Corticosterone Binding Globulin (SerpinA6) in Broilers and Broiler Breeders

by

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(Under the Direction of Adam J. Davis)

ABSTRACT

In poultry the stress hormone is corticosterone, and blood levels of corticosterone increase with fasting. Management of broiler breeders for improved reproductive performance involves them experiencing daily fasting periods. As plasma transport protein for corticosterone, corticosteroid binding globulin may play a vital role in the stress response by releasing corticosterone at specific tissues in response to stress as only released or free corticosterone can enter a target cell and cause a cellular response. The main objective of this research was to determine if corticosteroid binding globulin is expressed by developing follicles and if fasting altered its expression. Corticosteroid binding globulin is expressed by granulosa cells of the largest preovulatory follicles and its expression is increased by fasting. The results suggest locally produced corticosteroid binding globulin may act to prevent elevated corticosterone from inducing follicular atresia of the largest follicles during short term fasting periods.

INDEX WORDS: theca, granulosa, roosters, broiler breeder hens

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B.S., University of Georgia, 2019

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

MASTERS OF SCIENCE

ATHENS, GEORGIA

2020

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May 2020

DEDICATION

To my wonderful parents, Blake and Traci Thomason, without whose love and support I would not be where I am today and to my sister Blakely who was always willing to lend advice and support.

ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Davis for all of his assistance and support. I would not be where I am without his tireless efforts to support my progress. I would also like to thank Liz Freeman. Without her patience and guidance this project would not have succeeded. Lastly, I would like to thank my committee members, Dr. Fairchild and Dr. Benson for being willing to work with me to help further my education and love of learning.

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Chapter 1

Avian Reproduction and Feed Restriction

1.1 The Avian Ovary

The ovary of the mature laying hen consists of an easily identifiable hierarchy of follicles relative to size and time to ovulation. In the laying hen, there are commonly four to six large yellow yolk – filled follicles, termed hierarchical follicles, that are approximately 12 - 40 mm in diameter. These follicles are accompanied by several smaller 5 to 12 mm diameter follicles in which yellow yolk deposition has begun and a large number of small white follicles that are less than 5 mm in diameter. The large yolk filled follicles are named according to size and length of time before ovulation. The largest follicle is termed the F1 follicle and will ovulate within the next 24 hours. The next largest follicle is named the F2 follicle, and it will ovulate 24-26 hours after the ovulation of the F1 follicle. The remaining large yolk filled follicles are named in successive order following the same pattern. Following the ovulation of the F1 follicle and the forward advancement in the hierarchy of the remaining hierarchical follicles, one of the pool of small yellow prehierarchical follicles is selected and develops into the smallest hierarchical follicle. Additionally, several small white follicles will begin the uptake of yellow yolk and proceed into the pool of small yellow follicles. Only 5% of the developing prehierarchical follicles will grow to reach a size of 6-8 mm in diameter (Gilbert et al., 1983). The fate for the vast majority of the prehierarchical follicles is follicular atresia, during which the individual cells of the follicle undergo apoptosis (Johnson et al., 1996).

1.2 Avian Follicular Tissues and Follicular Maturation

Each preovulatory follicle has distinct tissue layers that surround the yolk – filled oocyte. The developing oocyte is first surrounded by its plasma membrane, then the inner perivitelline layer, followed by the granulosa cell layer, a basement membrane, and theca tissue layers. The theca tissue is highly vascularized, in contrast to the avascular granulosa cell layer, and facilitates the transfer of yolk precursors from the plasma to the developing follicles in the ovary (Etches et al., 1981).

In general terms, follicular maturation can be characterized by the accumulation of yolk and the development of endocrine capabilities within the follicular tissues (Huang and Nalbandov, 1979). Follicular maturation is regulated primarily by two anterior pituitary glycoprotein hormones, luteinizing hormone and follicle stimulating hormone, and is mediated partly by the expression of luteinizing hormone and follicle stimulating hormone receptors in granulosa tissue. Before granulosa cells mature and become luteinizing hormone dependent, they are sensitive to follicle stimulating hormone. During follicular development, follicle stimulating hormone promotes granulosa cell proliferation and maturation (Davis et al., 2001), helps maintain the follicular hierarchy through the prevention of atresia (Johnson et al., 1996), induces luteinizing hormone receptor, steroidogenic acute regulatory protein (StAR), and P450SCC enzyme expression in granulosa cells for subsequent steroid production (Li and Johnson, 1993; Johnson and Bridgham, 2001; Johnson et al., 2004) and stimulates progesterone production (Calvo and Bahr, 1983; Robinson et al., 1988; Davis et al., 1999; Davis et al., 2001). Taken together, these results suggest that the prehierachial follicle which has granulosa cells that are the most responsive to follicle stimulating hormone avoids atresia, and becomes increasingly responsive to luteinizing hormone, thus acquiring the capability of producing steroids and

ultimately being able to produce enough progesterone to generate a luteinizing hormone surge for ovulation.

For laying hens, the coordinated development and maintenance of the follicular hierarchy allows for a high rate of ovulation and egg production with most laying hens producing an egg every day through peak egg production. However, this is not the case for the broiler breeder hens that produce the fertilized eggs for broiler chicks. Through genetic selection and better bird management, today's broilers reach a market weight of 2 to 2.5 kilograms in 5 to 6 weeks after hatch. An undesired effect from the genetic selection for rapid growth and meat yield in poultry breeders has been an associated decline in fertility (Barbato, 1999; Pollock, 1999; Brillard, 2004). Additionally, to support their rapid growth rate broilers have nearly insatiable appetites. These appetites and rapid growth rates, however, are problematic for optimal reproductive performance in the genetically similar parent stocks of broilers. Overeating and rapid growth are not conducive to proper reproductive function, leaving broiler breeder hens to suffer various ovarian dysfunctions including but not limited to follicular atresia, internal ovulations, multiple ovulations, and the production of double-yolked eggs (Leeson and Summer, 2000; reviewed by Walzem and Chen, 2014). Improvement in broiler breeder reproductive function has been observed only with the implementation of feed restriction programs designed to limit feed intake and maintain optimal body weight.

1.3 Effects of Feed Restriction in Hens

Optimum reproductive efficiency in broilers is dependent in large part on attaining an ideal body weight to support reproduction, consuming a nutritionally adequate diet, and being photostimulated. Although the ideal body weight for reproduction in broiler breeder hens is similar to the market size of broilers achieved at 5 to 6 weeks of age, the optimum sensitivity to

photostimulation for reproduction in broilers has traditionally been thought not to occur until about 20 weeks of age. Thus, to prevent broiler breeder pullets from growing too quickly and becoming obese by the photosensitivity-based sexual maturity that occurs at 20 weeks of age, their dietary intake is severely restricted. Typically, feed allocations are 60-80% less during the rearing period and 25-50% less during the laying period than what the breeder pullets/hens would consume ad libitum. Feed restriction of broiler breeder hens is a successful management tool in increasing the reproductive efficiency of these birds. Feed restricting broiler breeder hens delays sexual maturation (Robbins et al., 1986; Yu et al., 1992; Heck et al., 2004; Bruggeman et al., 2005; Hocking and Robertson, 2005; Onagbesan et al., 2006) and decreases mortality (Robbins et al., 1986; Katanbaf et al., 1989; Heck et al., 2004; Bruggeman et al., 2005). Additionally, feed restriction during the rearing and the laying period reduces the development of an abnormally high number of large follicles on the ovary of broiler breeder hens (Hocking et al., 1987; 1989; Heck et al., 2004; Hocking and Robertson, 2005). But more importantly, broiler breeder hens which have been feed restricted produce more eggs (Yu et al. 1992; Heck et al., 2004; Bruggeman et al., 2005; Onagbesan et al., 2006) because they lay longer sequences (Robinson et al., 1991a), persist in lay longer (Fattori et al., 1991), lay fewer abnormal eggs and have fewer multiple ovulations in a single day (Fattori et al., 1991; Yu et al., 1992; Heck et al., 2004) compared to full-fed broiler breeder hens. Broiler breeders that have been prevented from growing too large or from becoming overweight will also have better fertility because the reduced locomotive capabilities, as well as physical difficulty in successful natural copulation, are alleviated in smaller feed restricted birds (Duff and Hocking, 1986). Even when artificial insemination is used, fertility in overweight broiler breeder hens is reduced (Brake and McDaniel, 1981), which may be a consequence of their fat or hormones derived from fat making

successful insemination more difficult, blocking the sperm storage tubules, or inhibiting sperm movement (Hocking, 1987).

Despite the improvement of egg production in broiler breeder hens resulting from feed restriction that limits body weight gain, these hens still produce over 100 eggs less than commercial laying hens even though they have the follicles to produce just as many eggs. Even with feed restriction, follicular maturation and ovulation is still plagued by an unacceptable incidence of atresia of large yellow follicles and internal ovulations. This abnormal follicular development and lack of egg production may be related to the industry's feed restriction practices for broiler breeder hens. Broiler breeder hens are typically fed every other day during rearing and then provided a restricted level of feed once a day after photostimulation for reproduction. The feed provided is quickly consumed, and the hens are left to fast until the next feeding period. Morris and Nalbandov (1961) suggested that the lack of gonadotropin secretion from the pituitary was responsible for the loss of egg production in fasted birds. Subsequently, Scanes et al. (1976) reported that plasma luteinizing hormone concentrations were significantly depressed in 6-week-old male chicks fasted for 12 hours compared to control fed cockerels. In addition, fasted laying hens have lower plasma concentrations of luteinizing hormones after 48 hours of fasting and lower estradiol and progesterone concentrations after 24 hours of fasting compared to ad libitum fed control hens (Tanabe et al., 1981).

Previous research supports the idea that the fasting periods created by poultry industry feed restriction practices depresses total egg production in broiler breeder hens. In commercial settings, a skip-a-day feeding program is sometimes continued until the broiler breeder flock reaches 5 percent egg production. This is done to control flock body weight uniformity and to help control body weight gain since even a very slight excess of body weight prior to peak

production results in a significant decrease in total egg production as reviewed by Robinson et al. (1991b). Gibson et al. (2008) reported that initiating an everyday feeding regimen after photostimulating broiler breeder hens for reproduction increased total egg production by about 19 eggs per bird by the end of 65 weeks of age, compared to continuing the skip-a-day feeding regime until 5 percent egg production was reached. Gibson et al. (2008) also reported that plasma estrogen levels were increased and plasma progesterone levels were decreased for the entire breeding period in the hens that had been fed on a skip-a-day basis until 5 percent egg production compared to the hens that were fed every day after being photostimulated for reproduction.

The research reported by Gibson et al. (2008) suggested the significant fasting period the broiler breeder pullets experienced between meals on a skip-a-day feeding program after photostimulation for reproduction might be detrimental to normal ovarian development. This hypothesis was explored further in subsequent research. Spradley et al. (2008) completed research that was very similar to Gibson (2008) except when the pullets were photostimulated for reproduction they were fed either once a day (equivalent to the everyday treatment of Gibson (2008) or twice a day. The pullets in both feeding treatment groups received the same total amount of daily feed, but the duration of fasting between meals was reduced for the pullets fed twice a day. Feeding the hens twice a day significantly improved the total number of eggs produced per hen through 41 weeks of age by 5 eggs and significantly improved the overall percent hen day egg production through 59 weeks of age by 2 percent. However, these gains in egg production were lost on a hen housed basis at 59 weeks of age due to a higher level of mortality associated with feeding broiler breeder hens twice a day. Cumulative mortality for the hens fed once a day and twice a day from 23-59 weeks of age was 12 and 18%, respectively.

