greatest in the prehierarchical follicles (Lovell *et al*., 2003).

Although inhibin and activin production have been well characterized in highly productive laying hen strains, little focus has been given to production of these hormones in poultry species with poor egg production. Although the inhibin α- and β_A- subunits have been cloned in the turkey (Ahn *et al*., 2001), a comprehensive examination of the expression of the inhibin subunits during follicular development has not been completed. The Ohio Agriculture Research and Development center maintains two lines of turkey hens differing greatly in body weight and egg production. E-line or egg line has been selected for 180 day egg production for over 53 generations and F-line or growth line has been selected for 16 week body weight for over 44 generations. The E-line originated from a randombred control established by crossing four popular large white turkey strains (McCartney *et al*. 1968).

The F-line was developed from a randombred control line that was started in 1966 from reciprocal crosses of two commercial strains of turkeys (McCartney et al. 1968; Nestor et al. 1969). Higher rates of egg production have been reported for the E-line hens than for the F-line hens (Nestor and Noble, 1995). The hens selected for rapid growth rate have been noted to have an increased number of hierarchical ovarian follicles and simultaneous ovulations (Nestor *et al*., 1970) in comparison to E-line hens.

Research utilizing the E- and F-line hens provides a unique opportunity to characterize mRNA expression of the inhibin/activin subunits and follistatin in birds with poor production compared to laying hens as well as the opportunity to compare expression of these subunits in hens of the same species with vastly different egg production rates. Therefore, the current study was conducted to characterize the follicular hierarchy of these two genetic lines of turkey hens and to characterize mRNA expression of the inhibin/activin subunits and follistatin in the ovaries of these hens.

MATERIALS AND METHODS

Animals

E-line and F-line poults were hatched at the Ohio Agriculture Research and Development Center and immediately shipped to the North Carolina State University turkey educational unit. The turkeys were raised in floor pens and provided 10 hours of light per day until 25 weeks of age when the hours of light were reduced to 8. At 31 weeks of age the turkey hens were moved to breeding pens (6 birds per pen) and photo-stimulated for reproduction by providing them 14 hours of light per day. Each breeding pen was equipped with a nest box.

The turkeys were provided with free access to appropriate commercial diets and water at all times through rearing and production. All animal procedures were approved by the North Carolina State University Animal Care and Use committee.

Characterization of Follicular Hiearchies

At 45 and 58 weeks of age six hens from both the E- and F-lines were selected and weighed. The hens were killed by electrocution 2-4 hours prior to ovulation. The entire ovary was removed from each bird and follicles were separated into hierarchical follicles (>12mm), SYF (5-12mm), or LWF (2-5 mm). Weights were determined for individual hierarchical follicles, pooled SYF, and pooled LWF.

Tissue Collection

The F1-F4 follicles, SYF, and LWF were utilized for subsequent RNA isolation from each of the turkey hens at 58 weeks of age. The granulosa cell layer was manually separated from the theca cell layers of each of the F1-F4 follicles and saved (Huang and Nalbondov, 1979). The connective tissue was removed from the SY and LW follicles and then the yolk material was expelled from each follicle. Next, the combined theca and granulosa tissue layers from the SYF and LWF were pooled by size and placed in RNA*later* (Ambion, Austin, TX). Granulosa samples from the F1-F4 follicles were frozen and stored at -80ºC in 1 mL of guanidinium thiocyanate solution for subsequent RNA extraction while the theca/granulosa samples from the SYF and LWF were maintained in RNA*later* soluation at 4º C for subsequent RNA extraction.

RNA Extraction and Northern Analysis

Total RNA was extracted from the collected individual granulosa layers of each of the F1 through F4 follicles of each hen and from the combined theca and granulosa layers of the SYF and LWF follicles of each hen using a guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). For each sample, forty micrograms of total RNA was electrophoresed on a 1.5% agarose/formaldehyde gel and then transferred to a nylon membrane as previously described (Davis and Johnson, 1998). Eight Northern blots were produced. Six of the blots each contained the F1-F4 granulosa samples obtained from one of the 6 E-line and F-line hens. The final two blots each contained 3 SYF and 3 LWF samples from each of the genetic lines.

cDNA clones were prepared for the chicken inhibin α-subunit, inhibin/activin β_A- and β_B-subunits, follistatin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and labeled with ^{32}P for Northern blot analyses as previously described (Davis and Johnson, 1998). The hybridization and densitometry procedures also followed those described previously (Davis and Johnson, 1998). After each hybridization, the blots were subjected to a stringent wash and exposed to x-ray film as described previously (Davis and Johnson, 1998). The order of the hybridizations for each blot was follistatin, inhibin/activin β_B-subunit, inhibin/activin β_A-subunit, and inhibin α-subunit. To verify and correct for equality of RNA loading and transfer, a final hybridization of the blots was done with GAPDH. The relative mRNA expression of the inhibin α-, β_A-, and β_B-subunits was determined for the samples of each blot by calculating the signal intensity for each sample relative to the strongest signal which was assigned a value of 1.

Before calculating the relative inhibin α-, β_A-, and β_B-subunit mRNA expression levels, GAPDH mRNA expression was used to correct the inhibin α-, β_A-, and β_B-subunit values for equality of RNA loading and transfer for each blot.

Statistics

Data were subjected to ANOVA using the General Linear Model procedure with replicate and follicle size or genetic line as factors. Tukey's multiple-comparison procedure (Neter *et al*., 1990) was used to detect significant differences among the follicles. All statistical procedures were done with the Minitab Statistical Software package (Release 13, State College, PA). Differences were considered significant when p-values were <0.05.

RESULTS

Characterization of Follicular Hierarchies

E-line hens produced a greater number of eggs than F-line hens (Table 1). At 45 and 58 weeks of age, the total follicular weight relative to body weight was significantly greater in the E-line hens as compared to the F-line hens, even though the F-line hens possessed a significantly greater number of hierarchical follicles (Table 2). The F-line hens had more LWF than the E-line hens at both 45 and 58 weeks of age (Table 2). The number of SYF was greater in F-line hens than E-line hens at 45 weeks of age but not at 58 weeks of age. The size of the SYF and LWF relative to body size was significantly smaller (by 100 percent) for the F-line hens than the E-line hens (data not shown). The F1-F5 follicles of the E-line hens were significantly larger relative to body size and displayed a distinct size hierarchy compared to the F1-F5 follicles of the F-line hens at 45 (Figure 1.1) and 58 (Figure 1.2) weeks of age. In contrast, the F8-F10 follicles at 45 weeks of age and F8-F9 follicles at 58 weeks of age of F-line hens were significantly larger relative to body size than in the E-line hens.

Expression of the Inhibin α-Subunit

Within the F-line, the F4 follicle expressed a significantly lower amount of inhibin α-subunit mRNA as compared to the F1 and F2 follicles in that line while the inhibin α-subunit mRNA expression in the F3 follicle was not different from the F1, F2, or F4 follicles (Figure 1.3 and 1.4). Within the E-line hens, there was no significant difference in mRNA expression of the inhibin α-subunit between the F1- F4 follicles (Figure 1.3). Overall expression of the inhibin α-subunit was significantly greater in the E-line F1-F4 hierarchical follicles (0.80 ± 0.04) as compared to the F-line hens (0.60 ± 0.04). The relative mRNA expression of the inhibin α-subunit between the individual F1, F2, F3, and F4 follicles of E- and F-line hens was (mean ± SEM) 0.73 ± 0.09 versus 0.70 ± 0.04; 0.73 ± 0.08 versus 0.72 ± 0.06; 0.84 ± 0.05 versus 0.54 ± 0.10; and 0.91 ± 0.05 versus 0.37 ± 0.04, respectively. Thus, the higher overall expression of the inhibin α-subunit in the four largest follicles in the E-line hens compared to the F-line hens was based largely on the significantly higher expression of the inhibin α-subunit mRNA in the F3 and F4 follicles of the E-line hens compared to the F-line hens.

Expression of the Inhibin/Activin β_A-Subunit

In the F-line hens, the F4 follicle had significantly less inhibin/activin β_A-subunit mRNA expression than the F1, F2, and F3 follicles but there was no difference in β_A-subunit mRNA expression between the F1, F2, and F3 follicles (Figure 1.5). In E-line hens, the F2 and F3 follicles had significantly less inhibin/activin β_A-subunit mRNA expression than the F1 follicle but there was no difference in inhibin/activin β_A-subunit mRNA expression between the F2 and F3 follicles (Figure 1.5).

The overall mRNA expression of the inhibin/activin β_A-subunit was significantly greater in the F1-F4 hierarchical follicles from the F-line hens than the E-line hens due to the significantly higher relative mRNA expression of the inhibin/activin β_A-subunit in the F2 and F3 follicles of the F-line hens than the E-line hens. The relative mRNA expression of the inhibin/activin β_A-subunit between the individual F1, F2, and F3 follicles of E- and F-line hens was (mean ± SEM) 0.60 ± 0.20 versus 0.84 ± 0.09; 0.04 ± 0.03 versus 0.69 ± 0.15; and 0.03 ± 0.01 versus 0.61 ± 0.2, respectively.

Expression of Follistatin and the inhibin/activin β_B-subunit

Follistatin and the inhibin/activin β_B-subunit mRNA were detected in the combined theca and granulosa samples of the SYF and LWF of both E- and F-line hens (data not shown). Despite extended film exposure times, no mRNA expression for the inhibin/activin β_B-subunit and follistatin were detected in the hierarchical granulosa samples (data not shown).

DISCUSSION

The correlation between selection for rapid growth rate and a decline in reproductive fitness has been well-established in the poultry industry (Nestor *et al*., 1970, 1980; Jaap and Clancy, 1968). The negative impact of genetic selection for rapid growth rate on reproductive fitness has been observed in the form of decreased egg production and increased numbers of hierarchical follicles in meat strain turkeys as compared to egg strain turkeys (Nestor *et al.*, 1970, 1980). The increased numbers of hierarchical follicles has been attributed to increased yolk production in growth strains of turkeys which is believed to be an inherited trait (Nestor *et al*., 1970; Nestor and Bacon, 1972).

This theory is based on the premise that inheritance for rapid growth favors rapid protein anabolism and may also favor rapid formation of lipoprotein in the liver and therefore increased yolk production for ovarian follicular development (Jaap, 1969). According to this theory, selection for increased growth rates in turkeys should result in the production of greater amounts of yolk and more preovulatory follicles than selection for increased egg production.

In the present study, at both 45 and 58 weeks of age, F-line hens that have been selected for rapid growth rates had a significantly greater number of hierarchical follicles and non-hierarchical follicles (with the exception of SY follicles at 58 weeks of age) than E-line hens. These findings are in accordance with those of Nestor *et al*. (1970) and Nestor and Bacon (1972) and support the theory that selection for growth has led to increased yolk production (Jaap, 1969) when total follicular weights are considered.

In contrast to previous studies, the present study considered follicular weights relative to body weight which revealed that E-line hens actually had significantly greater relative total follicular weights and thus total yolk mass, at both 45 and 58 weeks of age when compared to F-line hens. Individual relative follicular weights for each of the five largest hierarchical follicles, and SYF and LWF of the E-line hens were significantly greater at 45 and 58 weeks than in the F-line hens. E-line hens also displayed a more distinct follicular size hierarchy compared to F-line hens. If yolk production is considered relative to body weight, E-line hens are, in fact, producing more yolk than F-line hens on a gram per gram basis which contradicts the theory of increased yolk synthesis capacity in hens selected for growth. In addition, on a per bird basis the F-line hens produce less than half the total number of eggs than the E-line hens which also indicates that yolk synthesis is actually greater in the E-line hens.

It is possible that the F-line hens produce and deposit more yolk into maturing follicles but suffer a greater incidence of large yellow follicle atresia. However, in the past three years our laboratory has examined the follicular hierarchies of over 50 hens from each genetic line and we have found no evidence of an increased incidence of hierarchical follicular atresia between the two lines of hens (data not shown).

It is interesting to note that the F6 and F7 follicles had equivalent follicular weights between the two lines at 45 and 58 weeks of age. As follicular maturation proceeded from this point forward, the deposition of yolk in the E-line hens proceeded in a very orderly fashion such that a distinct size hierarchy based on total yolk deposition was established. This was not the case for the F-line hens.

Schuster *et al.* (2004) reported that high levels of occludin expression in the tight junctions between granulosa cells of white follicles prevents the paracellular transport of lipid (VLDL particles) and thus yellow yolk deposition. Occludin levels decrease drastically just prior to the initiation of yellow yolk deposition (Schuster *et al*., 2004). Once the paracellular pathway was open for lipid transport Schuster *et al*. (2004) indicated that lipoprotein uptake by follicles would depend on the oocyte's receptor-mediated uptake capacity and the delivery of VLDL particles to the plasma membrane of the oocyte. The reasons for the failure of the F1-F5 follicles of the F-line hens to take up yolk at differential rates is unclear. It is possible that with the greater number of developing follicles that there is actually a lack of available VLDL from liver synthesis to meet the lipid demands of all the follicles. It is also possible that as the F6 follicle matures into an F5 follicle and beyond in the F-line hens versus the E-line hens that there is a severe depression in the receptor-mediated uptake capacity of lipid so that the follicle takes up very little additional lipid.

At 45 weeks of age, the F5 follicle in the F-line hens only gained 30 percent more weight as it developed for several days into an F1 follicle. In contrast, there was a 102 percent increase in follicular weight as the F5 follicle transitioned into the F1 follicle in the E-line hens.

Why the capacity for follicular lipid uptake would be down-regulated in the large follicles of the F-line hens is unclear. However, we would note that the production of hormones capable of paracrine and autocrine actions within the largest hierarchical follicles may be different. Specifically, the current research indicates that the production of inhibin and activin may be altered between the two genetic lines of turkey hens since the mRNA expression patterns for the inhibin α-subunit and the inhibin/activin β_A-subunit were significantly different between E- and F-line hens.

The pattern of mRNA expression of the inhibin/activin subunits in the granulosa layer during follicular development in the laying hen has been well characterized (Davis and Johnson, 1998). Specifically, the mRNA for the inhibin α-subunit was greatest in the F5 follicle and it significantly decreased with each advance in the hierarchy to the F3 follicle stage at which point it remained constant through ovulation (Davis and Johnson, 1998). In contrast, expression of the inhibin/activin β_A-subunit is greatest in the F1 follicle with considerably lower amounts detected in the other hierarchial follicles (Davis and Johnson, 1998). Results from protein expression studies are consistent with the mRNA expression patterns discerned for the inhibin/activin subunits in the ovarian follicles of the hen in that the F1 follicle granulosa cells produce by far the most inhibin-A and very little activin-A (Lovell *et al*., 1998, 2003).

