PLASMA CORTICOSTERONE CONCENTRATIONS AND FOLLICULAR GLUCOCORTICOID RECEPTOR mRNA EXPRESSION IN BROILER BREEDER HENS AS INFLUENCED BY DIETARY TRYPTOPHAN SUPPLEMENTATION OR FEEDING PROGRAM

by

DUARTE RIBEIRO E SILVA DE ALMEIDA NEVES

(Under the Direction of Adam Davis)

ABSTRACT

Tryptophan, as a precursor for serotonin, may alleviate behavioral stress when supplemented above the requirement for maintenance, growth and production in chickens. Broiler breeder hens were fed a diet supplemented with 0.05% crystalline tryptophan to determine if it would enhance reproductive performance and reduce stress as indicated by plasma corticosterone levels. At 29 weeks of age, 96 birds were split into 2 groups and placed into individual cages. At 35 weeks of age, one group was maintained on a broiler breeder diet containing 0.20% tryptophan while the other group was fed this diet supplemented with 0.05% tryptophan until 62 weeks of age. Tryptophan supplementation had no effect on egg production, but it decreased plasma corticosterone concentration. In another experiment, plasma corticosterone was measured in broiler breeder pullets fed during rearing either on a skip-a-day or everyday basis until they were 21 weeks of age. All pullets received the same amount of feed over each 48 hour period. At 21 weeks of age pullets were distributed into laying pens and placed on an everyday feeding schedule. At 28 weeks of age follicular mRNA expression of the glucocorticoid receptor was evaluated in the hens. Skip-a-day feeding elevated plasma corticosterone concentrations on the days the birds were not fed. After the hens were all placed on an everyday feeding schedule

plasma corticosterone concentrations did not differ between the two groups of hens. However, overall glucocorticoid receptor mRNA expression was lower in hierarchical follicles from skip-a-day hens relative to those that had been fed everyday during rearing. The results indicate that SAD feeding, during rearing, may lead to periods of stress as indicated by plasma corticosterone levels and this may lead to subsequent down-regulation of follicular glucocorticoid receptor mRNA expression. Dietary tryptophan supplementation may alleviate stress associated with restrictive feeding in broiler breeders.

INDEX WORDS: ovary, skip-a-day feeding, stress

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DEDICATION

I dedicate this dissertation to my parents Maria Filomena and Luis Manuel, and my siblings Maria Joana and Diogo.

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CHAPTER 1

AVIAN FEMALE REPRODUCTIVE PHYSIOLOGY

The Avian Ovary

The sexually active avian ovary contains a visually evident hierarchy of follicles arranged according to size and thus time before ovulation. In the laying hen, the four to six large, yolk-filled follicles that are between 12 to 40 mm in diameter are referred to as the hierarchical follicles. The large, yellow hierarchical follicles are named according to size with the largest one, which will typically ovulate within 24 hours, designated as the F1 follicle; the next largest follicle is the F2 follicle and will ovulate in about 48 hours, and so on for the other follicles. The development of these hierarchical follicles is tightly regulated with an interval of 24-26 hours between each consecutive ovulation.

After the F1 follicle ovulates, succeeding follicles advance one place in the hierarchy and an additional follicle is recruited into the hierarchy from the prehierarchical follicles. The prehierarchical follicles are also categorized by size and there are several follicles in each category. Small yellow follicles (SYF) are 5 to 12 mm in diameter, the large white follicles (LWF) are 2-5 mm in diameter, and the small white follicles (SWF) are less than 2 mm in diameter. With each ovulation, a follicle is recruited to the hierarchy from the pool of small yellow follicles. In turn, some of the small white follicles will begin the uptake of yellow yolk and advance to the pool of small yellow follicles. It is estimated that only 5% of the growing prehierarchical follicles will mature to reach a size of 6-8 mm in diameter (Gilbert et al., 1983b). The vast majority of prehierarchical follicles undergo follicular atresia with the individual cells of the follicle dying by apoptosis (Johnson et al., 1996b).

Avian Follicular Tissues and Follicular Maturation

Distinct tissue layers surround each yolk-filled oocyte of the avian ovary. In each hierarchical follicle, the yolk-filled oocyte is surrounded by its plasma membrane, followed in subsequent order by the zona pellucida or inner perivitelline layer (IPVL), the granulosa cell layer, a basement membrane, and lastly the theca cell layer. The theca layer can be further subdivided into two tissue layers, the theca interna and externa. The theca tissue is highly vascularized, unlike the avascular granulosa tissue layer, and allows the transfer of yolk precursors from plasma to the developing follicles in the ovary (Etches and Cheng, 1981).