Necropsy results indicated that only 25% of the hens that died on the once a day feeding treatment were in lay (normal ovarian hierarchy and or egg in oviduct) compared to 63% of the hens that died in the twice a day feeding treatment. Thus, while decreasing the fasting period between meals may have reduced metabolic stress and improved egg production, this was negated by increased mortality associated with increased reproductive stress. Feeding hens twice a day increased egg weight without compromising shell quality, increased hatching egg production by decreasing the production of dirty eggs, had no effect on fertility and improved flock body weight uniformity.

Similarly, in broiler breeder roosters, the onset of testosterone production is delayed as the degree of feed restriction is increased during rearing (Stevens, 2010). This research also indicated that the severity of current feed restriction programs in male broiler breeders could be lessened without hurting fertility. This agrees with other research that has suggested that broiler breeder males are over feed restricted during the end of the broiler breeder production cycle (Buckner et al., 1986; Sexton et al., 1989a, b; Cerolini et al., 1995; Bramwell et al., 1996, Romero-Sanchez et al., 2008).

The mechanisms by which the fasting period associated with feed restriction is negatively impacting follicular development and ovulation are not clear. However, the feed restriction that the breeder hens are subjected to can cause the bird higher than normal levels of stress. As will be discussed in the next chapter, the stress hormone in avian species is corticosterone and plasma corticosterone levels have been reported to be elevated in feed restricted breeder birds as compared to those fed ad libitum (Savory et al., 1996; Savory and Mann, 1997). Furthermore, in recent years a better understanding of how stress and corticosterone could be directly impacting reproduction in avian species has emerged. Gonadotropin inhibitory hormone is a neuropeptide

that was first isolated from the hypothalamus of the Japanese quail and it negatively regulates gonadotropin release from the anterior pituitary (Tsutsui et al., 2000). In Japanese quail, Son et al. (2014) found gonadotropin inhibitory hormone neurons in the hypothalamus express glucocorticoid receptor mRNA and determined that, when treated with corticosterone for 24 hours, these diencephalic tissues displayed an increase in gonadotropin inhibitory hormone mRNA expression. Additional research also indicates that stress in avian species can induce gonadotropin inhibitory hormone production and biological actions (Calisi et al., 2008; Ernst et al., 2016).

1.4 Summary

Follicular selection and maturation in the laying hen is well researched and allows a productive laying hen to produce an egg a day during much of her productive lifespan in industry. In contrast, follicular development in broiler breeder hens fed ad libitum is not well maintained, and these hens produce far fewer eggs. Feed restriction of broiler breeder hens has greatly improved the cohesiveness of follicular development and maintenance of the follicular hierarchy with a resulting significant increase in egg production. However, egg production in broiler breeder hens is still very inferior to laying hens and possibly could be improved by reducing the stress associated with feed restriction programs.

Chapter 2

The Avian Glucocorticoid System

2.1 Glucocorticoids

Corticosteroids are steroid hormones containing a 21-carbon cholesterol ring structure and are primarily produced in the cortex of the adrenal gland in mammalian species. Their synthesis is under the control of the hypothalamic-pituitary-adrenal axis which is stimulated by a variety of internal and external stressors/stimuli. Corticosteroids can be divided into glucocorticoids and mineralocorticoids. While cortisol is the predominant glucocorticoid in humans, corticosterone is the primary glucocorticoid secreted in birds as reviewed by Carsia and Harvey (2000). However, evidence obtained in Zebra finches suggests that during embryonic development through hatching cortisol may play a significant role as a primary glucocorticoid (Schmidt and Soma, 2008) in avian species. Glucocorticoids regulate a wide range of biologically essential functions such as metabolism, reproduction, immunity, and behavior (Carsia and Harvey, 2000). However, despite having a variety of effects upon multiple systems, the primary role of glucocorticoids is to facilitate glucose release for utilization during homeostatic challenges as reviewed by Carsia and Harvey, (2000) and Schoech et al. (2009).

Aldosterone, the primary mineralocorticoid, is mainly responsible for sodium absorption in the kidneys, salivary glands, and large intestine epithelia. Plasma aldosterone increases blood volume through sodium reabsorption followed by water reabsorption, or through the control of the rennin-angiotensin system. In birds, synthesis of aldosterone declines significantly with age,

contributing to an average ratio of basal circulating aldosterone to corticosterone of about 1:100 in mature birds (Carsia and Harvey, 2000).

Synthesis and Activation

Synthesis of the glucocorticoids is initiated by the activity of the steroidogenic acute regulatory protein (StAR) that is required for the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where cytochrome P450 side-chain cleavage enzyme (P450scc or CYP11A1) catalyzes the conversion of cholesterol to pregnenolone. With the enzymatic action of 3β-hydroxysteroid dehydrogenase (3βHSD) pregnenolone is converted to progesterone. Progesterone is then converted into 11-deoxycorticosterone by 21-hydroxylase (CYP21 or P450c21). Then 11β-hydroxylase (P450c11β or CYP11B1) converts 11-deoxycorticosterone into corticosterone. Aldosterone is synthesized from corticosterone by the action of 18 hydroxylase followed by 18-OH-dehydrogenase. The production of cortisol utilizes the same enzymes (21-hydroxylase and 11β-hydroxylase) used to make corticosterone. But for cortisol production, the initial substrate is 17α-hydroxyprogesterone which can be derived from progesterone or 17α-hydroxypregnenolone by the action of 17α-hydroxylase (CYP17 or P450c17 or 17,20-lyase). Once formed 17α-hydroxyprogesterone can be converted to 11 deoxycortisol by 21 hydroxylase and 11 deoxycortisol is converted to cortisol by 11β-hydroxylase.

The half-life of circulating corticosterone in avian species is about 15 minutes (Carsia and Harvey, 2000). Clearance results from intracellular binding to its receptors and sequestration within the cell or metabolism primarily by the liver. Corticosterone is primarily converted by 5α-reductase to the inactive metabolites 11-dehydrocorticosterone and 5α-tetrahydrocorticosterone.

Regulation of Secretion

The interactions between the hypothalamus, the anterior pituitary gland, and the adrenal glands entails the hypothalamic-pituitary-adrenal axis (Harvey et al., 1984) which promotes the control and adjustment of the neuroendocrine response of the body to stress and affects vital functions of the body such as digestion, immunity, reproduction, and metabolism of nutrients (Harvey and Hall, 1990). In response to several stimuli such as stress, hypothalamic neurons secrete corticotropin-releasing hormone (Harvey and Hall, 1990).

Released corticotropin-releasing hormone stimulates corticotrophic cells of the anterior pituitary gland to produce and secrete adrenocorticotropic hormone which is a protein hormone consisting of 39 amino acids. Adrenocortical function is activated by adrenocorticotropic hormone and results in the synthesis and secretion of corticosterone (Beuving and Vonder, 1978, 1986; Radke et al., 1985a,b) and aldosterone (Radke et al., 1985a, b). Both corticotropin-releasing hormone (Jozsa et al. 1984; 1986; Mikami and Yamada, 1984; Peczely and Antoni, 1984; Yamada and Mikami, 1985; Carsia et al., 1986; Ball et al., 1989; Romero and Wingfield, 1998; Romero et al., 1998a, b) and arginine vasotocin (Castro et al., 1986; Westerhof et al., 1992) stimulate adrenocorticotropic hormone secretion from the pituitary gland in response to changes in the physiological status of the bird.

Circulating levels of adrenocorticotropic hormone range from 20 to 150 pg/ml in unstressed chickens, turkeys and geese (Carsia et al., 1988; Harvey and Hall, 1990; Kovacks and Peczely, 1991; Hendricks et al., 1995; Kocsis et al., 1995a). Once it reaches the adrenocortical cells in the adrenal gland adrenocorticotropic hormone binds to G-protein coupled receptors which produce cAMP and activate protein kinases that stimulate mitochondrial steroidogenesis of glucocorticoids. In laying hens, Etches and Cunningham (1976) recorded an increase and decrease in plasma corticosterone concentration after administration of adrenocorticotropic

hormone and dexamethasone, respectively. Secreted corticosterone provides a negative feedback back to the hypothalamus and the anterior pituitary gland to dampen the production and secretion of corticotropin-releasing hormone and adrenocorticotropic hormone, respectively.

2.2 Glucocorticoid Receptor

In glucocorticoid-target tissues, glucocorticoids bind to an intracellular glucocorticoid receptor which is a type I nuclear receptor that belongs to a subfamily of nuclear receptors that includes the mineralocorticoid receptor, estrogen receptor, progesterone receptor and androgen receptor (Mangelsdorf et al., 1995). All members of this family are known as ligand-activated transcription factors and consist of 3 primary domains: an N-terminal domain that holds a transactivation region (AF-1/tau-1/enh2) (Giguere et al., 1986; Dieken and Miesfeld, 1992), a central DNA-binding domain which binds to a specific DNA element and promotes nuclear export (Giguere et al., 1986; Black et al., 2001; Kumar and Thompson, 2005), and a C-terminal ligand-binding domain that contains a ligand-dependent transcriptional activation function and is responsible for the dimerization of the receptor (Giguere et al., 1986; Tang et al., 1998). Because of their close structural similarity and the similarity of their ligands the mineralocorticoid and glucocorticoid receptors both can bind corticosterone and aldosterone with different affinities and in many tissues corticosterone actually binds with higher affinity to the mineralocorticoid receptor.

Glucocorticoid Receptor Binding and Cellular Activation

When not bound to its substrate, the glucocorticoid receptor remains mostly in the cytoplasm, where it participates in a multimeric chaperone complex consisting of heat-shock protein 90, heat-shock protein 70, heat-shock protein 90-binding protein p23, immunophilins, and other factors to prevent its degradation (Pratt and Toft, 1997; Cheung and Smith, 2000;).

Once bound to its ligand substrate, the glucocorticoid receptor crosses the nuclear membrane into the nucleus where it acts either as a homodimeric transcription factor, binding to the glucocorticoid response element in promoter regions of glucocorticoid-inducible genes or as a monomeric protein that works along with other transcription factors to induce transcription (De Bosscher and Haegeman, 2009; McNally et al., 2000).

2.3 Physiological Effects of Glucocorticoids

Metabolism

In vertebrate species, glucocorticoids are known to have a regulatory role on carbohydrate, lipid, and protein metabolism (Bamberger et al., 1996). In birds, glucocorticoids stimulate the utilization of energy stores by increasing lipolysis and glucose mobilization to maintain body homeostasis in the face of stressors (Harvey et al., 1986; Remage-Healey and Romero, 2001). Glucocorticoids stimulate a decrease in insulin-dependent glucose uptake and an increase in gluconeogenesis in the liver of birds (Remage-Healey and Romero, 2001; Yuan et al., 2008). Increased levels of glucocorticoids suppress anabolic processes such as growth in birds (Muller et al., 2009; Almasi et al., 2012). Glucocorticoids also inhibit the synthesis of triglycerides from non-esterified fatty acids in birds (Remage-Healey and Romero, 2001).