The expression of the inhibin α-subunit mRNA in F-line turkey hens observed in the present study was not characteristic of the pattern observed in chicken laying hens. Specifically, this pattern was reversed in the F-line hens such that the F4 follicle of the F-line hierarchy had significantly less inhibin α-subunit mRNA expression as compared to the F1 and F2 follicles. E-line turkey hens exhibited an expression pattern more similar to laying chicken hens whereby decreasing inhibin α-subunit expression was observed as follicles matured from F4 to F1. Although, there was no significant difference in inhibin α-subunit expression between the individual E-line follicles, a regression analysis indicates that inhibin α-subunit expression significantly decreased ($P<0.05$) as follicles matured from an F4 to an F1.

Expression of the inhibin/activin β_A-subunit in F-line hens was also atypical compared to the pattern observed in laying hens and in E-line hens. In E-line hens, mRNA expression followed the pattern of expression observed in chicken laying hens (Davis and Johnson, 1998) with significantly greater inhibin β_A-subunit mRNA expression detected in the F1 follicle as compared to the F2 and F3 follicles. In F-line hens, however, no significant differences in mRNA expression of the inhibin β_A-subunit in the F1-F3 follicles of the F-line were observed.

The abnormal expression patterns observed for the inhibin α-subunit and the inhibin/activin β_A-subunit may be responsible for the absence of a distinct follicular hierarchy observed in the F-line hens. Previous studies in hens demonstrated that a greater amount of bioactive inhibin was present in the granulosa layer of the F1 follicle than of the F3 follicle (Akashiba *et al*., 1988) and the high mRNA expression level of the inhibin/activin β_A-subunit in the F1 follicle was associated with the granulosa cells of this follicle being identified as the primary production site of inhibin-A in the ovary (Lovell *et al*., 1998).

However, the abnormal expression patterns of the inhibin α-subunit and the inhibin/activin β_A-subunit in the F-line turkey hens suggest that the F1 follicle may not be producing a greater amount of bioactive inhibin A than the F2 and F3 preovulatory follicles. Futhermore, total inhibin-A production may be much greater than normal if all three (F1-F3) follicles are secreting inhibin in the F-line hens. The abundant production of inhibin-A may be disrupting normal follicular development. Further research is needed to determine if production of inhibin-A is higher in the F-line hens than the E-line hens. In addition, further research is needed to determine why the five largest follicles in the F-line hens fail to accumulate yolk at rates similar to those of the E-line hens.

Table 1. Egg production and egg weights for E- and F-line turkey hens between 37 and 54 weeks of age. The hens were photostimulated at 37 weeks of age. Values are means ± SEM, n=37 (E-line), n=36 (F-line). Means for F-line with an asterisk differ ($P<0.05$) from the corresponding mean for E-line.

	Egg Line (E)	Growth Line (F)
Eggs/hen	103 ± 2	45 ± 2 *
Egg weight (g)	63.2 ± 0.47	91.2 ± 0.48 *

Table 2. Characterization of follicular weights relative to body weight. Values are means ± SEM, n=6 replicate hens. Means for F-line with an asterisk differ (P< 0.05) from the corresponding mean for E-line at each specific age.

	45 Weeks of Age		58 Weeks ofAge	
	E-line	F-line	E-line	F-line
Bird weight (g)	6179 ± 155	17120 ± 526*	5662 ±143	15727 ± 397*
Number of hierarchical follicles	9.14 ± 0.459	15.60 ± 1.03*	9.0 ± 0.26	13.5 ± 0.56*
Number of SYF	6.43 ± 1.56	20.60 ± 5.71*	9.3 ± 1.93	12.2 ± 1.25
Number of LWF	15 ± 2.83	28.40 ± 3.66*	12.6 ± 1.09	26.8 ± 4.73*
Total Follicular weight (g)	94.1 ± 5.8	218.2 ± 13.3*	79.58 ± 6.47	174.60 ± 7.50*
Relative total follicular weight (g/g)	0.015 ± 0.0006	0.012 ± 0.0006*	0.014 ± 0.0008	0.011 ± 0.0008*

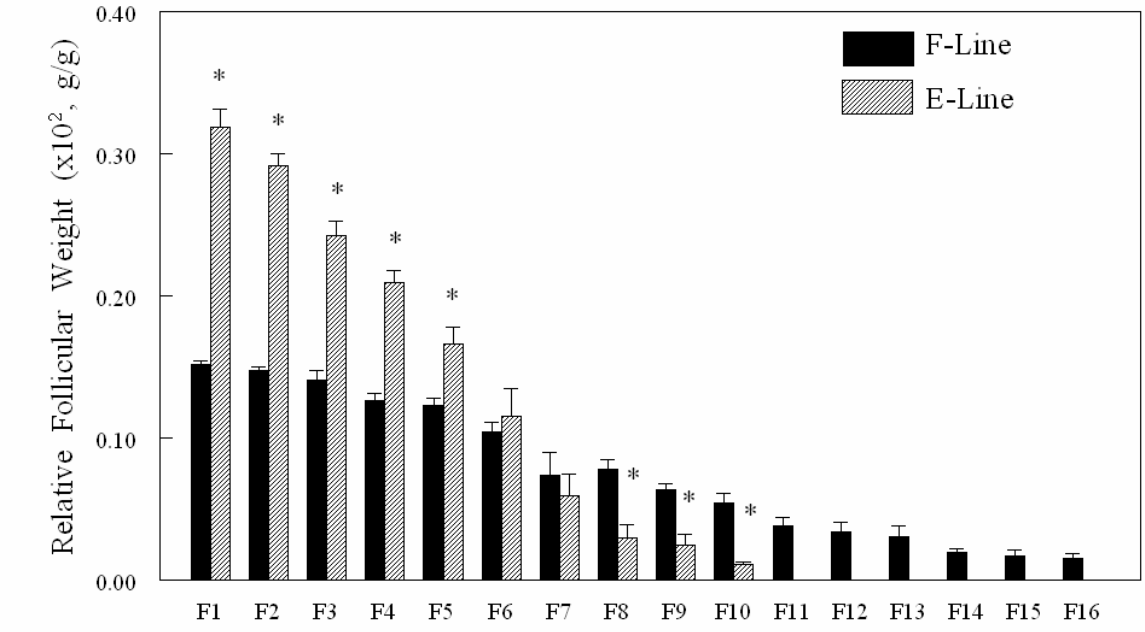

Figure 1.1. Relative follicular weights of hierarchical follicles in E- and F-line hens at 45 weeks of age. Values are means ± SEM, n=6 replicate hens. Means for E-line with an asterisk differ ($P < 0.05$) from the corresponding mean for F-line.

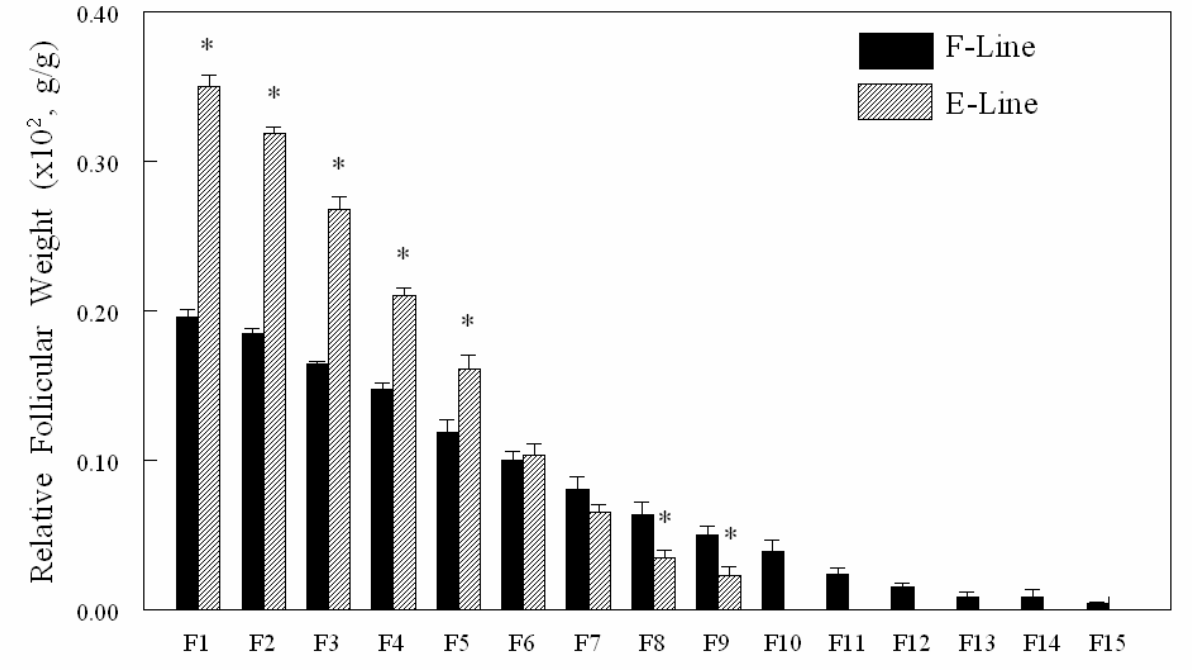

Figure 1.2. Relative follicular weights of hierarchical follicles in E- and F-line hens at 58 weeks of age. Values are means ± SEM, n=6 replicate hens. Means for E-line with an asterisk differ ($P< 0.05$) from the corresponding mean for F-line.

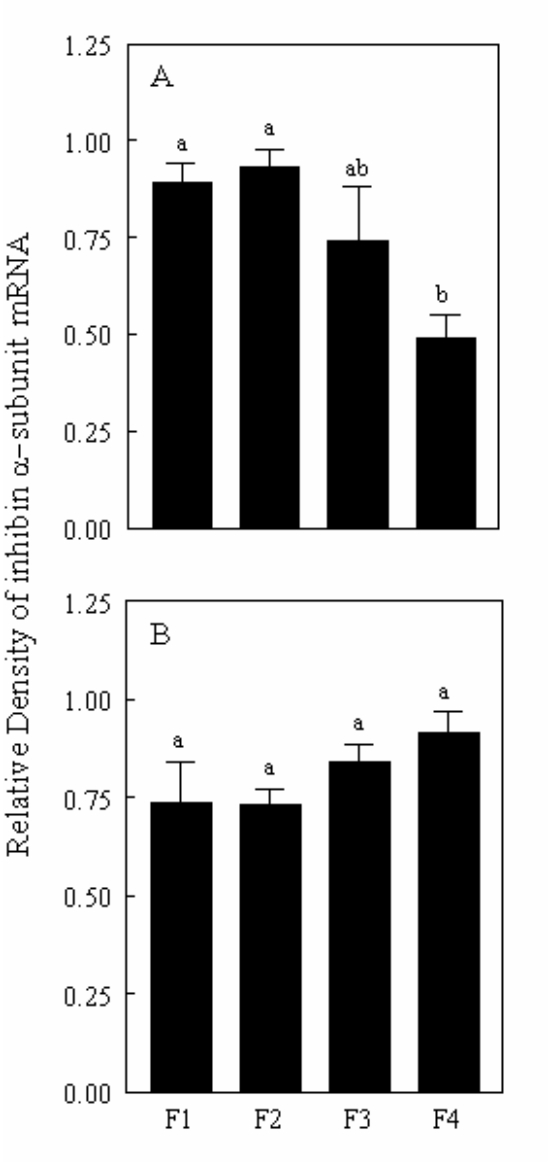

Figure 1.3. The relative density of the inhibin α-subunit mRNA in the individual F1-F4 hierarchical follicles of the F-line hens (A) and E-line hens (B). Values are means ± SEM, n=6 replicate hens. Means for each genetic line with different letters differ, $P < 0.05$. Note that the relative densities of inhibin α-subunit mRNA to one another are specific for each genetic line of turkey hen.

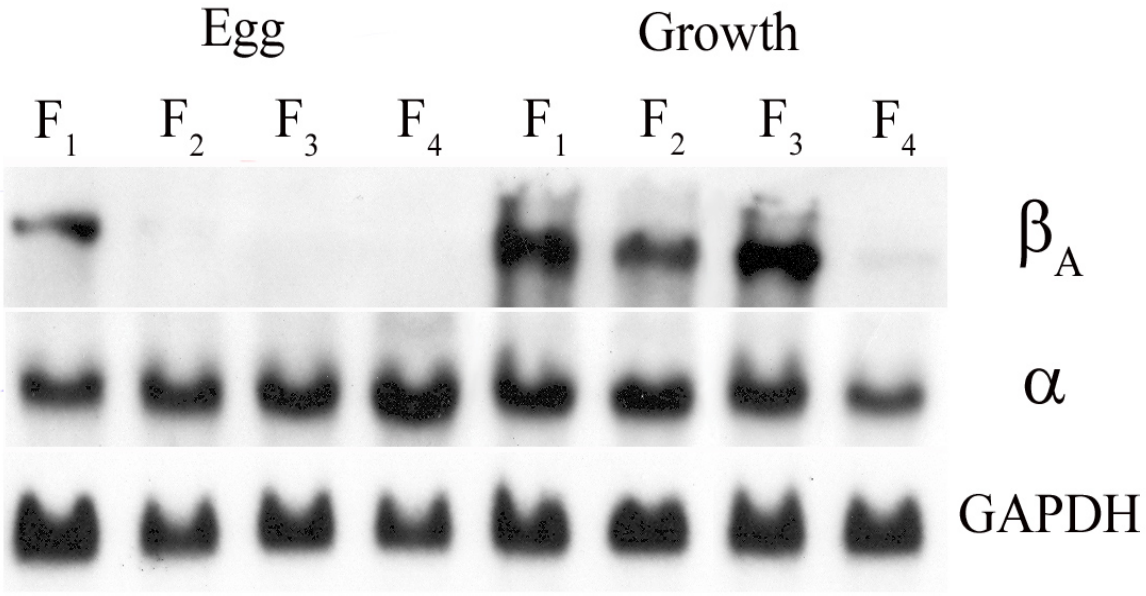

Figure 1.4. Autoradiograms from the Northern analysis of chicken inhibin α- and β_A-subunits from one of the replicate blots. Total RNA (40 μg) was loaded for each F1-F4 follicle sample from egg- and growth-line hens. Abbreviations: GAPDH, glyceraldehydes-3-phosphate dehydrogenase, α, inhibin α-subunit, β_A, inhibin β_A-subunit.