Follicular maturation is 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 pituitary glycoprotein hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), and is mediated in part by the expression of LH and FSH receptors in granulosa tissue. FSH receptor expression is highest in the granulosa cells of the SYF and the level of expression decreases with follicular maturation (Calvo and Bahr, 1983; Ritzhaupt and Bahr; 1987; You et al., 1996; Woods and Johnson, 2005). Theca cells express much fewer FSH receptors than granulosa cells and the level of FSH receptor expression in the theca cells does not vary significantly with follicular maturation (Etches and Cheng, 1981; Gilbert et al., 1985; You et al., 1996). In prehierarchical follicles, FSH promotes granulosa cell proliferation and maturation (Davis et al., 2000, 2001). FSH helps maintain the follicular hierarchy through prevention of atresia (Palmer and Bahr, 1992; Johnson et al., 1996b, 1999), induces LH receptor, steroidogenic acute regulatory protein (StAR) and P450 cholesterol side chain cleavage 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, 2001; Johnson et al., 2004). Collectively, these results suggest that the prehierarchical follicle that is more responsive to FSH, avoids apoptotic cell death, and is then recruited into the avian follicular hierarchy. From the pool of SYF, it is hypothesized that only one follicle has an upregulated level of FSH receptors, specifically in the granulosa layer, and it is likely the one that advances into the hierarchy (Woods and Johnson, 2005). However, this selective increase is the result, and not the cause, of follicular selection and very little is known about the nature of the mechanism responsible for up-regulating FSHR expression and responsiveness as reviewed by Johnson and Woods (2009).

As the selected follicle is recruited into the follicular hierarchy, the follicle transitions from being predominantly FSH dependent and becomes primarily LH dependent (Calvo and Bahr, 1983). LH receptor expression is highest in the granulosa cells of the large follicles, especially the F1 through F3 follicles (Calvo et al., 1981; Calvo and Bahr, 1983; Gilbert et al., 1983a, 1985; Johnson et al., 1996a). LH receptor mRNA expression in the theca tissue varies little with follicular development (Johnson et al., 1996a) and LH promotes steroidogenesis by the theca cells of prehierarchical and hierarchical follicles (Robinson et al., 1988; Kowalski et al., 1991). Decreased expression of the mRNA for the LH receptor in 3 to 5 mm diameter prehierarchical follicles is associated with atresia (Johnson et al., 1996a). LH promotes granulosa cell proliferation (Davis et al., 2000) and progesterone production (Davis et al., 1999; Johnson et al., 2004).

Avian Ovulation

The vascularized theca cells surrounding the SYF and LWF are steroidically competent and secrete androgens and estrogens via the Δ5 steroidogenic pathway (Lee et al., 1998) and these follicles are the primary source of plasma estrogen (Senior and Furr, 1975; Lee and Bahr, 1989, 1993). In contrast, the avascular granulosa cell layers of these SYF and LWF are steroidically incompetent due to a lack of P450 side chain cleavage enzyme activity (Li and Johnson, 1993). This enzyme initiates steroidogenesis by converting cholesterol to pregnenolone (Lee and Bahr, 1990; Tilly et al., 1991). Plasma concentrations of estrogen peak 4-6 hours before ovulation. Estrogen stimulates the hypothalamus and pituitary to express progesterone receptors (Wilson and Sharp, 1976). Estrogen also stimulates the liver to produce lipid and vitellogenin for yolk formation (Deeley et al., 1975) and aids in calcium metabolism for shell formation and medullary bone deposition (Etches, 1987). Estrogen increases the expression of its own receptor in the oviduct which increases estrogen's effects on oviductal functions (Takahashi et al., 2004). Estrogen also increases expression of the progesterone receptor in the ovary and oviduct, which promotes the formation of tubular secretory glands for albumen and shell secretion and the contractile activity of the myometrium (Yoshimura and Bahr, 1991).

Both the granulosa and theca cells of the hierarchical follicles are competent to produce steroids. The granulosa cells express P450 side chain cleavage enzyme activity (Li and Johnson, 1993; Kato et al., 1995) but very low levels of 17α-hydroxylase and thus utilize the Δ4 steroidogenesis pathway to produce progesterone (Etches and Duke, 1984; Robinson et al., 1988; Lee and Bahr, 1989, 1993; Johnson et al., 1996b; Lee et al., 1998). The theca cells of the hierarchical follicles also use the Δ4 pathway to produce progesterone, which they metabolize to androgens. The theca cells of the hierarchical follicles, with the exception of those of the F1

follicle, also metabolize the progesterone produced by the granulosa cells into androgens (Etches, 1990). In the F1 follicle, the theca cells do not metabolize the progesterone produced by the granulosa cells (Marrone and Hertelendy, 1985) and thus the F1 follicle is the primary source of plasma progesterone (Huang and Nalbandov, 1979).