Food intake and feed restriction

Corticosterone stimulates food intake in chickens (Bartov, 1985; Covasa and Forbes, 1995; El-Lethey et al., 2001). Even though there is significant research linking the effect of corticosterone on feed intake of chickens the neuroendocrine mechanism mediating this response is still not clear. The fact that corticosterone stimulates food intake is not surprising in light of research that indicates that feed restriction and fasting in birds increase plasma corticosterone levels. Scanes et al. (1980) reported that male White Leghorns fasted for 24 hours at an age of 4

days, 4 weeks, 6 weeks or as adults had had higher plasma corticosterone concentrations than non-fasted controls. Similarly, 7 to 8-week-old cockerels that were the progeny from Light Sussex crossed with Rode Island Reds had dramatically elevated plasma corticosterone levels after being deprived of food for 48 hours (Harvey and Klandorf, 1983). Nir et al. (1975) also reported that 10-day old crossbred New Hampshire X White Leghorn male chicks starved for either 1 or 3 days had elevated plasma corticosterone levels relative to fed controls. Interestingly, chicks that were bursectomized or sham operated at 1 day of age and then starved for 1 or 3 days at 10 days of age had no increase in plasma corticosterone. The authors suggested the stress of surgery helped the birds cope with the stress of starvation. However, this protective effect based on plasma corticosterone levels was not present when chicks were exposed to short-duration acute stress such as cold-water immersion (Nir et al., 1975).

The persistence of elevated corticosterone levels in poultry once the feed stress is removed varies. Beuving and Vonder (1978) reported that 40 week old White Leghorn laying hens that were feed deprived for 5 days and water-deprived for 2.5 days had elevated plasma corticosterone levels in the 2 days prior to returning water availability compared to the values for the two days after water availability was returned. In contrast, white Leghorn chicks that are protein-restricted have elevated plasma corticosterone levels, but more importantly relative to control chicks, the level of corticosterone remained elevated when measured 4 weeks after the chicks that had consumed a protein deficient diet for 4 weeks were returned to diets that were protein adequate (Weber et al., 1990). Finally, Light Sussex chicks that were feed restricted by 75 percent of the ad libitum feed consumption of controls from hatching to 8 weeks of age had plasma corticosterone concentrations that were 73 percent greater than controls at 1 week of age (Freeman et al., 1981). But the plasma levels of corticosterone progressively decreased each

subsequent week until 5 weeks of age when it was equal to the level found in the control birds. This suggested the birds were adapting to the stress over time.

Feed restriction in broilers can also cause an elevation in corticosterone. Hocking et al. (1996) reported that corticosterone levels were elevated in broiler breeder pullets feed restricted to gain 50 percent or less weight compared to ad libitum fed birds. De Jong et al. (2002) also found that plasma corticosterone was increased in broiler breeder pullets that were feed restricted relative to ad libitum fed controls. However, plasma corticosterone concentrations are not always elevated in feed restricted broiler breeders. Savory and Mann (1997) reported that 10-week old feed restricted broiler breeder pullets had elevated plasma corticosterone levels relative to ad libitum fed controls in blood samples collected within a couple hours of the morning feeding, but not when the samples were collected in the afternoon. In addition, Hocking et al. (1993) reported that feed restricted broiler breeder pullets had elevated plasma corticosterone concentrations relative to ad libitum controls at 8 and 12 weeks of age but not at 3 and 16 weeks of age. Savory et al. (1993) reported that there were no differences in plasma corticosterone concentration 5 hours after feeding in broiler breeder females fed a breeder guideline restriction amount, this amount doubled or fed ad libitum when measured at 5, 9 13, 17 and 21 weeks of age.

Mench (1991) reported that male broiler breeders feed restricted during rearing from 4 weeks of age to 15 weeks of age using a skip a day feeding regimen had elevated plasma corticosterone levels compared to ad libitum fed controls. In addition, the overall level of plasma corticosterone was greater in restricted cockerels on feed off days relative to the days when they were fed. De Beer et al. (2008) examined plasma corticosterone concentrations in broiler breeder pullets at 16 weeks of age that had been reared with an everyday or skip a day

feed restriction system in which the birds received the same total amount of feed over every 48 hour period. Overall, the skip a day birds had a higher plasma corticosterone level than the everyday fed birds with levels being consistently elevated in the period from 20 to 48 hours after the last feeding. Neves (2012) also reported similar results, with plasma corticosterone levels being elevated in skip a day fed broilers during the day they were not fed relative to the day they were fed or compared to pullets fed every day. Ekmay et al., (2010), reported that the overall plasma concentration of corticosterone over a 24 hour period was still greater in the skip a day hens versus the everyday hens at 26.4 weeks of age, even though all of the hens had started to be fed on an everyday basis at 24 weeks of age. This report like the Weber et al. (1990) report in Leghorns, suggests that alterations in corticosterone production can persist weeks after the original stressor has been removed.

Effect on Reproductive Tissues

Although avian ovarian cells lack the 21-hydroxylase enzyme necessary to synthesize glucocorticoids de novo, they are still subject to the peripheral influences of glucocorticoids due to the presence of glucocorticoid receptors in ovarian tissue (Kwok, 2007). Furthermore, a close interaction between the avian left ovary and the adrenal gland is strongly suggested since they are anatomically close to each other and the left ovary is extremely innervated by the adrenal gland (Etches et al., 1984a). The role of glucocorticoids in the direct regulation of ovarian function is poorly understood, but it is plausible that higher levels of corticosterone due to prolonged fasting stress may have a negative influence on ovarian development.

Research indicates that increasing levels of cortisol and corticosterone lead to a disruption in the normal ovarian function in birds as reviewed by Etches et al., (1984a) most likely due to a corticosterone stimulated decrease in circulating luteinizing hormone and follicle

stimulating hormone concentrations as seen in laying hens (Johnson, 1981; Etches et al., 1984a) and turkey hens (Rozenboim et al., 2004). Furthermore, ovarian regression decreased luteinizing hormone levels, decreased total number of large yellow-yolk filled hierarchical follicles and increased numbers of atretic pre-hierarchical follicles have all been associated with high levels of corticosterone, due to food and water deprivation or due to daily corticosterone administration (Etches et al., 1984b).

Based on research on Zebra finches, Salvante and Williams (2003) suggested that high levels of corticosterone shifted hepatic lipid metabolism away from yolk very low-density lipoprotein production. Liu et al. (2012) reported a 40% decrease in egg production and a significant decrease in egg mass in hens injected daily with corticosterone versus those injected with corn oil.

2.4 Corticosteroid-Binding Globulin

General Overview

Mammalian corticosteroid-binding globulin was first discovered independently by three separate scientists between 1957-1959. Bush (1957) used equilibrium dialysis of plasma to which increasing amounts of cortisol had been added. From this method, he was able to identify the presence of two binding molecules for cortisol. The first, albumin, had a high capacity but low affinity for cortisol. The second, corticosteroid-binding globulin, had a low capacity but a high affinity for cortisol. This discovery by Bush was subsequently confirmed by Sandberg et al. (1957) and Daughaday (1958).

Sandberg then took the research further and discovered that corticosteroid-binding globulin was an alpha globulin (Slaunwhite & Sandberg, 1959). They subsequently dubbed the protein, transcortin, a name which is still seen at times. Interestingly, corticosteroid-binding

globulin has little DNA sequence homology with other steroid carriers and is defined as a clade A serine proteinase inhibitor in the serpin family (Law et al., 2006). Serpins are the largest and most broadly distributed superfamily of protease inhibitors. At times, serpins perform a non-inhibitory function, for example, several human serpins function as hormone transporters and certain serpins function as molecular chaperones or tumor suppressors. Serpins are relatively large molecules (about 330-500 amino acids) in comparison with protease inhibitors. Over 70 serpin structures have been determined, and these data, along with a large amount of biochemical and biophysical information, reveal that inhibitory serpins are 'suicide' or 'single-use' inhibitors that use a unique and extensive conformational change to inhibit proteases (Law et al., 2006). In humans, the two largest clades of the 36 serpins that have been identified are the extracellular 'clade A' molecules and the intracellular 'clade B' serpins (Law et al., 2006).

Free Hormone Hypothesis and Free Steroid Regulation

Corticosteroid binding globulin is the major transport protein for glucocorticoids in the blood of almost all vertebrate species (Seal & Doe, 1965). More than 90 percent of the cortisol in human plasma is bound by corticosteroid-binding globulin with the remaining part being bound to albumin or present as "free" or active steroid (Siiteri et al., 1982). The free hormone hypothesis aids in understanding how steroids act at the target cell level by hypothesizing that only free steroids that are not bound by proteins can passively diffuse through the plasma membranes of cells (Mendel 1989). The strongest evidence to support the free hormone hypothesis comes from the fact that corticosteroid-binding globulin regulates metabolic clearance rates of plasma corticosteroid. The percentage of plasma corticosteroid bound to binding globulins is inversely proportional to the metabolic clearance rate (Malisch et al., 2010). While it is true that the "Free Hormone Hypothesis" is widely accepted, some believe, it is can

be overly simplistic. The "Free Hormone Hypothesis" does not account for the fact that the effects of steroid hormones are also mediated by target tissue type as well as what protein binds it.

With only the "free" portion of plasma corticosteroid seen as biologically active, it is easy to question the necessity of corticosteroid-binding globulin, but corticosteroid-binding globulin increases the half-life of corticosteroids and because of its lipophilic nature unbound corticosteroid would easily permeate all body cells and saturate corticosteroid receptors and maximize cellular responses to this stress hormone. With corticosteroid-binding globulin, corticosteroids can have a more sustained and regulated impact on body tissues (Malisch et al., 2010). In addition, corticosteroid-binding globulin also acts as a storage reservoir for corticosteroid so that it can be released when the need arises (Hammond, 1995).

The relationship between corticosteroid-binding globulin, albumin, and sex hormone-binding globulin

Along with corticosteroid-binding globulin and albumin, sex hormone-binding globulin also binds free steroid hormones in most vertebrates. As previously indicated, albumin binds all classes of hormones with a high capacity and a low affinity. Albumin's very high plasma concentrations and ligand-binding capacity allow it to buffer fluctuations in steroid hormone levels and their distribution between other steroid-binding proteins and the free fraction in plasma. Specific steroid hormones bind to corticosteroid-binding globulins and sex hormone-binding globulin with high affinity and specificity, with sex hormone binding-globulin preferentially binding the major androgens, estrogens (with androgens having higher binding affinity than estrogens) and to a lesser degree progesterone, and corticosteroid binding globulin preferentially binding the glucocorticoids and progesterone (with glucocorticoids having higher

affinity than progesterone) (Westphal 1986). Thus, corticosteroid binding globulin and sex hormone binding-globulin control the amounts of free steroids that passively diffuse into cells, (Hammond, 2016; Perogamvros et al. 2012). Although corticosteroid binding globulin and sex hormone binding-globulin are present in much lower concentrations in plasma than albumin, their high affinity and specificity for steroids allows them to have a much bigger role in determining and maintaining the free plasma concentrations of the steroid hormones they bind (Hammond, 2016).