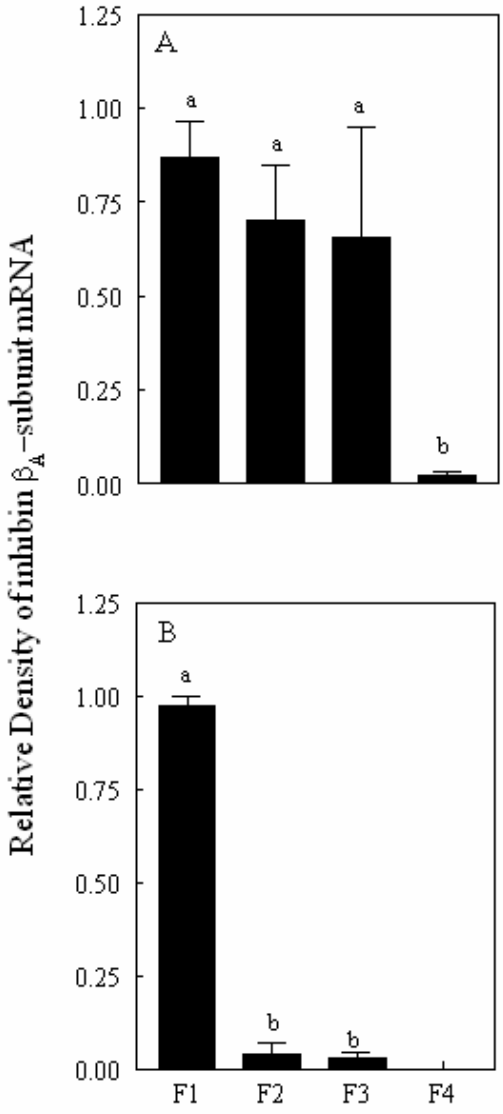

Figure 1.5. The relative density of the inhibin β_A-subunit mRNA in the individual F1-F4 hierarchical follicles of the F-line hens (A) and E-line hens (B). Values are means ± SEM, n=6 replicate hens. Means for each genetic line with different letters differ, $P < 0.05$. Note that the relative densities of inhibin α-subunit mRNA to one another are specific for each genetic line of turkey hen.

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CHAPTER 6

DETECTION OF IMMUNOREACTIVE INHIBIN α-SUBUNIT IN FEMALE AND MALE ASIAN AND AFRICAN ELEPHANTS WITH VARYING REPRODUCTIVE CHARACTERISTICS

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To be submitted to *Biology of Reproduction*

ABSTRACT

One of the greatest challenges impeding the captive breeding of elephants is the large proportion of the captive female population that is not cycling. Currently, there is a paucity of information on the reproductive role of the dimeric glycoprotein hormone inhibin in cyclic and acyclic captive female elephants. Elevated inhibin concentrations along with ovarian inactivity can be indicative of ovarian tumors in many species such as humans and horses. Inhibin has not previously been examined in the elephant population as a potential biomarker for ovarian inactivity and cancer. Using an inhibin α-subunit radioimmunoassay (RIA), this study explored the potential use of serum immunoreactive inhibin α-subunit concentrations as a biomarker for ovarian inactivity by comparing inhibin levels in cyclic and acyclic female African and Asian elephants. Serum immunoreactive inhibin levels were also examined in castrated and intact bull elephants. Analyses were conducted on blood samples collected from 3 cycling African elephants over the course of three ovulatory cycles (~36-48 weeks) and 2 Asian elephants over the course of one ovulatory cycle (~12-16 weeks). Analyses were also conducted on blood samples collected from 4 acyclic African elephants over a period of ~3-9 weeks. Additional analyses of serum immunoreactive inhibin α-subunit in 2 castrated African bull elephants, 3 intact Asian bull elephants, and 1 intact African bull elephant were performed. In the cycling African and Asian elephants, serum inhibin α-subunit concentrations were highest immediately after the ovulatory LH (ovLH) surge and then declined during the luteal phase of the estrous cycle with levels rising again immediately after the anovulatory LH (anLH) surge. Peak immunoreactive inhibin α-subunit concentrations in cycling elephants were significantly greater ($P<0.05$) than inhibin concentrations measured in acyclic elephants which remained at a steady low-level for consecutive weeks.

There was no significant difference between basal levels of inhibin in cycling elephants and the consistently low levels of inhibin produced by acyclic females. Two of the intact Asian bull elephants and the single intact African bull elephant produced a significantly greater amount of inhibin in comparison to the two castrated African bull elephants. The results suggest that cycling females produce varying amounts of inhibin α-subunit based on the stage of their estrous cycle while acyclic females show no variation in inhibin secretion which may be indicative of abnormal FSH secretion in these females but not of inhibin producing ovarian tumors.

Key Words*: inhibin, elephants, LH, acyclic*

INTRODUCTION

Despite the well-recognized need to establish self-sustaining populations of captive elephants (Olson and Wiese, 2000; Wiese, 2000), less than 20% of Asian and 10% of African captive elephants have produced offspring (Asian Elephant Studbook, 2000, 2001). In an effort to increase reproductive rates, the Elephant Taxon Advisory Group/Species Survival Plan instituted a reproductive endocrine monitoring program for captive elephants to learn more about their reproductive biology and to help identify viable breeding candidates. One of the findings of this effort is that many female elephants of reproductive age are not cycling normally (Brown, 2000). Termed "flatliners", these females exhibit ovarian inactivity, as evidenced by consistently stable, baseline serum progestin concentrations (Brown, 2000). A recent survey reported that up to 14% of Asian and 29% of African elephants in North America are not cycling normally (Brown *et al*., 2004a). The cause of this acyclicity is unknown, but may be related to a variety of reproductive problems such as reproductive tract pathologies, stress, hypothalamic-pituitary disruptions, hormone receptor dysfunctions, and neoplasias (Knobil and Neill, 1998). The probability that the etiology of acyclicity is the same for all females is unlikely; however, acyclic females may exhibit common endocrinological indices that could be used for diagnosing reproductive pathologies. Thus, it is important to elucidate what factors are related to reproductive dysfunction in elephants so that mitigating actions can be taken to prevent 'captive extinction.'

Inhibin is primarily produced in the granulosa cell layer of ovarian follicles in mammalian females (Erickson and Hsueh, 1978; Meunier *et al*., 1988a) and production has been shown to vary with the maturation of the follicles (Woodruff *et al*., 1988). Isolation and characterization of inhibin revealed the glycoprotein hormone to be a disulfide-linked heterodimer consisting of an α chain and one of two homologous β chains designated β_A and β_B. Both the α and β-subunit proteins initially exist as larger pro proteins that must undergo proteolytic cleavage in order to yield mature forms. In fact, the α-subunit may be detected in circulation in one of three forms: its monomeric pro-form, the fragment resulting from the cleavage of the pro-form, or bound to some form of the β-subunit. Inhibin is most biologically potent when the two mature forms of the α and β-subunits combine (Vale et al., 1988). Depending upon the combination of the specific β subunit with the α chain, inhibin A (α-β_A) or inhibin B (α-β_B) is formed. Both isoforms of inhibin act to suppress pituitary FSH synthesis and secretion (Ying 1988, De Kretser and Robertson 1989, Vale *et al*. 1990).

Currently, there is a paucity of information on the role of the glycoprotein hormone inhibin in cyclic and acyclic captive female elephants as well as in captive bull elephants. Studying this hormone in captive female elephants could prove to be important in determining causes of ovarian inactivity in these animals. Elevated inhibin concentrations along with ovarian inactivity can be indicative of ovarian tumors in species such as humans and horses (Burger *et al*., 1996; Christman *et al*., 1999). Elevated serum inhibin levels have been observed in women with granulosa cell tumors (GCTs) (Lappohn *et al*., 1989) as well as in mares where inhibin was elevated in approximately 90% of mares with GCTs (Christman et al., 1999). GCTs are hormonally active tumors derived from sex-cord stromal tissue and can be diagnostically detected through measurements of inhibin, testosterone, and progesterone.

In humans, variation in the inhibin form detected in serum exists between specific types of tumors and may even vary within a class of tumors (Robertson et al., 1999). For example, in some GCT's, dimeric inhibins, especially inhibin B, are the primary inhibin forms detected in serum, while in mucinous carcinomas the free inhibin α-subunit is primarily detected (Robertson et al., 1999). Thus, it is important to measure all inhibin α-subunit forms (inhibins A and B, and the free α-subunit) in order to detect all cancer types (Robertson *et al*., 1999)

Previously, serum concentrations of immunoreactive inhibin α-subunit were quantified throughout the estrous cycle of nine cycling Asian female elephants using antisera generated against a synthetic portion of the porcine inhibin-α-peptide (Brown et al., 1999). Serum concentrations of inhibin α-subunit were found to be negatively correlated with FSH during the non-luteal phase of the ovulatory cycle as would be expected due to inhibin's negative feedback regulation of FSH (Brown et al., 1999). The lack of specificity of this assay system to just intact dimeric inhibin prevented a definitive conclusive relationship between FSH and inhibin production in elephants. However, the subsequent advent and commercial availability of dimeric two-site ELISAs that allow for the measurement of inhibin A and inhibin B (Groome and O'Brien, 1993) may allow a definitive relationship to be established between serum FSH and inhibin concentrations in elephants.

Previous studies also found significant differences ($P<0.05$) in mean FSH levels between cycling and non-cycling Asian and African elephants, with non-cycling elephants continuously having FSH concentrations equivalent to the lowest levels produced by cycling elephants (Brown *et al*., 2004a). The depressed secretion of pituitary FSH in acyclic elephants may indicate the presence of elevated plasma concentrations of inhibin which could result from ovarian tumors.

Therefore, to further elucidate the role of inhibin in elephant reproductive biology the primary goals of this research were to determine if serum immunoreactive inhibin α-subunit concentrations were elevated in acyclic versus cyclic elephants and to determine if the commercially available two-site sandwich ELISAs for inhibin A and B could be utilized successfully to determine inhibin concentrations in elephant serum.

MATERIALS AND METHODS

Serum Sample Bank

Serum samples employed in this study were obtained from the elephant serum bank maintained by Dr. J.L. Brown (Conservation & Research Center (CRC), Front Royal, VA). These samples were collected, processed, and stored according to standard protocols followed by the CRC endocrine laboratory and the American Zoological Association (AZA). Intermittent samples were obtained from three cycling female African elephants for ~ 32-44 weeks, two cycling female Asian elephants for ~ 12-16 weeks, four acyclic African female elephants for ~ 3-6 weeks, three intact Asian bull elephants for ~ 4-5 years, one intact African bull elephant for ~ 17 weeks, and two castrated African bull elephants for ~ 5 weeks. The samples selected all had complete records regarding the care, maintenance, personal histories, and reproductive status of the donor elephants. In addition, the serum LH profiles for the cycling females utilized in this research were already determined by an RIA procedure previously reported (Brown *et al*., 1999).

Radioimmunoassay for Inhibin

An overnight double antibody RIA, employing rabbit antiserum raised to bovine 31 kDa inhibin (Pool P, Monash Institute, Australia) (McLachlan *et al*., 1986; Robertson *et al*., 1989; de Kretser and Robertson, 1989), highly purified bovine 31 kDa inhibin isolated from bovine follicular fluid (#1989, Monash Institute, Australia) (de Kretser and Robertson, 1989) for tracer, and equine granulosa cell tumor follicular fluid (courtesy of C.J. Munro, Clinical Endocrinology Lab, University of California, Davis, CA) for standards was utilized to measure immunoreactive inhibin α-subunit concentrations in the selected samples. Iodination and RIA procedures for the bovine inhibin were performed as described previously by Roser *et al*., (1994). Each serum sample was assayed in duplicate and the average intra-asay coefficient of variation was less than 10%.

Two-Site Dimeric Inhibin A and Inhibin B ELISAs

A two-site dimeric inhibin A 96-well plate ELISA kit (DSL-10-28100) and a two-site dimeric inhibin B 96-well plate ELISA kit (DSL-10-84100) (Diagnostic Systems Laboratories Inc., Webster, Texas) were utilized to measure dimeric inhibin A and B respectively, in selected elephant serum samples. Procedures for the dimeric ELISAs were performed following the manufacturer's protocols.

Statistics

Data were subjected to ANOVA using the General Linear Model procedure. All statistical procedures were done with the Minitab Statistical Software package (Release 13, State College, PA). Differences were considered significant when p-values were <0.05.

RESULTS

Detection of Inhibin α-subunit in Serum from Cyclic and Acyclic Elephants

A serum immunoreactive inhibin α-subunit profile was established in three cyclic African elephants throughout the course of three consecutive ovulatory cycles (32-44 weeks) (Figures 2.1-2.3) and in two cyclic Asian elephants (Figure 2.4) throughout the course of one ovulatory cycle (12-16 weeks). A similar pattern of immunoreactive inhibin α-subunit production was observed in all of the cycling elephants. The highest serum inhibin α-subunit concentrations were detected immediately after the ovLH surge and then declined during the luteal phase of the estrous cycle with levels rising again immediately after the anLH surge. Peak levels and basal levels of serum inhibin α-subunit from three cyclic African elephants over the course of three consecutive ovulatory cycles were compared, and peak levels of serum inhibin α-subunit (4.51 ± 1.36) were significantly greater ($P < 0.05$) than basal serum inhibin levels (0.457 ± 0.108). Immunoreactive inhibin α-subunit was also detected in serum samples from four acyclic African elephants over the course of ~3-9 weeks (Figure 2.5). There was no significant difference between basal levels of inhibin in cycling African elephants and the consistently low levels of inhibin produced by acyclic African females (Figure 2.6). Peak serum inhibin concentrations in African cycling elephants were, however, significantly greater than serum inhibin concentrations measured in acyclic African elephants (Figure 2.7).

Castrate vs. Intact Bull Elephants

Immunoreactive inhibin α-subunit was measured in the serum of two castrated African bull elephants, three intact Asian bull elephants, and one intact African bull elephant (Figure 2.8). The data for one of the intact Asian bull elephants (Pachy) shown in figure 2.8 was not included in statistical analyses due to his history as a failed breeder with an undiagnosed reproductive dysfunction. All other bulls were included in statistical analyses. Intact bull elephants had significantly higher levels of serum inhibin than castrated elephants (Figure 2.9).

Two-Site Dimeric Inhibin A and Inhibin B ELISAs

Elephant serum samples had no cross-reactivity with either the inhibin A or inhibin B dimeric ELISAs.

DISCUSSION

The premise of the present study was based on previous work in which the detection of elevated plasma inhibin concentrations along with ovarian inactivity in humans (Burger *et al*., 1996) and horses (Christman *et al*., 1999) was indicative of ovarian granulosa cell tumors (GCTs). Although high plasma immunoreactive inhibin concentrations were succesfully utilized as a potential indicator and as a biomarker for granulosa cell tumors in the ovaries of humans (Lappohn *et al*., 1989; Burger *et al*., 1996) and mares (Christman *et al*., 1999), concentrations of immunoreactive inhibin α-subunit in the serum of acyclic female elephants was not elevated as compared to cyclic females. In fact, the consistently low serum concentrations of immunoreactive inhibin α-subunit detected in acyclic elephants were not significantly different from the basal levels of inhibin detected in serum from cycling elephants during the middle portion of the luteal phase.