Encoding Genes

Corticosteroid binding globulin is found in a cluster of genes that exhibit a high degree of synteny between species. The serpin gene locus can be subdivided into three clusters of related genes, the most proximal of which is the SERPINA1 gene, then the SERPINA2 gene, then the corticosteroid binding globulin (SERPINA6) gene, and finally the SERPINA10 gene (Namciu et al., 2004). SERPINA1 encodes for an inflammatory response molecule such as (antitrypsin). The SERPINA2 gene encodes an antitrypsin-related protein and SERPINA10 encodes for a protein Z-dependent proteinase. The SERPINA6 gene encodes for the non-inhibitory hormone-transport molecule called corticosteroid binding globulin (Law et al., 2006). The transcriptional activation of this serpin gene cluster appears to be coordinately regulated by a locus control region upstream of the (SERPINA1) gene (Zhao et al., 2008), that responds to transcription factors like hepatic nuclear factor-1 and hepatic nuclear factor-4 involved in mediating important physiological responses in cases of shock or inflammation (Rollini and Fournier, 1999).

Unlike other SerpinA proteins encoded by genes within this syntenic gene cluster, corticosteroid binding globulin (SERPINA6) is not known to inhibit proteases (Hammond, 2016). Instead, the cleavage of corticosteroid binding globulin by proteases within a distinct

structural domain allows for the targeted delivery of corticosterone and other corticosteroid binding globulin ligands to their sites of action (Hammond et al., 1987). This concept has been confirmed in vitro (Pemberton et al., 1988; Hammond, 1990) and in vivo (Hammond, 1990) and provides a good example of how corticosteroid binding globulin may regulate the plasma distribution of its steroid ligands and also enhance their bioavailability at a site of action (Hammond, 1990).

Tissue distribution of corticosteroid-binding globulin

The primary production site for circulating corticosteroid binding globulin is the liver (Hammond et al., 1987). However, corticosteroid binding globulin mRNA has also been found to be expressed in several other tissues, including the kidney, pancreas, lungs, and testes (Hammond et al., 1987; Scrocchi et al., 1993). The biological significance of corticosteroid binding globulin production by these extra-hepatic tissues is not well understood, but it does not appear to contribute to plasma corticosteroid binding globulin levels and likely serves to control local tissue availability of corticosteroid (Scrocchi et al., 1993).

Steroid Hormone Binding and Release

In mammals, plasma corticosteroid binding globulin has five or six sites for N-glycosylation, depending on the species (Smith & Hammond, 1988). One of these sites is strictly conserved and appears essential for high-affinity steroid-binding activity (Li et al., 2012). In addition, like other serpins, corticosteroid binding globulin has an unstructured reactive center loop that is targeted by specific proteases (Li et al., 2012). Proteolysis of the reactive center loop in many serpins is an essential step for their subsequent inhibition of proteases. Proteolysis of the reactive center loop of human corticosteroid binding globulin by neutrophil elastase, chymotrypsin, or by exogenous bacterial proteases causes a conformational rearrangement in its

tertiary structure, as seen with other serpins (Vashchenko, 2016). Proteolysis of corticosteroid binding globulin causes a pronounced alteration of its conformation that is marked by an increase in thermostability (Pemberton et al., 1988), a behavior corticosteroid binding globulin has in common with other members of the serpin superfamily that undergo S to R structural transformations. The S to R transformations are a hallmark of serpin structures as they undergo conformational rearrangements as part of their biological function. The conformations they adopt are highly dependent on whether a surface-exposed loop, known as the reactive center loop or "proteinase bait" domain, is intact. Cleavage of the reactive center loop segment by proteinases usually causes a typical stressed to relaxed (S→R) transition in structure (Klieber et al., 2007). However, in corticosteroid binding globulin, this eliminates its ability to bind steroids (Hammond, 1990) and supports the belief that proteinase cleavage of corticosteroid binding globulin facilitates the targeted delivery of its anti-inflammatory steroids to sites of action (Hammond et al., 1987). As indicated previously, instead of subsequent inhibition of proteases, proteolysis of the corticosteroid binding globulin reactive center loop irreversibly disrupts its high-affinity steroid-binding activity and serves to promote the delivery of corticosteroid binding globulin bound ligands to locations where proteases are present (Klieber et al., 2007). Up to this point, all research evidence suggests that corticosteroid binding globulin binds glucocorticoids in the blood and acts as a storage depot that allows the rapid release of glucocorticoids at times of stress or inflammation in a targeted manner by proteolysis of the reactive center loop (Hammond, 1990).

Corticosteroid Binding Globulin in Times of Elevated Stress in Mammals

Corticosteroid binding globulin is unique from the other SERPIN gene products because corticosteroid binding globulin is cleaved by the serine proteases that the other serpins inhibit.

Activated neutrophils at sites of inflammation carry and secrete serine proteases. These proteases can then cleave corticosterone binding-globulin, releasing corticosterone and increasing the local concentration of free corticosterone over what is available in the general circulation (Breuner and Orchinik, 2002). Pemberton et al. (1988) hypothesized that corticosteroid binding globulin evolved as a carrier protein for corticosterone because of this characteristic, which allows for local delivery of free corticosterone to sites of inflammation. Plasma corticosteroid binding globulin levels change in varying ways under normal physiological conditions in a wide range of vertebrates (Westphal, 1986). However, during severe stress, there is a marked and rapid reduction in plasma corticosteroid binding globulin levels (Hammond, 1990; Garrel et al., 1983).

2.5 Avian Corticosteroid Binding Globulin

To date, most corticosteroid binding globulin research has been completed in mammals with limited research done in avian species, and with avian research focused primarily on songbirds. While the gene and protein structure of corticosteroid binding globulin is conserved across mammalian species, avian corticosteroid binding globulin has only about 45% sequence identity with mammalian corticosteroid binding globulins (Vashchenko et al., 2016). Surprisingly, even the ligand binding site shares less than 50% homology between avian and mammalian corticosteroid binding globulins (Vashchenko et al., 2016). Further complicating corticosteroid binding globulin biology in birds is the fact that avian species do not appear to have sex steroid binding globulin (Wingfield et al., 1984). As reviewed by Malisch and Breuner (2010), while avian corticosteroid binding globulin binds glucocorticoids with high affinity, it also binds progesterone with high affinity, testosterone, and dihydrotestosterone with lower, but physiologically relevant affinity and estrogen with negligible affinity. This means that an

increase in progesterone and testosterone and dihydrotestosterone can displace corticosterone that is bound to corticosteroid binding globulin and vice versa which could potentially cause an increase in free levels of the displaced hormone without changing the hormones total levels (Charlier, 2009). The fact that corticosteroid binding globulin binds progesterone and testosterone with high affinity in avian species may explain why it shares less homology with mammalian corticosteroid binding globulins (Vashchenko et al., 2016).

Corticosteroid Binding Globulin in Times of Elevated Stress in Avian Species

Restraint or tail-shock stress, social stress, and food deprivation have all been shown to decrease corticosteroid binding globulin binding capacity (Malisch, 2010; Fleshner et al., 1995; Spencer et al., 1996; Alexander & Irvine, 1998) in wild birds. While this does not appear to be a rapid mechanism for increasing free corticosterone, the regulation of corticosteroid binding globulin levels may enable an animal to maintain elevated free corticosterone levels when total corticosterone levels are no longer elevated. In many songbirds, the plasma concentrations of several steroids, such as testosterone and corticosterone, change seasonally, with high levels in the early breeding season and lower levels in the late breeding season and non-breeding season (Romero, 2002). In the Puget Sound white-crowned sparrow (Zonotrichia leucophrys pugetensis), aggressive interactions rapidly increase plasma total testosterone levels during the early breeding season (Wingfield and Wada, 1989; Wingfield and Hahn, 1994). Later in the breeding season, when gonadal testosterone synthesis is waning, aggressive interactions might be less effective at increasing total testosterone levels in plasma (Wingfield and Hahn, 1994), but a decrease in plasma corticosteroid binding globulin could account for the rapid increase in free testosterone. Also, an increase in total corticosterone or progesterone levels might lead to increases in free testosterone levels, through competitive interactions for the single steroid-

binding site per corticosteroid binding globulin molecule (Westphal, 1986; Swett and Breuner, 2008). There is increasing evidence that the environment modulates free corticosterone levels by varying the amount of corticosteroid binding globulin available. Lynn et al. (2003), reported that in Eurasian tree sparrows that after 18 hours of food deprivation total corticosterone levels were no longer significantly higher than the fed control birds, but a reduced corticosteroid binding globulin capacity was allowing for free corticosterone levels to remain high.

2.6 Summary

Corticosterone is the primary avian glucocorticoid hormone. Its production is increased under stress such as feed restriction. Although the avian ovary is not capable of producing corticosterone, it does express the glucocorticoid receptor. Thus, elevated levels of corticosterone that result in feed restricted broiler breeders could impact follicular maturation. Additionally, corticosterone can increase the expression of gonadotropin inhibiting hormone and potentially inhibit reproduction in avian species by inhibiting luteinizing hormone and follicular stimulating hormone production. Corticosteroid binding globulin has also been identified in undomesticated avian species. Because of corticosteroid binding globulin's ability to store, transport, and release glucocorticoids in a targeted manner, it is an influential part of the mammalian stress response, and corticosteroid binding globulin may also be critical in the response of poultry to stress as well.

Chapter 3

Statement of Purpose

Feed restriction of broiler breeders drastically improves egg production and fertility relative to ad libitum fed broiler breeders. However, despite the overall success of this management practice, its implementation at the farm level causes developing broiler breeders to have fasting periods that often exceed well over 24 hours in length and broiler breeders in production to have fasting periods that extend over 12 hours in duration. These fasting periods cause metabolic and behavioral stress that may negatively impact overall reproductive fitness. In poultry, the stress hormone is corticosterone, and blood levels of corticosterone increase with fasting. Although the avian ovary is not capable of producing corticosterone, it does express the glucocorticoid receptor. Thus, elevated levels of corticosterone that result in feed restricted broiler breeders could impact follicular maturation. Additionally, corticosterone can increase the expression of gonadotropin inhibitory hormone which can inhibit reproduction in avian species by inhibiting luteinizing and follicle stimulating hormone production. While most of the research focus on the stress response in poultry has involved corticosterone and the glucocorticoid receptor that mediates its action, corticosteroid binding globulin may also be a key regulator of the stress response. In mammalian species, corticosteroid binding globulin is an integral component of the stress response based on its ability to bind glucocorticoids with high affinity and thus providing transport of these steroid hormones in the blood, extending their half-life, providing a storage depot, and providing for their target release at tissues via activated proteases. The targeted release of glucocorticoids as tissue specific sites may be the most

important function of corticosteroid binding globulin given that only unbound or free glucocorticoids are able to diffuse across cell plasma membranes and bind to its receptor to mediate a cellular response. In avian species less is known about corticosteroid binding globulin biology but based on limited research in songbirds and other wild bird species, it does appear to regulate free glucocorticoid steroid availability. In poultry, the biology of corticosteroid binding globulin is unknown. Therefore, the main objective of this research is to characterize the expression of corticosteroid binding globulin in broilers in order to determine whether it is correlated with the overall stress response in poultry. Specifically, the current research will be conducted in order (1) to determine if corticosteroid binding globulin mRNA is expressed in a wide variety of non-reproductive tissues in broilers (2) to explore whether corticosteroid binding globulin mRNA is expressed in mature and/or immature testes, (3) to determine whether corticosteroid binding globulin mRNA is expressed in the theca and granulosa tissues of hierarchical and prehierarchical follicles, and (4) to investigate in broiler breeder hens whether fasting changes corticosteroid binding globulin mRNA expression in the developing follicles of the ovary and in the pituitary.

Chapter 4

Materials and Methods

4.1 Experiment 1-Tissue Distribution

The purpose of this experiment was to determine the tissue distribution of corticosteroid binding globulin mRNA in key metabolic and digestive tissues in growing broilers.

Animals

Cobb 500 X Cobb 500 fast feathering male broilers which had been hatched and vent sexed at the University of Georgia Poultry Research Center were reared from hatch to 35 days of age in floor pens using standard corn/soybean based starter, grower and finisher diets. The floor pens were in an environmentally controlled facility. Ambient temperature was set to 34°C on day 1 and decreased daily by 0.28°C until 24°C was reached and then maintained. A typical broiler industry lighting program was implemented with a lighting intensity of 20 lux for 24 hours (0 to 4 days of age), 20 lux for 20 hours (5 to 7 days of age), 10 lux for 16 hours (8 to 14 days of age), and 2 lux for 16 hours (15 to 35 days of age). Birds were provided with water and feed ad libitum. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Tissue collection

At 5 weeks of age, the broilers were killed by cervical dislocation for tissue collection. Approximately 150 mg of duodenum, jejunum, ileum, gizzard, liver, spleen, kidney, gastrocnemius (leg muscle), pectoralis major (breast muscle), and heart tissue was collected from 6 individual birds with the tissue from 2 birds being combined to create 3 replicate samples.

Immediately after collection each tissue was placed in 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA). Individual homogenized tissue solutions were frozen and stored at -80ºC for future RNA extraction.

4.2 Experiment 2- Immature and Mature Broiler Testes

The aim of the second experiment was to investigate corticosteroid binding globulin mRNA expression in the testicular tissues of sexually immature male broilers and sexually mature broiler breeder roosters.

Animals

Cobb 500 male broilers were obtained from the Cleveland, Georgia hatchery on day of hatch and placed into floor pens in an environmentally controlled facility at the University of Georgia Poultry Research Center, where they were reared until 42 days of age. The broilers were fed a standard corn/soybean-based starter diet, grower diet, and finisher diet for the first, second, and third two-week phases of the study, respectively. The broilers were provided with ad libitum access to both water and feed and were maintained under typical temperature and lighting programs described in Experiment 1 for the entirety of the 42-day experiment. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

For the mature testes samples, Ross broiler breeder roosters were obtained from a commercial broiler breeder farm in northeast Georgia at 62 weeks of age.

Tissue Collection

At 42 days of age, ten of the Cobb male broilers were selected for tissue collection and removed from the floor pens. The broilers were killed by cervical dislocation. From each sexually immature broiler, 300 mg of testicular tissue was collected (n = 10).

Ten of the 62-week-old Ross broiler breeder roosters were killed by carbon dioxide asphyxiation. From each rooster, the left testicle was removed. The removed testicle was cut in half vertically, and a 300 mg sample of testicular tissue from the center of the testicle was collected (n = 10).

Each replicate sample of immature and mature testicular tissue was placed into 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The tissue solutions were stored at -80ºC for subsequent RNA extraction. After RNA extraction the RNA from 2 roosters in each age group were combined to create a total of 5 replicate samples (n = 5) for each age group.

4.3 Experiment 3- Broiler Breeder Hen Pituitary

The purpose of the third experiment was to examine the expression of corticosteroid binding globulin mRNA in the pituitary tissue of fed and fasted broiler breeder hens.

Animals

Cobb 500 fast feathering broiler breeder pullets were reared in floor pens from day 1 of age at the University of Georgia Poultry Research Center. They were provided a standard broiler breeder pullet diet on a skip a day feed restriction program. Ten percent of the pullets were randomly selected and weighed once per week in the rearing phase to determine feed allocation. This method was used to ensure that the body weight gain of the pullets matched the recommended guidelines of the primary breeder (Cobb-Vantress, 2005a). From placement on

day 1 until 21 weeks of age, the pullets received 8 hours of light. The lighting program was adjusted to provide 14 hours of light per day for photostimulation at 21 weeks of age. At time of photostimulation, the hens were provided a standard broiler breeder layer diet on an every day basis. The amount of feed provided to the hens every day was determined using the guidelines of the primary breeder (Cobb-Vantress, 2005a; Cobb-Vantress, 2005b), which are based on the weekly body weight measurements and egg production rates of the hens. At 40 weeks of age, a group of hens were removed from floor pens and placed into individual cages, where egg laying was monitored and feed continued to be provided on a daily basis until pituitary tissue was collected roughly two weeks later. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Tissue Collection

Twenty-eight caged hens were selected for tissue collection based on records indicating consistent egg laying patterns. The hens were divided into two treatment groups. The fourteen hens placed in the fed treatment group continued to receive their daily feed allotment, and in the fasted treatment group, the remaining fourteen hens did not receive any feed. At the time of tissue collection, the broiler breeder hens were 42 weeks of age. In each hen, the cranial portion of the skull was carefully removed to display the area beneath the hypothalamus, which allowed for collection of the pituitary gland. Within each treatment, the pituitary gland tissue collected from one hen was combined with the pituitary gland tissue from another hen, meaning that each replicate tissue sample contained the pituitary gland tissues from two hens in the same treatment. Each replicate sample of pituitary gland tissue was placed into 1.5mL of guanidinium isothiocyanate solution (Chomczynski, 1987) and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The tissue solutions were stored at -80ºC

for subsequent RNA extraction. A total of seven replicate samples of pituitary gland tissue was collected for each treatment (n=7).

4.4 Experiment 4- Follicular Tissue

The aim of the fourth experiment was to investigate the expression pattern of corticosteroid binding globulin mRNA in the granulosa and theca tissue layers of hierarchical and prehierarchical follicles of fed and fasted broiler breeder hens. This experiment was completed in two parts. Part one investigated expression levels in fed follicular tissues and part two investigated the relative differences in corticosteroid binding globulin expression between fed and fasted granulosa tissue.

Animals

Management of the Cobb 500 fast feathering broiler breeder pullets and hens was as described in Experiment 3. At 42 weeks of age, 40 hens were placed into individual cages to monitor individual egg production.

Tissue Collection

Tissue was collected from the hens when they were between 45 and 52 weeks of age. At each collection, 4 hens were utilized that had been fed either 5 or 72 hours earlier. Hens were killed by cervical dislocation 2 to 4 hours prior to ovulation, as evidenced by the presence of a hard-shelled egg in the shell gland. From each hen, the ovary was removed. The four largest hierarchical follicles, F1, F2, F3, and F4, the small yellow follicles (SYF, >5 to 12 mm in diameter), and the large white follicles (LWF, <2-5 mm in diameter) were gathered from each collected ovary. In each hierarchical follicle (F1-F4), the granulosa cell layer was manually separated from the theca cell layer (Huang and Nolbandov, 1979). However, in the prehierarchical follicles (SYF and LWF), the theca and granulosa cell layers were separated enzymatically (Davis et al., 2000). Within each treatment, the individual theca and granulosa

tissues for each follicle size collected from one hen were combined with the corresponding samples from another hen, meaning that each replicate tissue sample contained the tissues from two hens in the same treatment. The theca layers collected from the hierarchical follicles and the theca layers enzymatically separated from the individual pools of prehierarchical follicles were placed into 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The single cell layer of granulosa tissue from each hierarchical follicle and the granulosa cells enzymatically separated from the individual pools of prehierarchical follicles were placed into 2 mL of guanidinium isothiocyanate solution and vortexed for 20 seconds. The tissue solutions were stored at -80ºC for subsequent RNA extraction. This collection procedure was repeated until a total of three replicate follicular tissue sets were obtained for each treatment (n=3).

4.5 RNA Extraction

Total RNA was extracted from the tissue samples using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski, 1987). Samples of isolated RNA were stored at -80°C. The integrity of each RNA sample was assessed by the presence of intact bands for 28S and 18S rRNA on a 1.5% agarose gel stained with ethidium bromide.

4.6 Real Time RT - PCR

To remove any potential genomic DNA contamination, the extracted RNA samples were DNase treated using the TURBO-DNA-free kit (Ambion, Austin, TX). Taqman minor groove binding (MGB) probes and primers designed to specifically detect chicken SerpinA6 (corticosteroid binding globulin, GenBank accession # KU180444), and the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession # M11213) were generated using Primer Express software version 2.0 [Applied Biosystems, Foster City, CA].

The forward primer sequence for SerpinA6 (corticosteroid binding globulin) was 5' CTTTTGCCTATGGCCAGCTT-3' while the reverse primer sequence was 5'GGTCTTTAGGTTCATTTGGATCGT-3'. The probe sequence for SerpinA6 was 5'AGCCAGCCACTACAA-3'. The forward primer for GAPDH was 5'GACGTGCAGCAGGGAACACTA-3' and the reverse primer was 5'CCTCTGTCATCTCTCCACAGC-3'. The probe sequence for GAPDH was 5'TGACCACTG TCCATGCCAT-3'. The primers and Taqman MGB probes were synthesized by Applied Biosystems. Each probe was labeled at the 5' end with FAM (6-carboxyfluorescein), the reporter dye, and at the 3' end with TAMRA (6-carboxy-N, N, N', N'-tetramethylrhodamine), the quencher dye. Validation of the primer and MGB probe sets for real-time PCR was completed by determining the optimal amplification efficiency and primer/probe concentration as described by the manufacturer (Applied Biosystems).

Synthesis of cDNA by reverse-transcription reactions was completed using the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol. Two step real-time PCR, amplification of SerpinA6 utilized 400 ng of cDNA generated from duodenum, jejunum, ileum, gizzard, liver, spleen, kidney, gastrocnemius (leg muscle), pectoralis major (breast muscle), heart, pituitary, testicular, and ovarian follicular RNA samples. Amplification of GAPDH utilized 75 ng of cDNA from all tissue samples. All PCR reactions utilizing 400 ng of cDNA were performed in a 50 uL volume, and PCR reactions utilizing 75 ng were also performed in a 50 uL volume. Both reaction volumes consisted of reaction buffer containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the appropriate primer pair, and 25 nM of the appropriate probe. An ABI 7500 Thermocycler (Applied Biosystems) was used to complete the real-time RT-PCR reactions. 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 reactions for each sample were performed in duplicate for SerpinA6 and GAPDH assays. The CT (the cycle number at which the fluorescence exceeds the threshold level) was determined for each reaction using the Sequence Detection software (version 1.2.2, Applied Biosystems), and quantification was completed using the 2-ΔΔCt method (Livak and Schmittgen, 2001). The CT values for SerpinA6 were determined for each sample and, subsequently, normalized to the GAPDH CT value from the same sample (SerpinA6 CT - GAPDH CT = ΔCT). After the ΔCT values for all reactions were obtained for an experimental replicate, the ΔCT values for each individual SerpinA6 reaction were compared to the sample within the replicate that had the highest mRNA expression for SerpinA6 using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Therefore, all data for SerpinA6 is expressed as the fold-difference relative to the sample with the highest expression.