Cycling elephants actually had significantly greater amounts of serum immunoreactive inhibin α-subunit than acyclic elephants when their peak immunoreactive inhibin α-subunit concentrations were compared to the serum concentrations detected in acyclic elephants. Thus, serum immunoreactive inhibin α-subunit concentrations were not indicative of GCTs in the acyclic elephants examined. In fact, the results indicate that baseline serum inhibin α-subunit concentrations could be used clinically to identify acyclic elephants and that the low levels of FSH previously reported in acyclic elephants (Brown *et al*., 1999) is not due to elevated serum inhibin concentrations depressing pituitary production of FSH. The lack of substantial inhibin α-subunit serum concentrations indicate that follicular development is not proceeding since the granulosa cells of developing preovulatory follicles are the primary source of circulating inhibin α-subunit (Rokukawa *et al*., 1986; Merchenthaler *et al*., 1987; Meunire *et al*., 1988; Schwall *et al*., 1990; Engelhardt *et al*., 1993; Otsuka *et al*., 1997; Nagamine *et al*., 1998).

A consistent pattern of serum immunoreactive inhibin α-subunit was detected in all cycling elephants examined. Specifically, serum inhibin α-subunit concentrations were highest immediately after the ovLH surge and then declined during the luteal phase of the estrous cycle with levels rising again immediately after the anLH surge. This pattern may be the cause of the unique pattern of FSH secretion that occurs in the female elephant (Brown *et al*., 1991; Brown *et al.*, 1999). Unlike other mammals, FSH concentrations peak at the end of the luteal phase which coincided with the lowest serum immunoreactive inhibin α-subunit levels detected in the current research. After peaking, serum FSH concentrations decrease progressively during the non-luteal phase which coincided with the progressive increase in immunoreactive inhibin α-subunit concentrations detected in the present study.

Thus, it seems likely that the detected serum concentration of immunoreactive inhibin α-subunit is strongly correlated with plasma inhibin levels and that inhibin produced by the ovary is actively suppressing FSH production by the anterior pituitary gland. Regretably, the commercially available two-site sandwich ELISAs for inhibin A and B were not successful in measuring inhibin concentrations in elephant serum.

Both the inhibin A and B ELISA assays only detect dimeric inhibin due to their unique two-site sandwich ELISA protocol (Groome *et al*., 1996). Specifically these assays were developed such that an antibody raised against the human inhibin α-subunit is bound to the bottom of microtitre plate wells. The samples are added and free alpha and dimeric inhibins bind to the antibody. The wells are then washed and a second antibody is added, that is raised against the human inhibin β-subunit. This antibody will bind to dimeric inhibin but not the free alpha subunit which are bound to the plate by the first antibody. If the antibody raised against the α-subunit for the dimeric assay lacks epitopic specificity with the α-subunit in the serum of the species being assayed, no cross-reactivity will occur. While, the β-subunit is highly conserved among a variety of species including: humans, cows, rats, and sheep (Vale *et al*., 1990), the α-subunit is not as highly conserved among species (Ying, 1988) and it appears that the anti-human inhibin α-subunit antibody in the assay does not react with elephant inhibin α-subunit. Nevertheless, because no cDNA clones exist for the inhibin subunits in elephants, a definitive analysis of the inhibin α-subunit cDNA sequences between human and elephant inhibin cDNA sequences cannot be made. The antibody produced against native bovine 31kD inhibin in the Monash assay is compatible with the elephant inhibin α-subunit, as was observed in the RIA conducted in the present study.

This study was the first to examine production of immunoreactive inhibin α-subunit in castrated and intact bull elephants. Although it was not surprising that intact bulls produced signficantly greater concentrations of immunoreactive inhibin α-subunit as compared to castrated bulls, it was interesting to note the differences in production levels that existed between intact males. The serum levels of inhibin α-subunit in an intact African elephant in musth was 7.13 ng/mL and was only 5.00 when this bull was not in musth.

In addition, "Pachy," (Figure 2.8) an intact Asian bull elephant examined in this study had immunoreactive inhibin α-subunit levels equivalent to the two castrated bulls in this study. Interestingly, "Pachy" was previously determined to be infertile which may suggest that a strong correlation exists between low concentrations of immunoreactive inhibin α-subunit levels and infertility in bull elephants. This relationship has been well-defined and confirmed in humans where inhibin concentration is higher in men with apparently normal fertility than in those with infertility and abnormal spermatogenesis (Anwalt *et al*., 1996; Illingworth *et al*., 1996; Jensen *et al*., 1997). In fact, serum Inhibin B is the direct serum marker of spermatogenesis in men with testicular disorders and there is 100% predictive power of detecting abnormally low sperm counts among men with significantly decreased concentrations of serum inhibin B and FSH (Anwalt *et al*., 1996; Illingworth *et al*., 1996; Jensen *et al*., 1997). Thus, serum concentrations of immunoreactive inhibin α-subunit may be a useful diagnostic tool to predict fertility potential in bull elephants as it is in other species. In summary, the current results indicate that inhibin may play a critical role in elephant reproduction. Futhermore, the results indicate that serum inhibin concentrations could have a strong diagnostic value in assisting reproduction success in captive elephants.

In order to maximize the use of serum inhibin concentration as a diagnostic tool in female and male elephants and to determine the specific biological roles of inhibin and the closely related hormone activin in elephant reproduction, it is necessary to clone the inhibin α- and β-subunits in elephants.

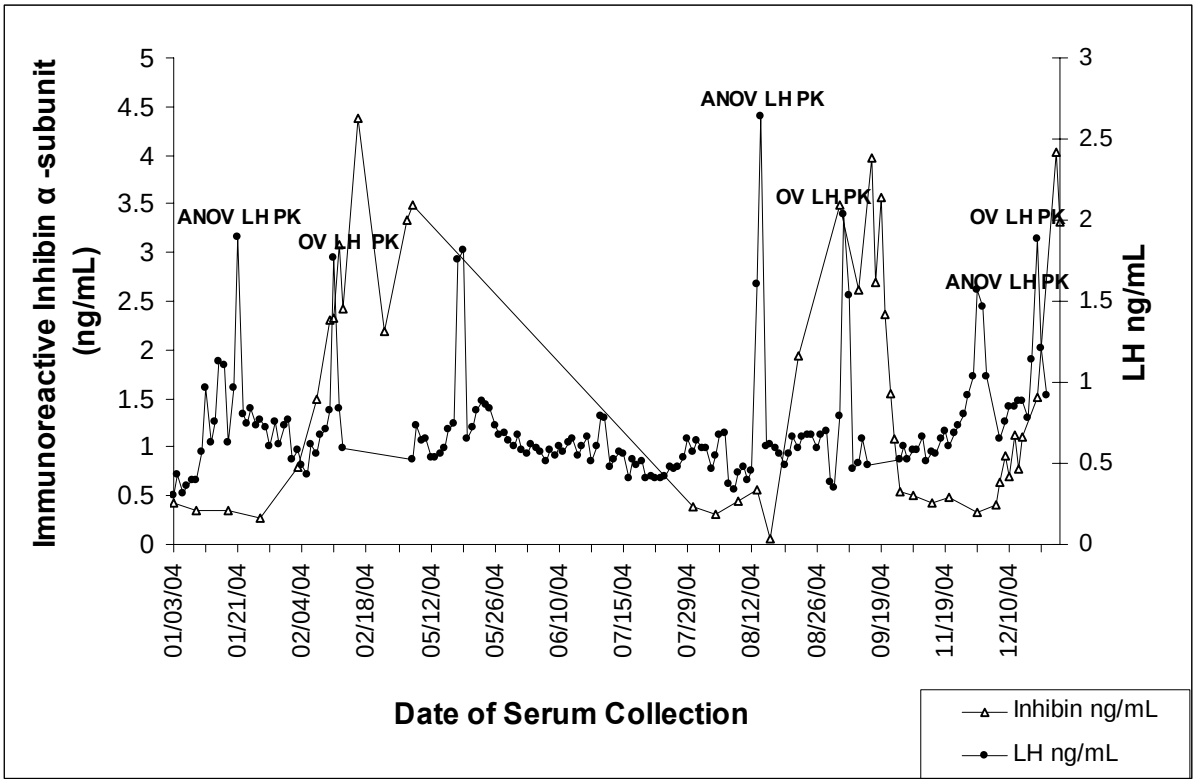


Figure 2.1. Serum immunoreactive inhibin α-subunit and LH concentration profiles for a cycling African female elephant (Dolly) during the course of three ovulatory cycles (~ 44 weeks). ANOV LH PK denotes the first LH peak which is anovulatory and OV LH PK denotes the second LH peak during which ovulation occurs.

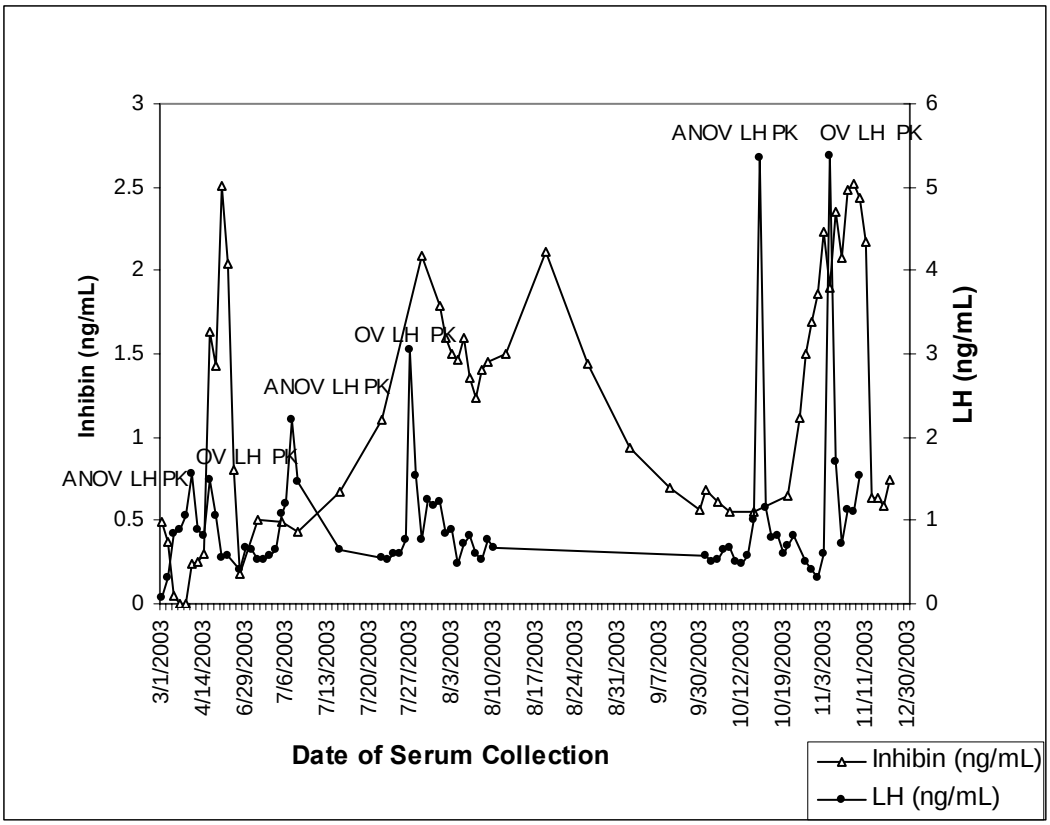


Figure 2.2. Serum immunoreactive inhibin α-subunit and LH concentration profiles for a cycling African female elephant (Timba) during the course of three ovulatory cycles (~ 36 weeks).

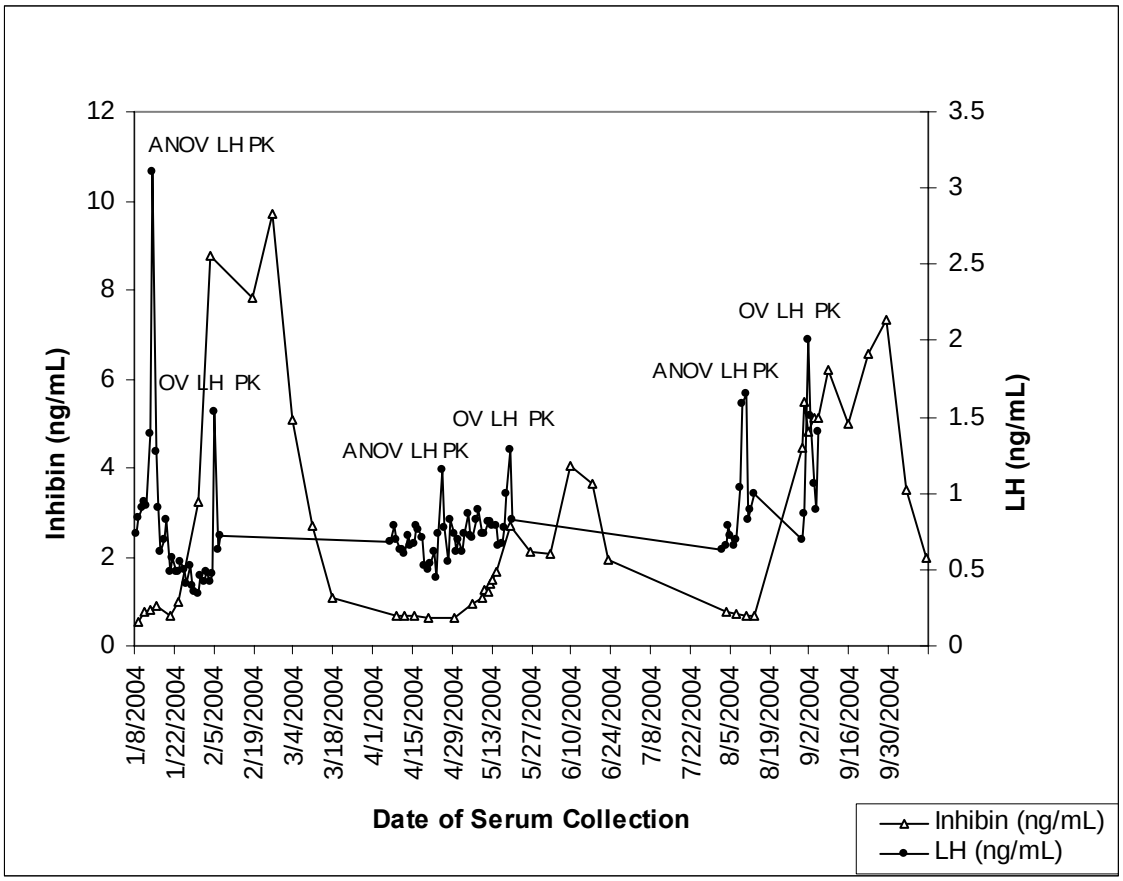
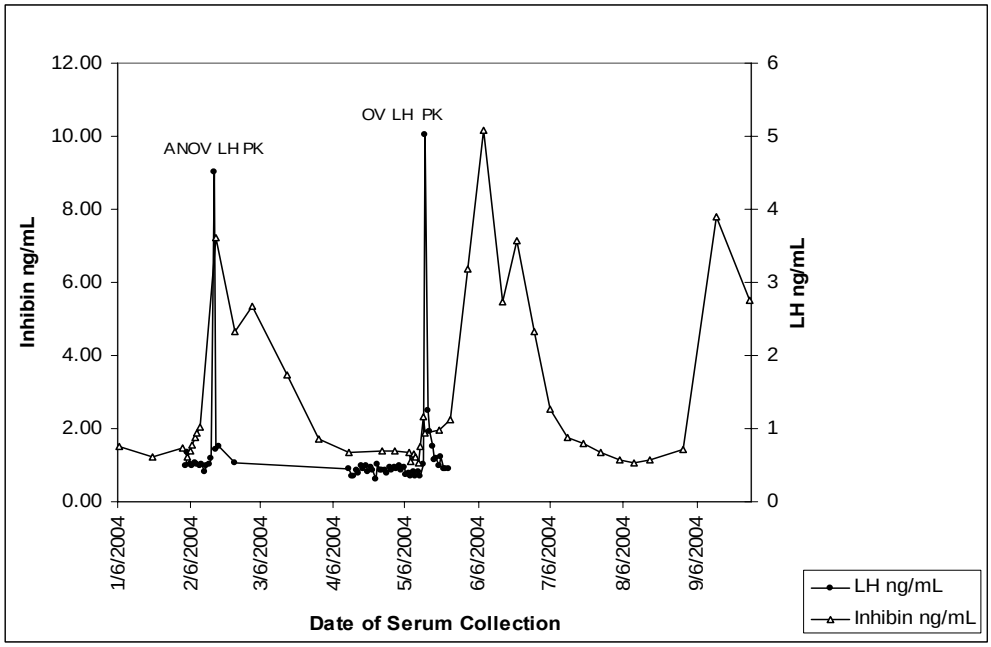


Figure 2.3. Serum immunoreactive inhibin α-subunit and LH concentration profiles for a cycling African female elephant (Kiba) during the course of three ovulatory cycles (~ 32 weeks).