4.7 Statistics

For each experiment, the data were subjected to ANOVA using the General Linear Model procedure. Tukey's multiple-comparison procedure (Netter et al., 1990) was used to detect significant differences among individual tissues and follicle sizes. Differences were considered significant when $P < 0.05$. All statistical procedures were completed with the Minitab statistical software package (Release 17, State College, PA).

Chapter 5

Results

5.1 Experiment 1

The purpose of this experiment was to determine the tissue distribution of corticosteroid binding globulin (SerpinA6) mRNA in key metabolic and digestive tissues in growing broilers. Hepatic tissue contains significantly more corticosteroid binding globulin mRNA than any other tissue tested (Table5.1). When the abundant hepatic expression of corticosteroid binding globulin was excluded from the statistical analysis and the mRNA expression of corticosteroid-binding globulin was compared in the remaining tissues, ileum had greater expression of corticosteroid binding globulin mRNA than the gizzard and kidney (Figure 5.1).

5.2 Experiment 2

The aim of the second experiment was to investigate corticosteroid-binding globulin mRNA expression in the testicular tissues of sexually immature male broilers and sexually mature broiler breeder roosters. The mRNA transcript for SerpinA6 was expressed in mature testes tissue of broiler roosters, but no expression was detected in the immature testes of broilers (Table 5.2 and Figure 5.2).

5.3 Experiment 3

The purpose of the third experiment was to examine the expression of corticosteroid-binding globulin mRNA in the pituitary tissue of fed and fasted broiler breeder hens. The mRNA transcripts encoding for SerpinA6 were not detected by real time RT-PCR in the pituitary tissue of fed or fasted broiler breeder hens.

Table 5.1. The relative fold expression of SerpinA6 mRNA in the tissue of broilers at 35 days of age[1].

Tissue	Relative fold expression
Liver	794.26 ± 282.03[a]
Gizzard	0.12 ± 0.06[b]
Duodenum	0.22 ± 0.08[b]
Jejunum	0.30 ± 0.15[b]
Ileum	0.49 ± 0.02[b]
Kidney	0.17 ± 0.06[b]
Spleen	Not detectable
Cardiac muscle	Not detectable
Pectoralis major muscle	Not detectable
Gastrocnemius muscle	Not detectable

[1]The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). [a-b]Values with different superscripts for a given tissue differ, ($P < 0.05$).

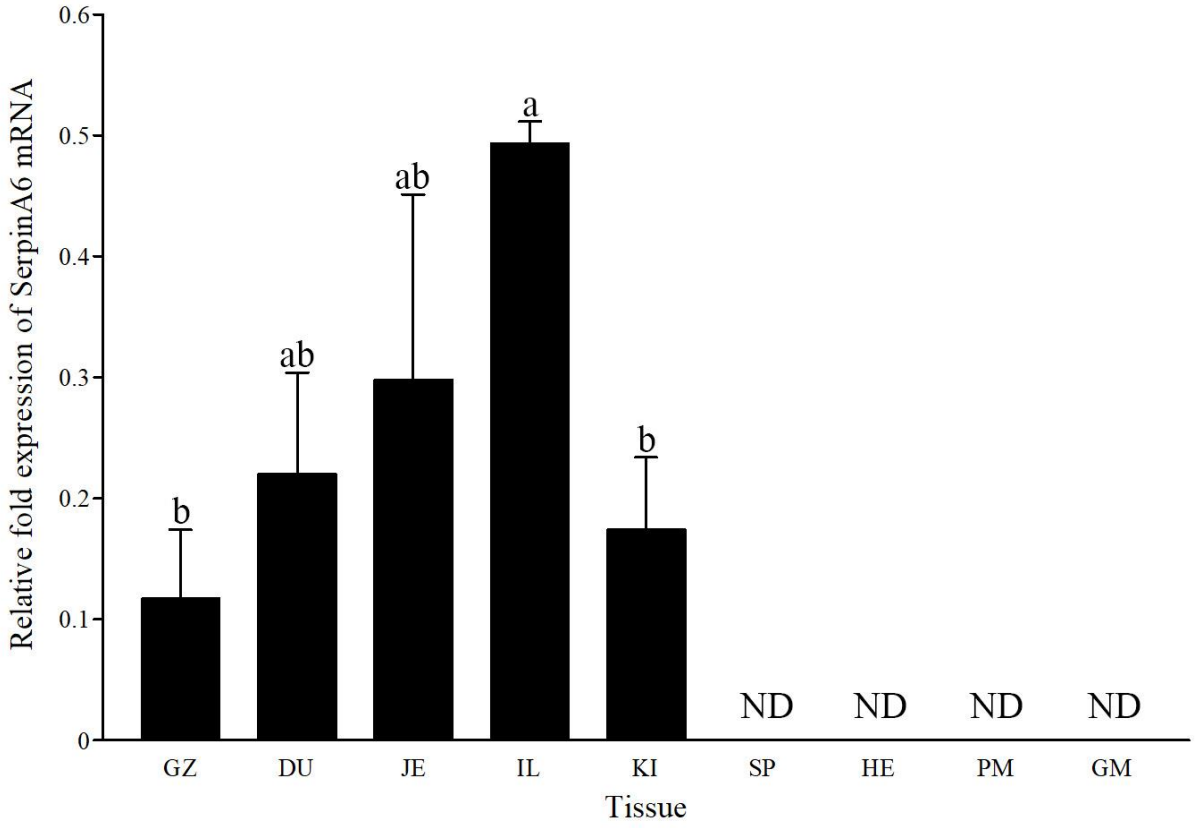

Figure 5.1. The relative fold expression of SerpinA6 mRNA in the tissue of broilers at 35 days of age when the expression found in liver is not included. The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). $^{a-b}$Values with different superscripts for a given tissue differ, ($P < 0.05$). Abbreviations: GZ (gizzard), DU (duodenum), JE (jejunum), IL (ileum), KI (kidney), SP (spleen), HE (heart), PM (pectoralis major muscle) GM (gastrocnemius muscle) and ND (not detectable).

Table 5.2. The relative fold expression of SerpinA6 mRNA in immature and mature broiler testes[1].

Tissue	Relative fold expression
Mature testes	0.40 ± 0.16
Immature testes	Not detectable

[1]The values are means ± SEM, n = 5 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$).

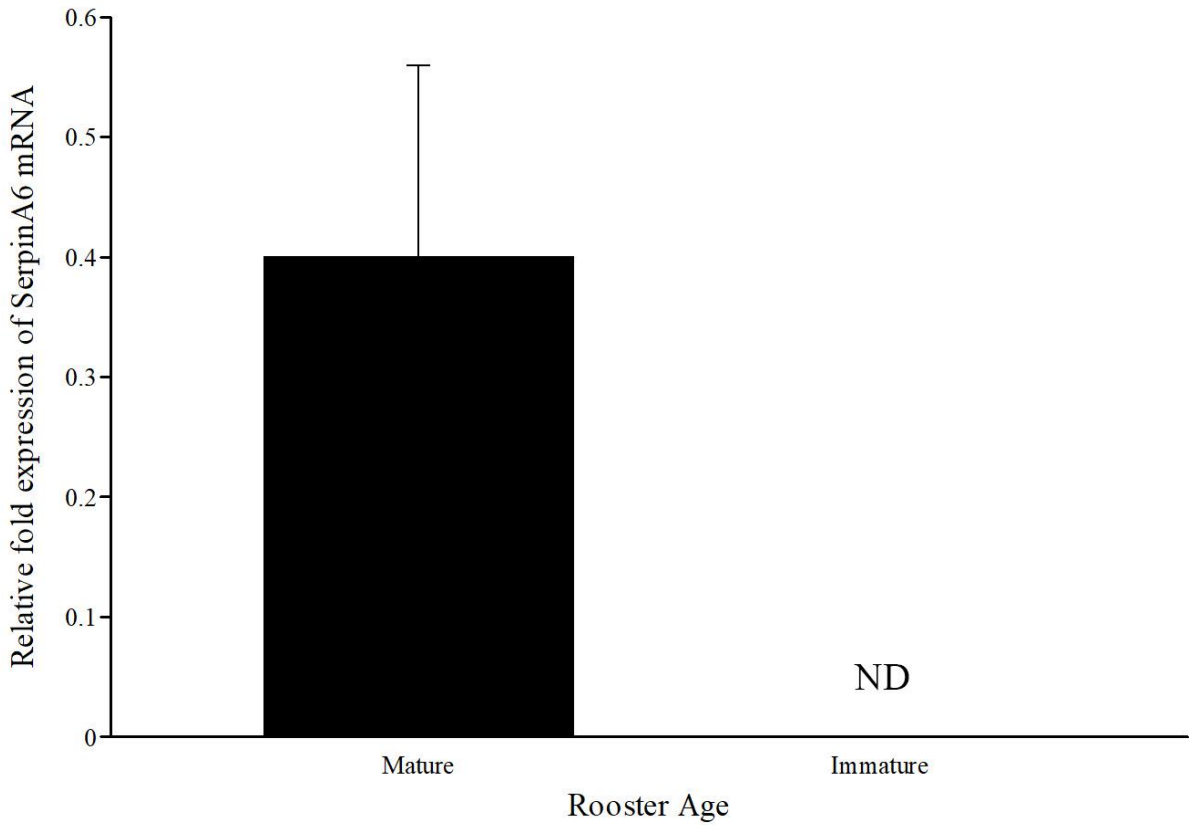

Figure 5.2. The relative fold expression of SerpinA6 mRNA in mature and immature broiler testes. The values are means ± SEM, n = 5 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). Abbreviation: ND (not detectable).

5.4 Experiment 4: Part 1

The aim of part 1 for the fourth experiment was to investigate the expression patterns of SerpinA6 mRNA in the hierarchical and prehierarchical follicular tissues of fed broiler breeder hens. The mRNA transcript encoding for SerpinA6 was not detected in the granulosa or theca tissue of the prehierarchical follicles or the theca tissue of the hierarchical follicles (Table 5.3 and Figure 5.3).

5.5 Experiment 4: Part 2

The aim of part 2 of the fourth experiment was to investigate the relative differences in SerpinA6 mRNA expression in follicular tissue from fed and fasted broiler breeder hens. Similar to what was found in fed broiler breeder hens, the mRNA transcript encoding for SerpinA6 was not detected in the granulosa or theca tissue of the prehierarchical follicles or the theca tissue of the hierarchical follicles of fasted hens. However, the overall expression of SerpinA6 mRNA transcript in the granulosa tissue of the four largest follicles (F1-F4) was significantly greater than in fed hens (Table 5.4 and Figure 5.4). When examining the expression of corticosteroid binding globulin mRNA in the granulosa layer of different sized prehierarchical follicles, it was greater in the F1, F3 and F4 follicles of fasted broiler breeder hens than fed broiler breeder hens (Table 5.5 and Figure 5.5).