A.



B.

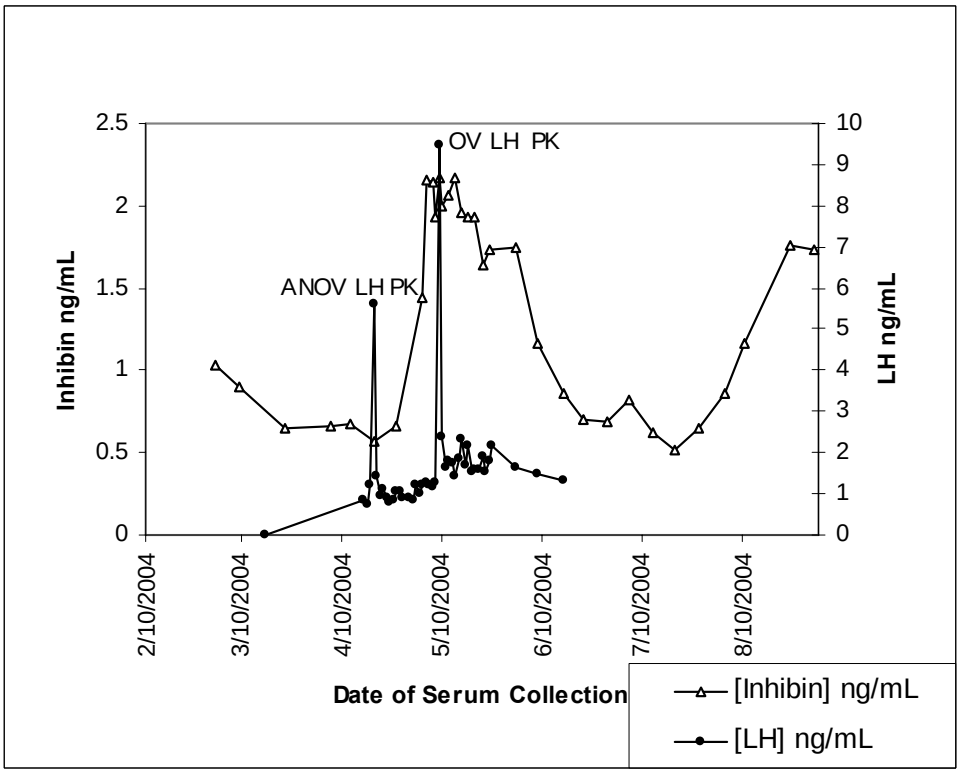
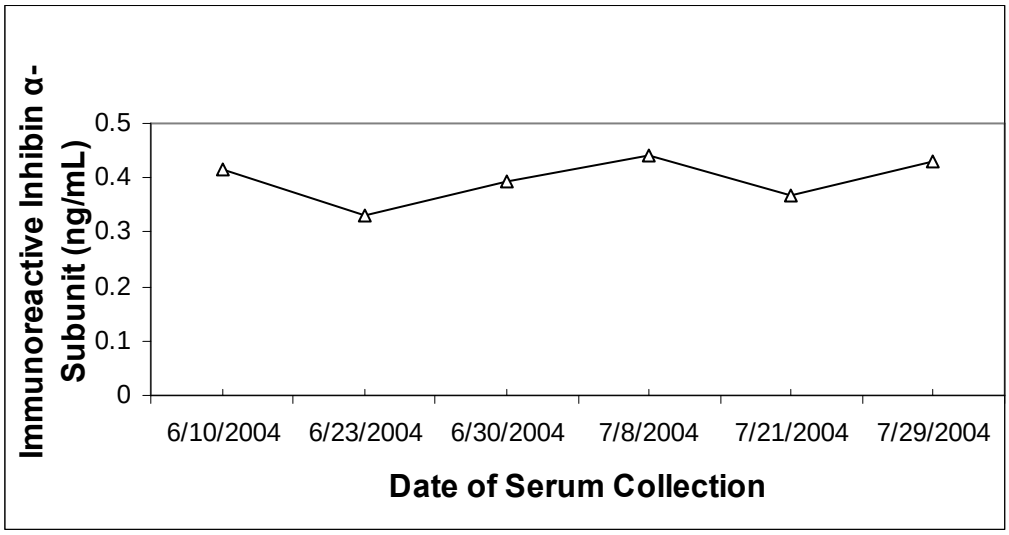


Figure 2.4. Serum immunoreactive inhibin α-subunit and LH concentration profiles for two cycling Asian female elephants A- Vaigai, B-Mari) during the course of one ovulatory cycle (~12-16 weeks)

A.



B.

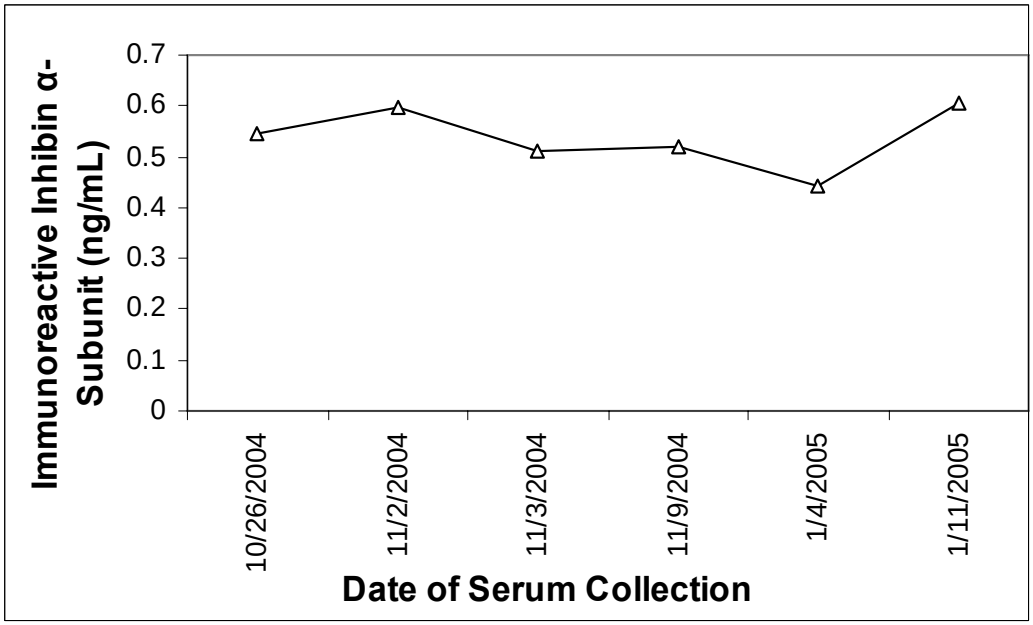


Figure 2.5. Serum immunoreactive inhibin α-subunit concentration profiles for two representative acyclic African female elephants (A-Monica, B-#885040).

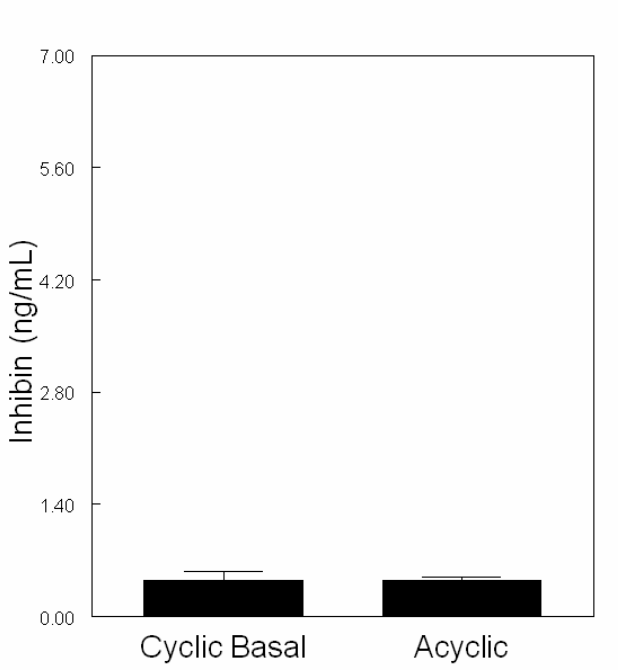


Figure 2.6. The serum concentration (mean ± SEM) of immunoreactive inhibin α-subunit for three cycling African elephants at the nadir of their plasma inhibin α-subunit cycle and for four acyclic African elephants.

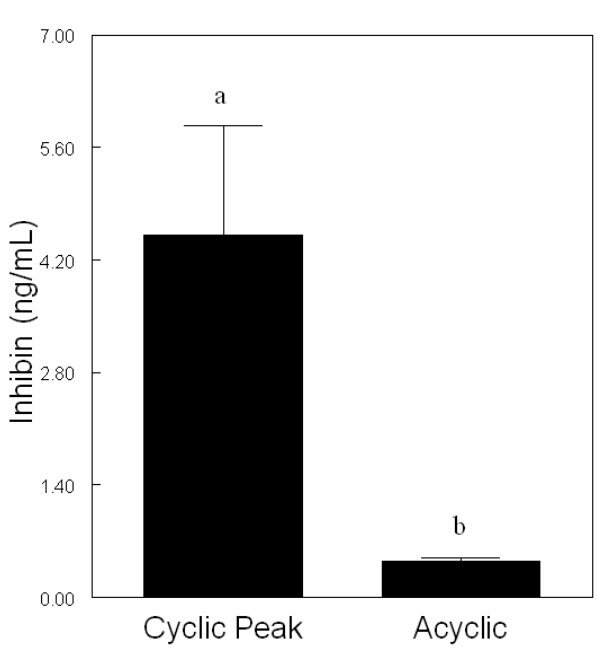


Figure 2.7. The serum concentration (mean ± SEM) of immunoreactive inhibin α-subunit for three cycling African elephants at the peak of their plasma inhibin α-subunit cycle and four acyclic African elephants. Values with different letters differ, $P < 0.05$.

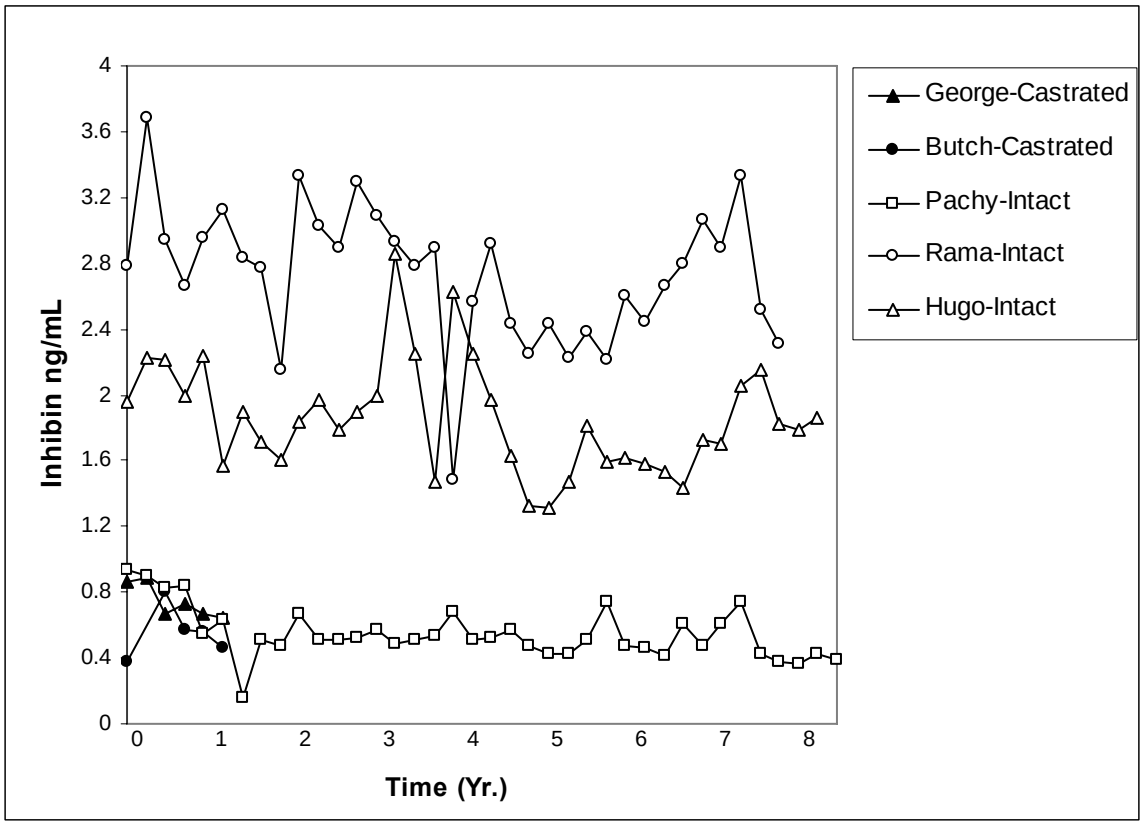


Figure 2.8. Serum immunoreactive inhibin α-subunit profiles for three intact Asian bull elephants and two castrated African bull elephants. Note that although Pachy is an intact male he is infertile.

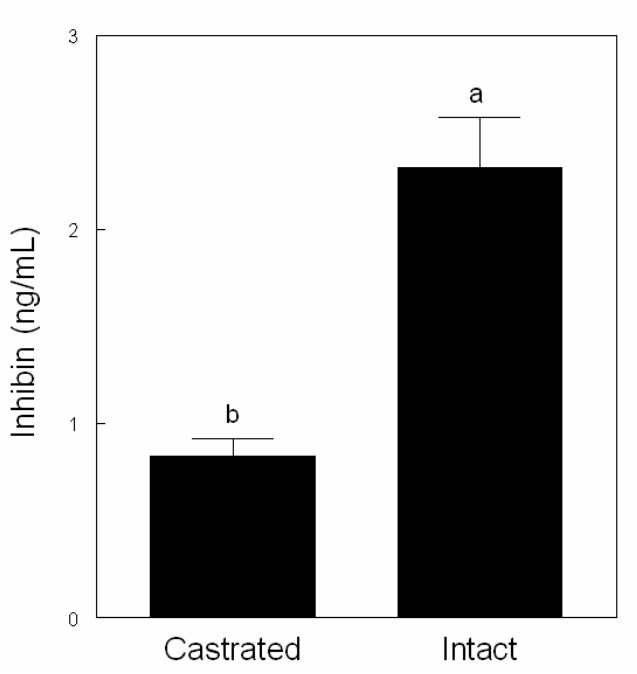


Figure 2.9. The serum concentration (mean ± SEM) of immunoreactive inhibin α-subunit for two castrated sexually mature African elephants and for three intact sexually mature elephants (two Asian and one African). Values with different letters differ, $P < 0.05$.

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CHAPTER 7

EXPRESSION AND HORMONAL REGULATION OF THE ACTIVIN TYPE IA AND IB RECEPTORS DURING FOLLICULAR DEVELOPMENT IN BROILER BREEDER HENS

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To be submitted to *Biology of Reproduction*

ABSTRACT

The expression of mRNA for the activin type I receptors (ActRIA and ActRIB) was determined in the individual granulosa and theca layers of the F1 through F4 follicles and in the granulosa cells from the small yellow follicles and the large white follicles from 6 broiler breeder hens. The isolated theca and granulosa layers were combined from 2 birds to create 3 replicate granulosa and theca samples for each follicle size. Additionally, gonadotropin (LH and FSH) and steroid hormone (estrogen and testosterone) regulation of both ActRIA and ActRIB mRNA expression was investigated in cultured chicken granulosa cells, which were isolated from the F1, F3, or small yellow follicles (SYF) from three broiler breeder hens for each replicate experiment. Isolated and dispersed granulosa cells from each follicular size were cultured in the absence or presence of 50 ng/mL of LH or FSH (4 replicate experiments), or in the absence or presence of 1×10^{-6} M testosterone or 17 β-estradiol (4 replicate experiments). For all experiments, cell cultures were terminated 24 hours after plating. Total RNA was extracted from all samples for subsequent real-time RT-PCR analyses of these receptors with GAPDH serving as the endogenous control. Relative expression of both ActRIA and ActRIB was significantly greater in the theca than in the granulosa of the hierarchical follicles. In granulosa cells from hierarchical follicles, the gonadotropins inhibit the mRNA expression of ActRIA and ActRIB. Estrogen had no effect on the mRNA expression of ActRIA and ActRIB. The addition of testosterone to the granulosa cell cultures only decreased the mRNA expression of ActRIB in F1 granulosa cells. Previously, our laboratory reported that ActRIIA mRNA expression could be detected by Northern blot analysis in both the theca and granulosa layers of a follicle of any size.

Therefore, when the current data is combined with our previous findings, it suggests that ActRIA acts as the predominant type I receptor to form a complex with ActRII in developing follicles and that theca tissue of the hierarchical follicles may be more sensitive to local activin effects than granulosa tissue.

Key Words: *activin receptors, broiler breeder hens, theca, granulosa, LH, FSH, estradiol, testosterone*

INTRODUCTION

The glycoprotein hormone activin consists of two similar but distinct β subunits, β_A and β_B, that can combine to form the homodimers activin A (β_A-β_A) and activin B (β_B-β_B), as well as the heterodimer activin AB (β_A–β_B) (Ying 1988, De Kretser and Robertson 1989, Vale *et al*. 1990). Activin stimulates FSH synthesis and secretion from the anterior pituitary (Ying 1988, De Kretser and Robertson 1989, Vale *et al*. 1990). Besides stimulating FSH secretion, activin has been associated with a multitude of diverse functions in reproductive and non-reproductive cell types as reviewed by Mather *et al*., 1992; Woodruff, 1998; Woodruff, 2002; Chapman *et al*., 2003. The biological actions of activin are antagonized by inhibin, a closely related dimeric glycoprotein that consists of the inhibin-specific α-subunit combined with one of the activin β-subunits (Lewis *et al*., 2000; Chapman *et al*., 2002).

Activin's cell surface receptor is composed of a complex of two single membrane spanning serine-threonine kinases, type I and type II (ActRI and ActRII, respectively). The known type I receptors include ActRIA and ActRIB, while the type II receptors include ActRIIA and ActRIIB (Woodruff, 1998). Additionally, the ActRIIB gene undergoes alternative splicing which results in at least four isoforms of this receptor in the mouse (Attisano *et al*., 1992). The cooperative manner in which the activin receptors interact to bind activin and elicit activin's downstream intracellular signal is now fairly well established. In general, activin binds to a type II receptor (ActRIIA or ActRIIB) which then recruits a type I receptor (ActRIA or ActRIB) to form an activin receptor complex.

The activin type II receptor then transphosphorylates a glycine-serine rich domain in the type I receptor and then the type I receptor phosphorylates downstream intracellular MAD-related (mothers against decapentaplegic) proteins (SMAD) which act as mediators of transcriptional activation for members of the TGF-beta superfamily (Kaivo-oja *et al*., 2006).

Both the ActRIIA and ActRIIB receptors have been cloned in the chicken (Ohuchi *et al*., 1992; Nohno *et al*., 1993; Stern *et al*., 1995). The mRNA for the type II receptors has been detected by in situ hybridization in embryonic chicken spinal cord (Ohuchi *et al*., 1992) as well as in ciliary (Kos and Coulombe, 1997) and dorsal root ganglion neurons (Kos *et al*., 2001), while the expression of ActRIA protein has been detected in chick embryo atrioventricular endocardial cells (Lai *et al*., 2000).

Slappey and Davis (2003) examined the expression of the activin type II receptors in granulosa and theca samples from the follicles of broiler breeder hens by Northern analysis. They detected ActRIIA mRNA in granulosa and theca samples but were unable to detect ActRIIB by Northern analysis (Slappey and Davis, 2003). Through the use of quantitative real-time polymerase chain reaction (PCR), Lovell *et al*. (2006) detected and quantified the expression of ActRIA, ActRIIA, and ActRIIB in granulosa and thecal layers of all the hierarchical preovulatory and prehierarchical follicles of the laying hen. Expression of the mRNA for all three receptor types was detected in all the theca and granulosa samples examined. They suggested that their co-localization supported a local autocrine/paracrine role of activins in modulating ovarian follicle development in the domestic fowl.

To futher establish the potential for activin to have significant autocrine and paracrine roles within the hen ovary, the present research expands on previous work by examining the expression of ActRIB in the theca and granulosa tissues of the follicular hierarchy of the broiler breeder hen. In addition, since follicular development is regulated in a large part by pituitary gonadotropins and ovarian steroids, the regulation of the mRNA expression of ActRIA and ActRIB by LH, FSH, estradiol, and testosterone will be investigated in granulosa cells isolated and cultured form the F1, F3, and small yellow follicles.

MATERIALS AND METHODS

Animals

Ovarian tissue was obtained from 50- to 62-week old, individually caged Cobb slow-feathering broiler breeder hens. The hens were maintained on 15 hours of light, were fed according to commercial broiler breeder guidelines, and had free access to water. Hens were killed by cervical dislocation 2-4 hours prior to ovulation based on daily egg records and palpating a hard shell egg in the egg shell gland. The Instructional Animal Care and Use Committee of the University of Georgia approved all animal procedures.

Experiment 1: Follicular mRNA Expression of ActRIA and ActRIB

The four largest follicles (F1-F4), (SYF) (>5-10mm diameter), and large white follicles (LWF) (>2-5 mm diameter) were removed from six individual birds and placed in ice-cold Krebs solution. The granulosa cell layer was manually separated from the theca cell layers of each hierarchical follicle (Huang and Nalbondov, 1979), and the theca and granulosa cells were enzymatically separated in the LWF and SYF as previously described (Davis *et al.* 2000).

The granulosa and theca samples from two individual birds were pooled to generate three replicate samples of theca and granulosa tissue for the F1-F4 follicles and granulosa samples for the SY and LW follicles. The theca tissue from the SYF and LWF was not utilized because of the potential that it contained residual granulosa cells. All isolated granulosa cell and theca cell samples were frozen and stored at -80 ˚C in 1 mL of guanidine isothiocyanate solution for subsequent RNA extraction.

Cell Culture

The F_1, F_3 and SYF were removed from three hens for each replicate cell culture experiment. Granulosa cells were isolated from each of the follicles as previously described (Davis *et al.* 2000) and subsequently cultured in 6-well tissue culture plates as previously described (Davis *et al.* 2000) except that the lipoprotein supplement was not added to the M199 culture media. Cell number and viability were estimated using a hemocytometer with trypan blue exclusion. Cell viability was greater than 95%.

Experiment 2: Gonadotropin Regulation of ActRIA and ActRIB

Ovine LH (Lot AFP8468A) or human recombinant FSH (Lot AFP5551B) (both generously provided by Dr. A. F. Parlow of the National Hormone and Peptide Program, Torrance, CA) were added at doses of 0 or 50 ng/ml of culture media. The cultures were terminated 24 h after the cells were plated. After termination, the medium was saved for RIA and the granulosa cells were collected in solution D (Chomczynski and Sacchi, 1987) and frozen at -80 ˚C for future RNA extraction. Each treatment consisted of three wells and the cells were combined from these three wells prior to RNA extraction. This experiment was replicated four times.

Experiment 3: Steroid Regulation of ActRIA and ActRIB

The same protocol that was used in examining gonadotropin regulation of ActRIA and ActRIB was followed to examine steroid regulation of ActRIA and ActRIB, except the isolated and dispersed granulosa cells were cultured in the absence or presence of 1 x 10^{-6} testosterone (Steraloids, Newport, RI) or 17 β-estradiol (Sigma, St. Louis, MO).

Primer/Probe Design

Primers and TaqMan probes specific for chicken AcRtIA, ActRIB, and GAPDH (Table 3) were generated using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA). Primers for reverse transcriptase polymerase chain reaction were designed based on reported chicken cDNA sequences for ActRIA (GenBank accession # U38622), ActIB (Slappey, 2001), and GAPDH (GenBank accession # M11213). The chicken GAPDH assay was the only assay designed to span an exon / exon junction. Each probe was labeled at the 5' end with FAM (6-carboxyfluorescein) as the reporter dye and at the 3' end with TAMRA (6-carboxy-*N,N,N'*,*N'*-tetramethylrhodamine) as the quencher dye. Primer and probe sets were validated for real-time PCR by determining the optimal amplification efficiency and primer/probe concentrations as described by the manufacturer (Applied Biosystems).

RNA Extraction and Two-Step Real-Time Polymerase Chain Reaction

Total RNA was extracted from the granulosa and theca samples using a guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). A TURBO-DNA-*free* kit (Ambion, Austin, TX) was used following the manufacturer's protocol to remove any genomic DNA contamination in each RNA sample. Reverse-transcription cDNA synthesis reactions were performed using the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol.

For two-step real-time PCR, 100 ng of cDNA was used for each sample. The reactions were preformed in a 25 µl volume of reaction buffer containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems) and 900 nM of either ActRIA, ActRIB, or GAPDH primer pairs and 25 nM of the appropriate probe. The reactions were completed in an ABI 7500 Thermocycler (Applied Biosystems). The thermocycler conditions were 10 minutes at 95 ˚C and 40 cycles each of 15 seconds at 95 ˚C and 1 minute at 60 ˚C. The C_T (the cycle number at which the fluorescence exceeds the threshold level) was determined for each reaction (run in duplicate) using the Sequence Detection software (version 1.2.2, Applied Biosystems), and quantification was completed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Briefly, the ActRIA and ActRIB C_Ts were determined for each sample, then normalized to the GAPDH C_T from the same sample (GAPDH C_T subtracted from the ActRI protein C_T yields the ΔC_T). These values were then compared to control levels using the $2^{-\Delta\Delta C_T}$ method and data was expressed as the fold-difference relative to sample with the highest expression.

Progesterone Radioimmunoassay (RIA)

Media progesterone concentrations were determined by radioimmunoassay (RIA) for experiments 2 and 3. The progesterone RIA was completed with a Coat-A-Coat Progesterone kit (Diagnostic Products Corporation, Los Angeles, CA) following the manufacturer's protocol.

Statistics

Data from each experiment were subjected to ANOVA according to the General Linear Model (GLM) procedure. Tukey's multiple comparison procedure (Neter *et al.* 1990) was used to detect significant differences between samples. Differences were considered significant when P values were less than 0.05. All statistical procedures were completed with the Minitab statistical software package (Release 13, State College, PA).

RESULTS

ActRIA and ActRIB mRNA Expression in Broiler Breeder Hen Follicles

The mRNA expression for both ActRIA and ActRIB was detected by real time PCR in the theca and granulosa tissues of the F1-F4 follicles as well as in the granulosa tissue of the SY and LW follilcles (Figure 3.1). ActRIA relative mRNA expression was significantly greater ($P<0.05$) in the theca tissue (0.863 ± 0.080) than in the granulosa tissue (0.141 ± 0.019) of the F1-F4 hierarchical follicles. Similarly, ActRIB mRNA expression was significantly greater in the theca tissue (0.762 ± 0.099) than in the granulosa tissue (0.332 ± 0.075) of the F1-F4 hierarchical follicles. The ΔC_T values of ActRIA and ActRIB for each sample were also compared and ActRIA expression was 6.24, 2.08, and 5.87 fold greater than ActRIB in F1-F4 theca, F1-F4 granulosa, and SYF and LWF granulosa, respectively.