5.6 Summary of relative tissue expression

This mRNA expression study was completed to provide the relative expression level of SerpinA6 mRNA between reproductive tissue and digestive tissue. The mRNA expression level of SerpinA6 is equivalent in broiler ileum and broiler breeder testes and F2 granulosa tissue of fed hens (Table 5.6 and Figure 5.6).

Table 5.3. The relative fold expression of SerpinA6 mRNA in theca or granulosa tissue collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles, and the largest white follicles from 45 to 52 week old broiler breeder hens fed daily[1].

Tissue	Relative fold expression
F1 theca	Not detectable
F1 granulosa	0.23 ± 0.07
F2 theca	Not detectable
F2 granulosa	0.11 ± 0.06
F3 theca	Not detectable
F3 granulosa	0.02 ± 0.01
F4 theca	Not detectable
F4 granulosa	0.34 ± 0.33
Small yellow follicle theca	Not detectable
Small yellow follicle granulosa	Not detectable
Large white follicle theca	Not detectable
Large white follicle granulosa	Not detectable

[1]The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$).

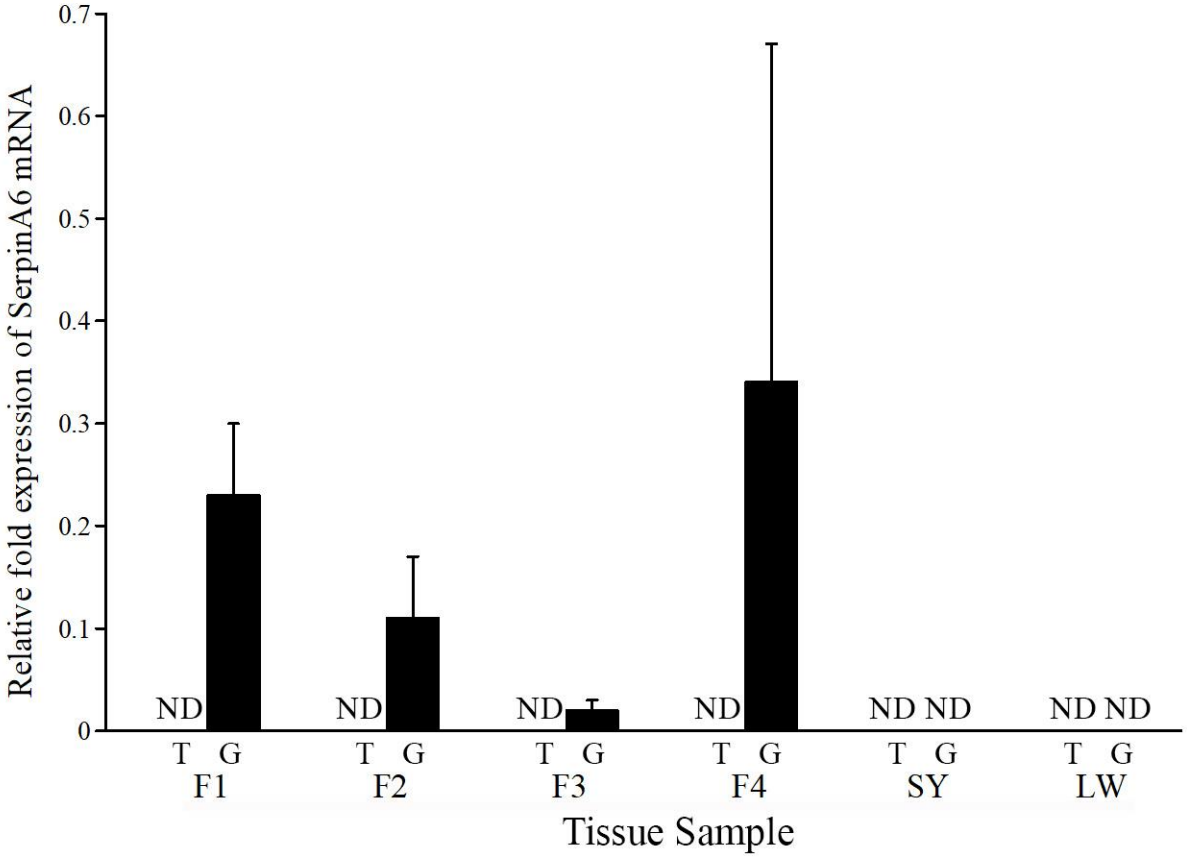

Figure 5.3. The relative fold expression of SerpinA6 mRNA in theca (T) or granulosa (G) tissue collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles, and the largest white follicles from 45 to 52 week old broiler breeder hens fed daily. The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). Abbreviation: ND (not detectable).

Table 5.4. The overall relative fold expression of SerpinA6 mRNA in the granulosa tissue collected from the four largest follicles (F1 through F4) from 45 to 52 week old broiler breeder hens fed daily or fasted for 72 hours[1].

Feeding state	Relative fold expression
Fed	0.09 ± 0.04^{b}
Fasted	0.69 ± 0.27^{a}

[1]The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n =12 (3 samples from each of the 4 follicle sizes). [a-b]Values with different superscripts for a given feeding state differ, ($P < 0.05$).

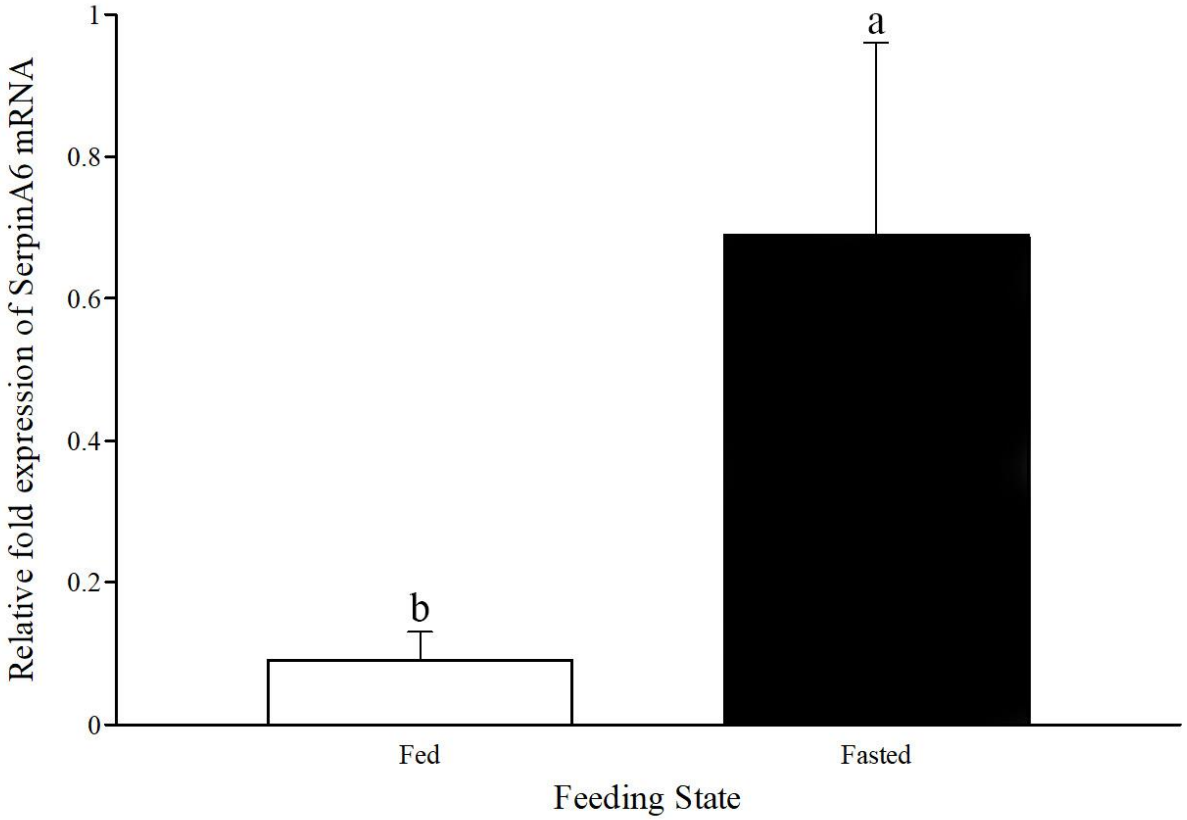

Figure 5.4. The overall relative fold expression of SerpinA6 mRNA in the granulosa tissue collected from the four largest follicles (F1 through F4) from 45 to 52 week old broiler breeder hens fed daily or fasted for 72 hours. The values are means ± SEM, n =12 (3 samples from each of the 4 follicle sizes). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). $^{a-b}$Values with different superscripts for a given feeding state differ, ($P < 0.05$).

Table 5.5. The relative fold expression of SerpinA6 mRNA in the granulosa tissue collected from the four largest follicles (F1 through F4) from 45 to 52 week old broiler breeder hens fed daily or fasted for 72 hours[1].

Tissue and feeding state	Relative fold expression
F1 granulosa fed	0.16 ± 0.08^{b}
F1 granulosa fasted	0.77 ± 0.12^{a}
F2 granulosa fed	0.08 ± 0.03^{b}
F2 granulosa fasted	0.27 ± 0.16^{ab}
F3 granulosa fed	0.04 ± 0.02^{b}
F3 granulosa fasted	0.42 ± 0.08^{a}
F4 granulosa fed	0.06 ± 0.02^{b}
F4 granulosa fasted	0.81 ± 0.19^{a}

[1]The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broiler breeder hens. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). [a-b]Values with different superscripts for a given parameter differ, ($P < 0.05$).

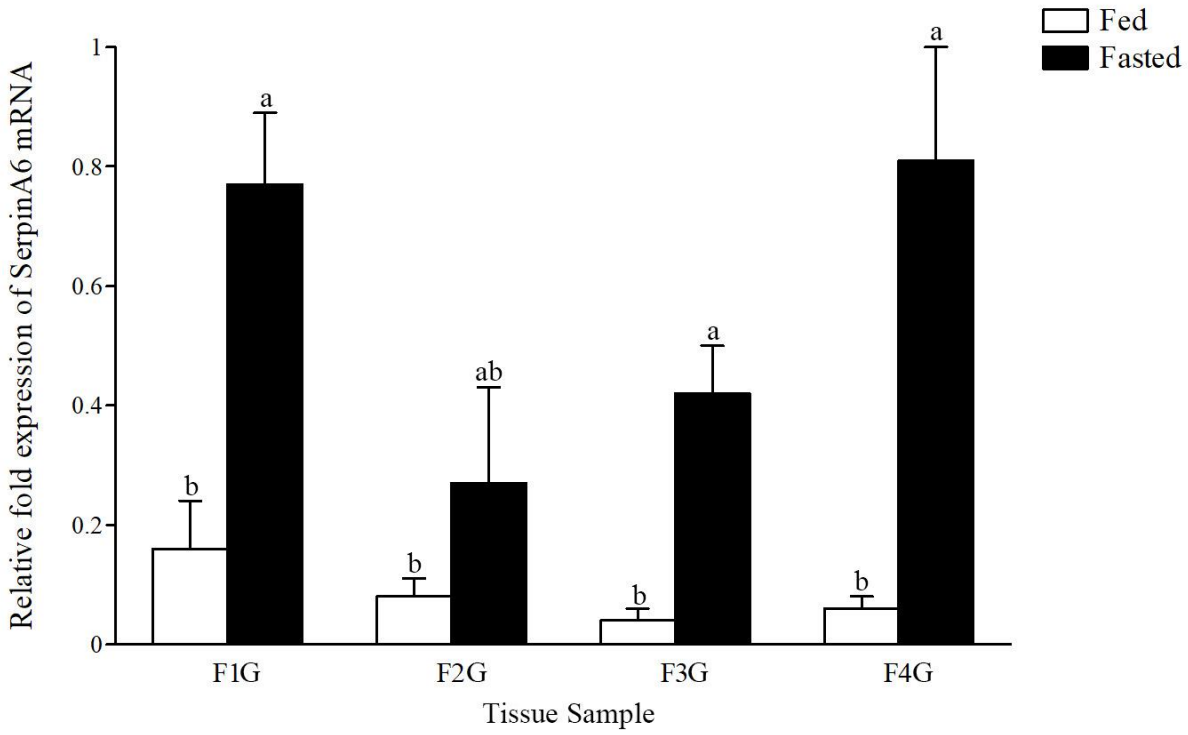

Figure 5.5. The relative fold expression of SerpinA6 mRNA in the granulosa (G) tissue collected from the four largest follicles (F1 through F4) from 45 to 52 week old broiler breeder hens fed daily or fasted for 72 hours. The values are means ± SEM, n =3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). $^{a-b}$Values with different superscripts for a given tissue differ, ($P < 0.05$).