The Effect of LH and FSH on ActRIA and ActRIB mRNA Expression

The mRNA expression of ActRIA in untreated granulosa cells from both the F1 and F3 follicles following 24 hours of culture in M199 was significantly increased, but was significantly decreased in granulosa cells for the SY follicles relative to ActRIA mRNA expression in freshly isolated granulosa cells from these follicles (Figure 3.2). Addition of LH or FSH to granulosa cell cultures from the F1 and F3 follicles significantly decreased the mRNA expression of ActRIA in comparison to the untreated control cultures. However, no change in the mRNA expression of ActRIA was detected in granulosa cells from the SYF cultured with LH or FSH. The mRNA expression of ActRIB in untreated granulosa cells following 24 hours of culture in M199 was significantly reduced in both the F1 and SY follicles relative to ActRIB mRNA expression in freshly isolated granulosa cells from these follicles (Figure 3.3).

Addition of LH to F1 granulosa cells resulted in a significant decrease in ActRIB mRNA expression whereas the addition of FSH to these cells did not result in any change in ActRIB mRNA expression relative to untreated F1 granulosa cells (Figure 3.3). Both LH and FSH reduced ActRIB mRNA expression in cultured granulosa cells from the F3 follicles. Finally, the addition of LH or FSH to granulosa cells from the SYF did not cause any change in ActRIB mRNA expression (Figure 3.3).

Progesterone accumulation in the media of the granulosa cells cultured with LH was higher than the progesterone accumulation in the untreated cell cultures for all follicle sizes except the SYF (Table 4). The addition of FSH to the granulosa cell culture media significantly increased the accumulation of progesterone for the granulosa cells from all follicle sizes (Table 4).

The Effect of Testosterone and 17-β Estradiol on ActIA and ActIB mRNA Expression

The mRNA expression of ActRIA in untreated granulosa cells from F1, F3, and SY follicles following 24 hours of culture in M199 was significantly greater than ActRIA mRNA expression in freshly isolated granulosa cells from these follicles (Figure 3.4). Addition of 17-β estradiol to the F1, F3, and SYF granulosa cell cultures did not cause any change in ActRIA mRNA expression relative to untreated granulosa cells from these follicles (Figure 3.4). Similarly, addition of testosterone to the SYF of F1 granulosa cells resulted in no change in ActRIA mRNA expression. However, adding testosterone to the granulosa cells from the F1follicle caused a significant decrease in ActRIA mRNA expression relative to untreated F1 granulosa cells (Figure3. 4). The mRNA expression of ActRIB in untreated granulosa cells from the SYF but not from F1 and F3 follicles following 24 hours of culture in M199 was significantly decreased relative to ActRIB mRNA expression in freshly isolated SYF granulosa cells (Figure 3.5).

The addition of 17-β estradiol or testosterone to granulosa cells from the F1, F3, and SY follicles did not alter ActRIB mRNA expression relative to untreated controls (Figure 3.5).

Progesterone accumulation in the media of granulosa cells cultured with testosterone was significantly higher than in control granulosa cell cultures (Table 5). In contrast, the addition of 17-β estradiol to the cultured granulosa cells had no effect on progesterone production (Table 5).

DISCUSSION

The present study is the first to detect and quantify the mRNA expression of ActRIB in the developing follicles of the domestic chicken. ActRIB mRNA expression was lower than the mRNA expression of ActRIA in the theca and granulosa cells of the preovulatory follicles of the hen ovary. Previously, Slappey and Davis (2003) and Lovell *et al*. (2006) reported that the mRNA expression of ActRIIA is greater than ActRIIB in the granulosa and theca cells of the hen preovulatory follicles. If protein expression is similar to the mRNA expression then the results suggest that the activin receptor complexes formed in the theca and granulosa cells of the hen ovary will preferentially consist of ActRIIA and ActRIA.

Although the mRNA for ActRIB was detected in both the granulosa and theca cells, expression was significantly higher in the theca tissue of the hierarchical follicles. Similar differences in the mRNA expression of ActRIA between the granulosa and theca tissue were seen in the current research and in previous research (Lovell *et al*., 2006). Lovell *et al*., (2006) also reported that the expression of ActRIIA and ActRIIB were both significantly lower in the granulosa cells than the theca cells of the preovulatory follicles of the hen ovary. Activin-A production is mainly confined to the theca cells of the hierarchical follicles of the hen ovary (Lovell *et al.,* 1998, 2003).

The current research combined with the previous findings of Lovell *et al*. (1998, 2003 and 2006) suggests that while paracrine actions of activin in the granulosa cells are possible, that autocrine actions of activin in the theca cells may predominate.

The regulation of the expression of the mRNA for the activin receptors has not been investigated. Since the gonadotropins along with the ovary-derived steroids estradiol and testosterone play a significant role in regulating ovarian development, it seemed reasonable that these hormones might influence the mRNA expression of ActRIA and ActRIB in granulosa cell cultures. The gonadotropins depresses the mRNA expression of ActRIA and ActRIB in the cultured granulosa cells from the F1 and F3 follicles but had no effect on the expression of these receptors in the granulosa cells from SYF. The reason for a lack of a response in the granulosa cells from the SYF compared to the hierarchical follicles is not clear, but is likely related to initial expression levels of the mRNA for ActRIA and ActRIB. The granulosa cells from the small yellow follicles express the highest level (2 times greater) of ActRIA compared to all other follicles (Lovell *et al*., 2006). ActRIB mRNA expression also tends to be higher in the granulosa cells of nonhierarchical follicles versus hierarchical follicles. In addition, the expression of ActRIA and ActRIB were both significantly lower in untreated granulosa cells after 24 hours of culture than in freshly dispersed granulosa cells from the SYF. This was not the case for the F1 and F3 follicles. Thus, some endocrine factor was missing in the cell culture media that normally supports the level of mRNA expression for the activin type I receptors *in vivo*. It is possible that this factor is either provided by the SYF theca cells or that expression of this factor's receptor is high in the SYF granulosa cells and not in the hierarchical follicles since the mRNA expression of the activin type I receptors was either the same or elevated in untreated granulosa cells cultured for 24 hours compared to freshly dispersed cells.

The decreased expression of ActRIA and ActRIB in the granulosa cells treated with either gonadotropin from the F1 and F3 follicles is interesting especially for ActRIA since it is the more abundantly expressed activin type I receptor in hen granulosa cells. The expression of ActRIA was significantly increased in the untreated granulosa cells from the F1 and F3 follicles cultured for 24 hours compared to freshly dispersed granulosa cells. The addition of FSH or LH returned the expression of ActRIA to the levels observed in freshly isolated cells. Lovell *et al*. (2005) also noted that in the pituitary, ActRIIA mRNA levels peaked roughly 6 hours prior to an expected ovulation of the F1 follicle, but then the level declined over a 100 percent within the next 4 hours. ActRIA mRNA expression in the pituitary had a similar pattern but the decrease was not significant. However, the data strongly suggests that peak levels of LH also potentially regulate activin receptor mRNA expression in vivo as well. Finally, the elevation of ActRIA in cultured granulosa cells and thus a potentially an increased sensitivity to activin, may shed some light on why the expression of the inhibin/activin β_B subunit was induced by the addition of activin-A to granulosa cell cultures from the F1 and F3+F4 follicles (Johnson *et al*., 2006) even though this message (Davis and Johnson, 1998) cannot be detected in these follicles in freshly isolated granulosa cells.

Steroid regulation of the mRNA for the activin type I receptors was less than what was observed for the gonadotropin regulation of the mRNA for these receptors. In fact, 17-β estradiol did not affect mRNA expression of either receptor in the granulosa cells cultured from the F1, F3 or SYF. Testosterone did cause a decrease in ActRIA mRNA expression in the F1 follicle. The addition of testosterone to the granulosa cell cultures did increase the accumulation of progesterone in the cell culture media from all follicle sizes.

This finding agrees with previous studies performed in granulosa cell cultures from the F1 and F3 follicles of laying hens (Phillips *et al*., 1985) and in granulosa cell cultures from the three largest follicles of quail (Sasanami and Mori, 1999). In addition, immunization of laying hens against testosterone (Rangel *et al*., 2005) and treatment of laying hens with a testosterone antagonist (Rangel *et al.*, 2006) blocked ovulation and the preovulatory surges of progesterone. Thus, the current research provides further support to the idea that testosterone plays a vital role in progesterone production in the hen ovary.

In summary, the mRNA expression for ActRIB was detected in the granulosa and theca cells of preovulatory follicles. Theca expression of this message as well as that of ActRIA was greater in theca cells than in granulosa cells. The gonadotropins appear to play a critical role in suppressing the expression of the activin type I receptors in granulosa cells from the large hierarchical follicles, which suggests that the activin-A produced by the theca cells of these follicles may be more important as autocrine rather than a paracrine factor in the large preovulatory follicles of the hen.

Table 3. Sequences for the oligonucleotide primer pairs and probes for real time PCR

Product	Primer	Oligonucleotide Primer and Probe Sequence	Product Size (bp)
ActRIA	Forward	5'-GGC CTC GCA GTC ATG CA-3'	
	Reverse	5'-GGG TTG TTC CCC ACA TCC A-3'	56
	Probe	5'-CCC AAA GCA CGA ACC A-3'	
ActRIB	Forward	5'-AGC CTG GGA TTG CTC ACA GA-3'	
	Reverse	5'-CGT GCC ATT CTT CTT CAC CAA-3'	56
	Probe	5'-ACC TGA AAT CCA AGA ACA-3'	
GAPDH	Forward	5'-TTG GCA TTG TGG AGG GTC TTC -3'	
	Reverse	5'-GGG CCA TCC ACC GTC TTC -3'	70
	Probe	5'-TGA CCA CTG TCC ATG CCA T -3'	

Table 4. Progesterone concentrations in granulosa cell culture media from F_1, F_3 or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 50 ng/ml culture media of LH or FSH.

	Progesterone concentration[1] (ng/ml)
F1	
Control	356 ± 35 [a]
LH	3600 ± 390 [b]
FSH	1800 ± 346 [c]
F3	
Control	134 ± 9 [a]
LH	1900 ± 179 [b]
FSH	1460 ± 154 [c]
SY	
Control	0.168 ± 0.08 [a]
LH	0.959 ± 0.46 [ab]
FSH	1.275 ± 0.54 [b]

[a-c] Values with different superscripts for a given follicle size differ ($p < 0.05$).
[1] Values are means ± SEM, n = 4 replicate experiments.

Table 5. Progesterone concentrations in granulosa cell culture media from F_1, F_3 or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 1 x 10^{-6} M of estrogen or testosterone

	Progesterone concentration[1] (ng/ml)
F1	
Control	335 ± 39 [a]
Estrogen	297 ± 40 [a]
Testosterone	619 ± 68 [b]
F3	
Control	150 ± 16 [a]
Estrogen	137 ± 12 [a]
Testosterone	679 ± 36 [b]
SY	
Control	0.40 ± 0.1 [a]
Estrogen	0.29 ± 0.1 [a]
Testosterone	3.24 ± 0.49 [b]

[a-c] Values with different superscripts for a given follicle size differ ($p < 0.05$).
[1] Values are means ± SEM, n = 4 replicate experiments.

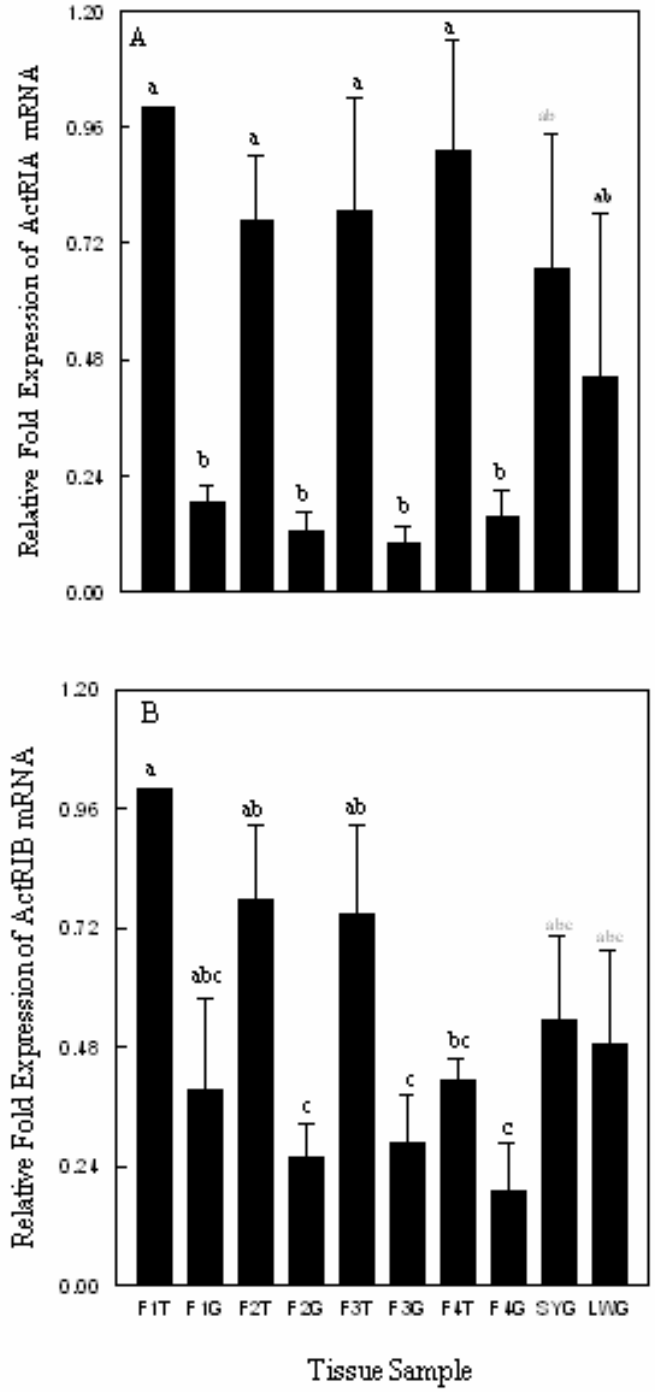

Figure 3.1. The relative expression of ActRIA (A) and ActRIB (B) mRNA as determined by real time PCR using total RNA isolated from the theca and granulosa tissues of the F1-F4 hierarchical follicles and the granulosa tissue of the SY and LW follicles. Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T$ ± SEM, n=3. Means for follicle samples with different letters differ, $p < 0.05$.

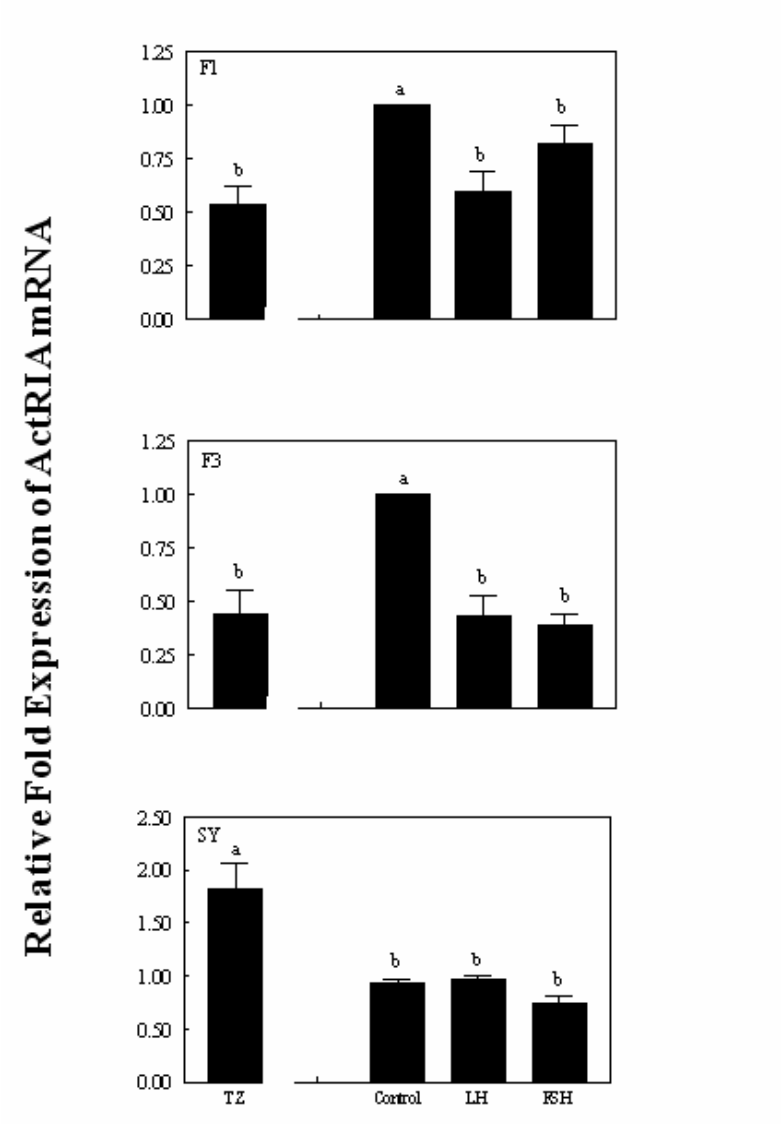

Figure 3.2. The relative fold expression of ActRIA mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/ml culture media of LH or FSH. Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n=4. Means for a follicle size with different letters differ, $p < 0.05$.

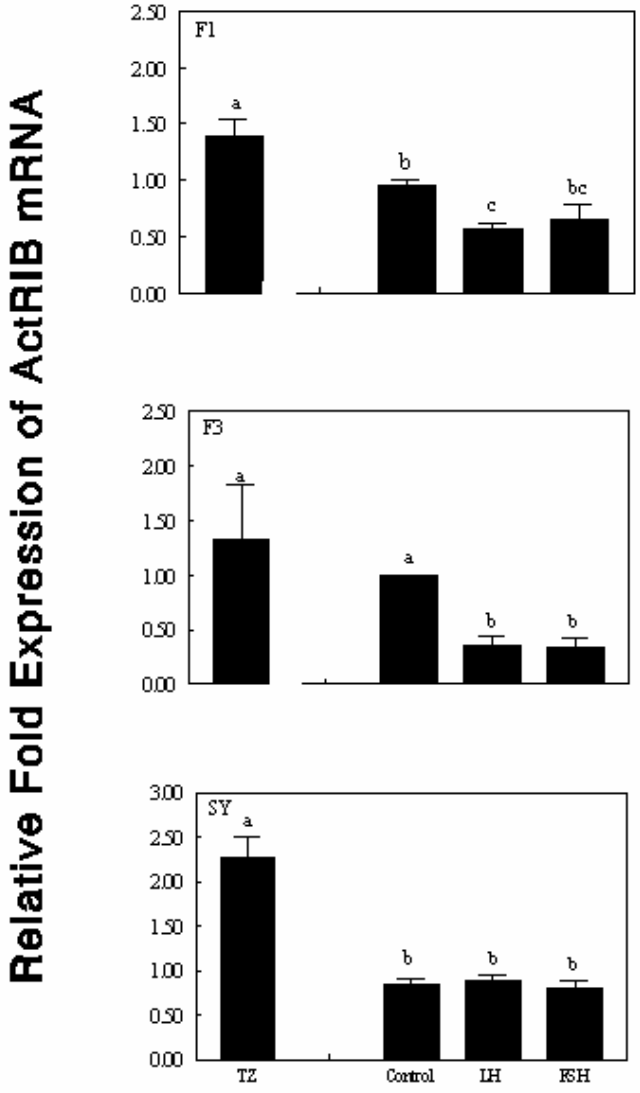

Figure 3.3. The relative fold expression of ActRIB mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/ml culture media of LH or FSH. Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n=4. Means for a follicle size with different letters differ, $p < 0.05$.

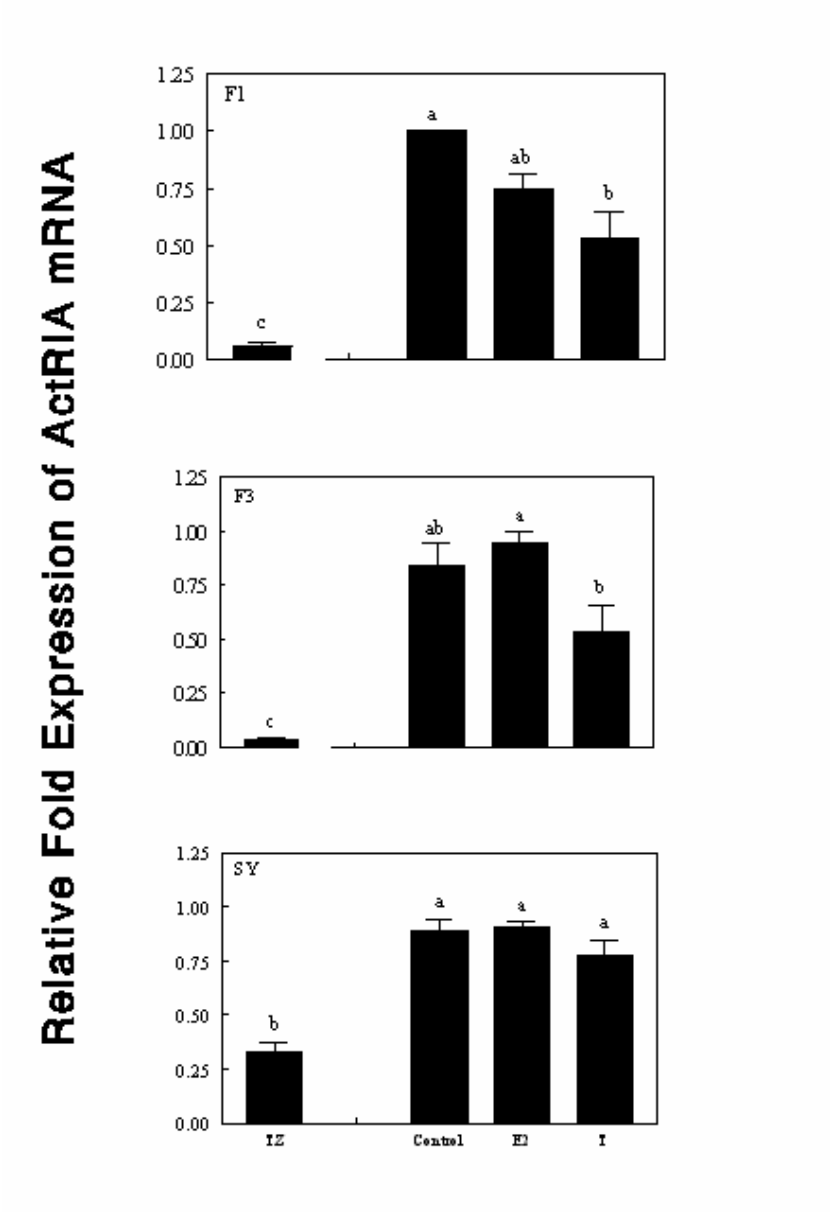

Figure 3.4. The relative fold expression of ActRIA mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 0 (time zero, TZ) or 24 hours in the presence of 0(control) and 1 x 10^{-6} M culture media of 17-β estradiol (E2) or testosterone (T). Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n=4. Means for a follicle size with different letters differ, $p < 0.05$.

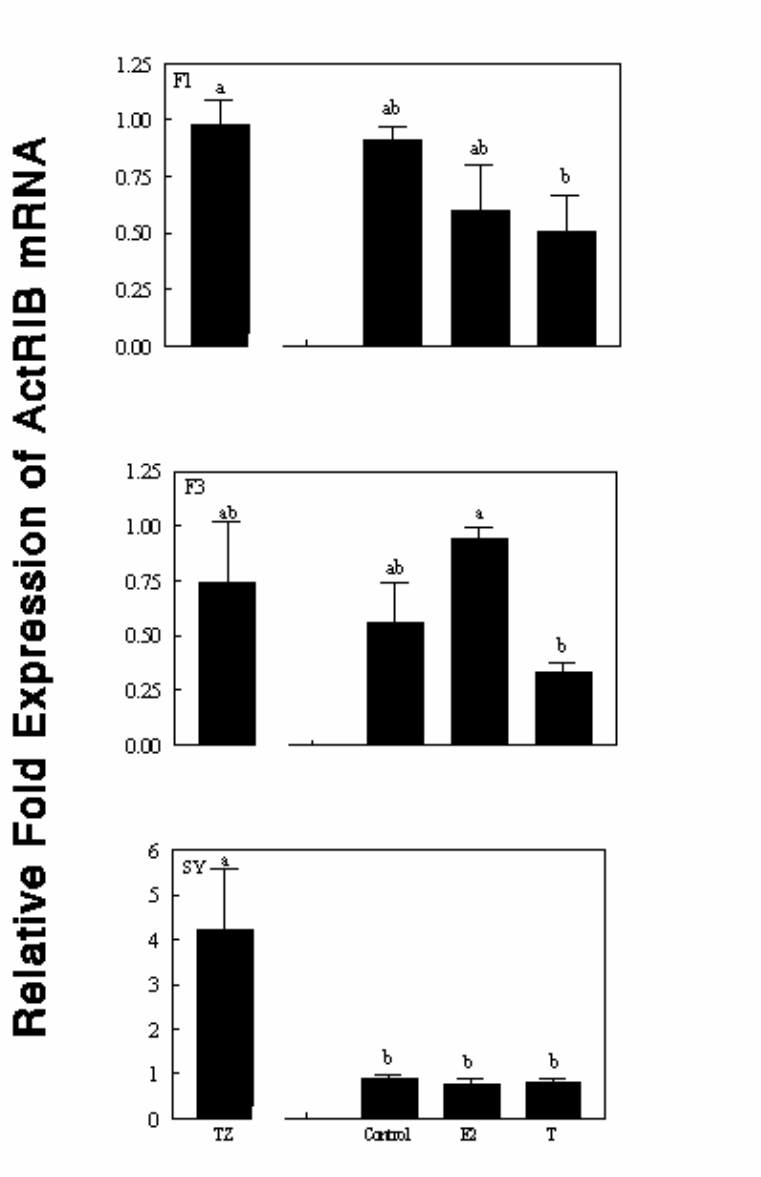

Figure 3.5. The relative fold expression of ActRIB mRNA in granulosa cells from the F_1, F_3 and small yellow (SY) follicles cultured for 0 (time zero, TZ) or 24 hours in the presence of (control) and1 x 10^{-6} M culture media of 17-β estradiol (E2) or testosterone (T). Data were normalized to GAPDH and expressed as the mean fold difference $\Delta\Delta C_T \pm$ SEM, n=4. Means for a follicle size with different letters differ, $p < 0.05$.

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CHAPTER 8

GENERAL CONCLUSIONS

Regulation of follicular development is a complex phenomenon that relies upon the synergistic interactions between hypothalamic, pituitary, and gonadal factors. Through the hypothalamohypophysial portal system gonadotropin-releasing hormone (GnRH) stimulates the anterior pituitary to produce the gonadotropins, LH and FSH. Inhibin and activin, which are produced abundantly by the gonads, regulate FSH secretion from the anterior pituitary gland. Inhibin suppresses FSH secretion while activin stimulates FSH secretion. Inhibin's ability to suppress FSH production and secretion is based on its ability to antagonize activin function by interfering with the formation of the activin receptor complex. Both inhibin and activin are members of the TGFβ superfamily which has been recognized as serving a variety of endocrine, autocrine, and paracrine roles in regulating follicular maturation. Although much has been discovered regarding the function, expression, and regulation of inhibin and activin, much is still left to be understood about this family of proteins. The purpose of these studies was to add to our understanding of the roles of inhibin and activin in the reproductive functions and dysfunctions observed in mammalian and avian species.

Avian species have contributed much to our understanding of the biology of activin and inhibin. The structure of the hen's ovary provides an excellent paradigm for studying the roles of inhibin and activin in modulating follicular growth and development. In avian species, as in mammalian species, follicular development is a highly regulated process. However, avian

species differ from mammalian species due to their consecutive ovulations and follicular hierarchy which consists of pools of follicles at different developmental stages.
By examining differences in mRNA expression of inhibin and activin subunits in follicles of different maturational stages, we are beginning to understand the degree of complexity and integration that exists in regulating follicular development.

The integration of inhibin into our current understanding of reproductive biology has revealed the usefulness of this hormone in diagnosing reproductive dysfunctions. Elevated inhibin levels are currently used to diagnose various types of ovarian tumors and are also indicative of ovarian activity or inactivity. Similarly, inhibin is a good indicator of testicular function and fertility in males. The development of assays specific for inhibin types in different species is continuously emerging especially with the advent of dimeric ELISAs.

The present studies add to our knowledge of the inhibin/activin family in mammalian and avian species. Specifically, the current work indicates that genetic selection for increased growth has altered the expression of the inhibin α- and β_B-subunit in the largest preovulatory follicles of the turkey hen ovary. These changes undoubtedly participate in the lower egg production seen in the turkey hens selected for growth instead of egg production. Furthermore, the current research indicates that activin can have local effects on ovarian development in the hen ovary since all the necessary components for active signaling are present in the granulosa and theca cells of the developing follicles. The current research, however, suggests that activin's influence may be greater in the theca cells than in the granulosa cells. In addition, the current research indicates that monitoring the inhibin family of hormones in the serum of elephants could be an important diagnostic tool in determining reproductive fitness in the captive elephant population which is facing extinction due to reproductive inefficiency.

Finally, although elucidating the functions and regulation of a hormone system with multiple ligands, binding proteins, and receptors is a significant challenge, meeting this challenge could enhance reproductive efficiency in endangered species, food animals, and of course humans suffering from infertility.