Table 5.6. The relative fold expression of SerpinA6 mRNA in reproductive tissue and intestinal tissue[1].

Tissue	Relative fold expression
Mature Testes	0.56 ± 0.23
F2 granulosa	0.20 ± 0.04
Pituitary	Not detectable
Ileum	0.49 ± 0.02

[1]The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$).

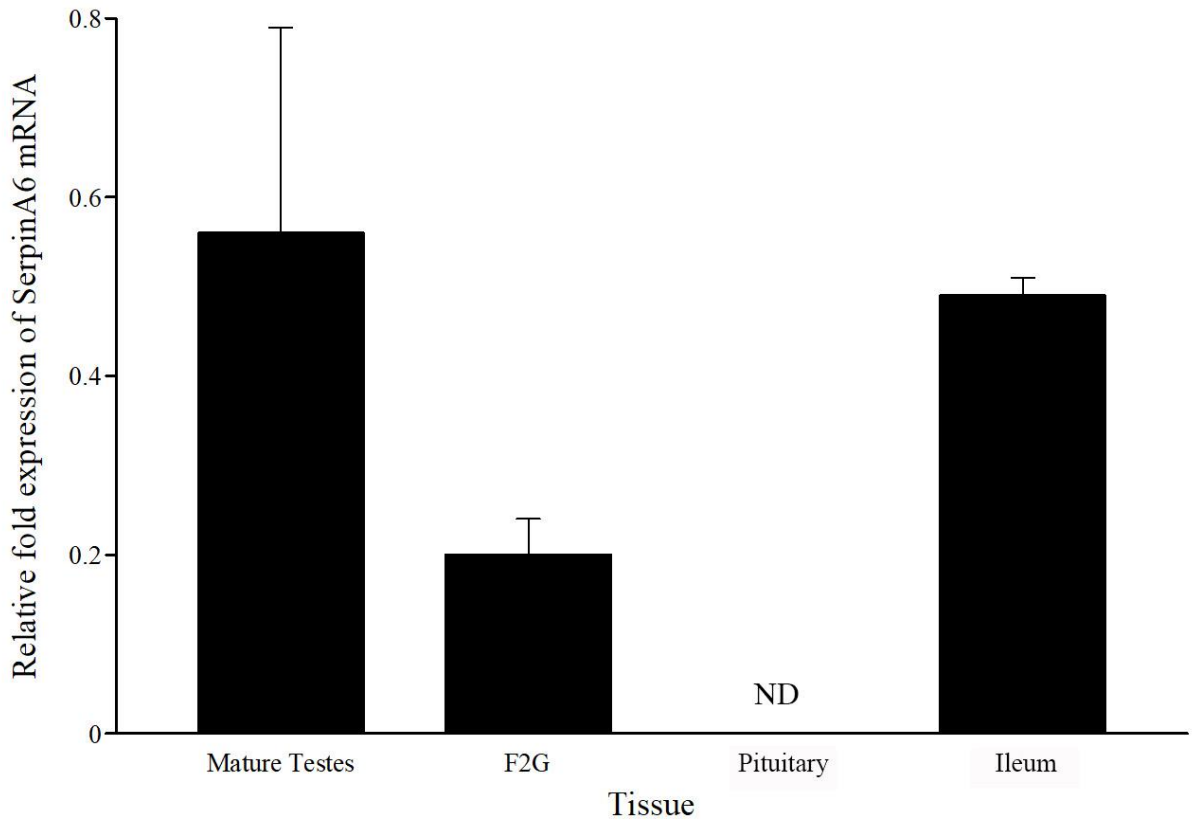

Figure 5.6. The relative fold expression of SerpinA6 mRNA in reproductive tissue and ileum from boilers. The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$).

Chapter 6

Discussion

The present research is the first to characterize the mRNA expression of SerpinA6 in poultry. Vashchenko et al. (2016) reported that in adult Zebra finches the mRNA expression of SerpinA6 was about 1000 times greater in hepatic tissue than kidney, ovary, testis, spleen, and lung, with no mRNA expression being detected in muscle. In the current research, the SerpinA6 mRNA expression profile was similar in broilers but was also expanded to include digestive tissues which expressed the SerpinA6 transcript. In addition, in the current research ovarian expression was investigated in much greater detail than the previous whole ovary expression (Vashchenko, 2016), and this revealed that it is the granulosa cells rather than the theca cells of the developing follicle that expresses SerpinA6 mRNA. Furthermore, SerpinA6 mRNA expression is limited to the largest hierarchical follicles and not detected in the prehierarchical follicles. Similarly, there was a lack of expression of corticosteroid binding globulin mRNA in immature testes, while expression was found in mature testes. The expression of SerpinA6 in several tissues throughout the body in poultry was not unexpected because it has been found in similar tissues in mammalian species (Hammond et al., 1987; Scrocchi et al., 1993). Furthermore, in mammalian species, SerpinA6 mRNA expression is very abundant in hepatic tissue relative to other tissues. (Hryb et al., 1986; Singer et al., 1988; Strel'chyonok and Avvakumov, 1991; Maitra et al., 1993).

The role of glucocorticoids in mediating the stress response in vertebrate species is well established. In birds, corticosterone is the major functional glucocorticoid regulating the stress

response and metabolism. Corticosteroid binding globulin binds corticosterone in birds but also binds with high affinity testosterone and progesterone. Plasma corticosteroid binding globulin is predominately synthesized by the liver. With corticosterone's role in metabolism and stress along with testosterone and progesterone being critical to reproductive function, it is not surprising that corticosteroid binding globulin's capacity to bind these hormones varies across bird species, bird populations, season, life history, and sex as reviewed by Malisch and Breuner (2010). However, it is well established that stress in both birds and mammals decreases corticosteroid binding globulin and increases free corticosteroid levels within minutes to hours following acute stress or the onset of chronic stress (Fleshner et al., 1995; Spencer et al., 1996; Deak et al., 1999; Tinnikov, 1999; Lynn et al., 2003; Malisch and Breuner, 2010; Li et al., 2017) which could make corticosteroid binding globulin a critical regulator of metabolism, reproduction, and stress in feed restricted broiler breeders that needs further investigation based on the current research findings.

Although the liver is the predominant source of plasma corticosteroid binding globulin, it is found intracellularly in the cells of several tissues, and it is unclear if it is presence there is the result of local synthesis, or endocytosis (Hammond et al., 1987; Scrochi et al., 1993a,b; Berdusco et al., 1995; Hammond, 1995; Misao et al., 1995; 1999; Seralini, 1996; Benassayag et al., 2001). Binding sites for the corticosteroid binding globulin complexed with it ligand have been located on cell membranes in multiple tissues (Hryb et al., 1986; Singer et al., 1988; Maitra et al., 1993) which could facilitate the endocytosis of these complexes into the cytoplasm. Given the widespread tissue distribution of the mRNA for corticosteroid binding globulin in the current research local production seems very plausible. Corticosteroid binding globulin synthesized within a cell may function to bind free corticosteroids entering the cell, and thus preventing it

from activating intracellular receptors. Berdusco et al. (1995) reported that corticosteroid binding globulin in ovine pituitary cells interferes with corticosteroid negative feedback. It is possible that a similar phenomenon is occurring in the granulosa cells of the hierarchical follicles of fasted broiler hens. Caloric insufficiency from fasting induces follicular atresia of the hierarchical follicles within 24 to 36 hours in laying hens and 72-96 hours in broiler breeder hens (Freeman, 2008). In addition, fasting also increases plasma corticosterone levels after 24 hours (Neves, 2012) in broiler breeders. Thus, the increase in corticosteroid binding globulin mRNA expression in the granulosa cells of fasted hens may be binding increased levels of corticosterone entering the granulosa cells, and thereby preventing/delaying corticosterone from initiating apoptosis and subsequent follicular atresia in case caloric intake resumes.

The lack of corticosteroid binding globulin mRNA expression in the pituitary of fed or fasted broiler breeder hens was surprising given that it had been detected in the ovine pituitary (Berdusco et al., 1995). In addition, Stephens (2017) reported an increase in gonadotropin inhibitory protein mRNA expression in the pituitary of broiler breeder hens fasted for 72 hours and corticosterone is a positive regulator for the production of gonadotropin inhibitory hormone in avian species. Gonadotropin inhibitory hormone is a neuropeptide that was first isolated from the hypothalamus of the Japanese quail and it negatively regulates gonadotropin release from the anterior pituitary (Tsutsui et al., 2000). In Japanese quail, Son et al. (2014) found gonadotropin inhibitory hormone neurons in the hypothalamus express glucocorticoid receptor mRNA and determined that, when treated with corticosterone for 24 hours, these diencephalic tissues displayed an increase in gonadotropin inhibitory hormone mRNA expression. Additional research also indicates that stress in avian species can induce gonadotropin inhibitory hormone production and biological actions (Calisi et al., 2008; Ernst et al., 2016). The current research

suggests that corticosterone action on gonadotropin inhibitory hormone synthesis in the anterior pituitary would not be regulated by local production of corticosteroid binding globulin.

6.1 Summary

In avian species, corticosteroid binding globulin plays a unique physiological regulatory role because it binds with high affinity the major stress hormone (corticosterone) as well as the critical sex hormones testosterone and progesterone. As a transport protein, it extends the half-life of these hormones and provides for the targeted release of these hormones at target tissues allowing the free hormones to diffuse into cells to cause biological responses. The current research suggests that localized granulosa cell production of corticosteroid binding globulin may serve to prevent free corticosterone that enters these cells from binding to its receptor and triggering a response such as atresia. Based on the current research and other reported research in avian species, it is becoming clear that corticosteroid binding globulin plays a large and active role in the stress response and reproduction, and deserves further research.

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