Plasma progesterone produced by the F1 follicle binds to progesterone receptors in the hypothalamus to increase production and release of LHRH I, which increases the release of LH from the anterior pituitary as reviewed by Advis and Contijoch (1993). The released LH travels through the circulatory system to the ovary and binds to its receptors on the granulosa cells of the F1 follicle to stimulate more progesterone production thus creating a positive feedback loop that leads to a surge in LH and progesterone production that induces ovulation (Wilson and Sharp, 1976; Robinson and Etches, 1986). Plasma concentrations of progesterone peak 4 to 6 hours before ovulation. Progesterone and LH bind to their receptors in the cells along the stigma of the F1 follicle, which activates them to produce enzymes such as collagenase that degrade the tissue along the stigma and allow the rupture of the F1 follicle for ovulation (Isola et al., 1987; Yoshimura and Bahr, 1991).

Effects of Feed Restriction in Broiler Breeder Hens

Through genetic selection and better bird management, today's broilers reach a market weight of 2.5 to 3 kilograms in 5 to 6 weeks. To support this rapid growth rate, broilers have nearly insatiable appetites. These voracious appetites and rapid growth rates, however, are problematic for optimal reproductive performance in the genetically similar parent stocks of broilers. Optimum reproductive efficiency in broiler breeders 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 is a little less than

market size, the optimum sensitivity to photostimulation for reproduction in broiler breeders does not occur until about 20 weeks of age.

To prevent broiler breeder pullets from growing too quickly and becoming too large and obese by the photosensitivity-based sexual maturity that occurs at 20 to 21 weeks of age, their dietary intake is severely restricted. Typically, feed allocations are 60-80 percent less during the rearing period and 25-50 percent less during the laying period than what the breeder pullets/hens would consume ad libitum (Renema and Robinson, 2004). 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., 1992a; Heck et al., 2004; Bruggeman et al., 2005; Hocking and Robertson, 2005; Onagbesan et al., 2006), promotes flock body weight uniformity (Bennett and Leeson, 1989; de Beer and Coon, 2007) 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, 1987; Hocking et al., 1989; Heck et al., 2004; Hocking and Robertson, 2005). But more importantly, broiler breeder hens which have been feed restricted produce more eggs (Leeson and Summers, 1985; McDaniel et al., 1981; Yu et al., 1992a; 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., 1992a; Heck et al., 2004) compared to full-fed broiler breeder hens. Overweight broiler breeders have compromised fertility due to reduced locomotion and their physical difficulty in successfully copulating (Duff and Hocking, 1986). Fertility is reduced in overweight hens even when artificial insemination is

used (Brake and McDaniel, 1981) and this may be due to the fact that the fat may actually make the insemination more difficult or may block the sperm storage tubules or inhibit sperm movement (Hocking, 1987).

Despite the success of feed restriction in broiler breeder hens, these hens still produce annually over 100 eggs less than their Leghorn laying hen counterparts and follicular maturation and ovulation is still plagued by an unacceptable incidence of atresia of follicles and internal ovulations. It is currently unknown why broiler breeders fed ad libitum produce excessive numbers of follicles at one time and how feed restriction controls this process and improves egg production. Without this basic knowledge, it is not surprising that the degree of feed restriction and the timing and duration of feed restriction varies greatly among commercial broiler breeder management programs.

There are also conflicting research reports on the optimum timing and duration of feed restriction. Pym and Dillon (1974) reported that severe restriction during the rearing period followed by ad libitum feeding during the laying period could be the best feeding regimen for broiler breeder hens. Yu et al. (1992b) suggested that feed restriction should occur during both the rearing and breeding periods for optimum performance. Robbins et al. (1986, 1988) reported that restricting feed intake during the rearing period followed by ad libitum feeding during part or all of the laying period, increased egg production compared to birds which were feed restricted during both periods. Robinson et al. (1991b) reported however, that ad libitum feeding during the breeding period resulted in lower egg production. Finally, Bruggeman et al. (1999) indicated that ad libitum feeding from 1 to 7 weeks of age followed by feed restriction from 7 to 15 weeks of age followed by ad libitum feeding to first egg, resulted in improved reproductive

performance compared to any other combination of ad libitum or restricted feeding during the rearing period.

In the United States broiler breeder pullets are typically provided feed once every other day during rearing (skip-a-day feed regimen) and then once a day at photostimulation for reproduction, when the flock produces its first egg or reaches 5% egg production. This feed is quickly consumed by the birds and as a result they will fast for a significant portion of each day. 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 LH 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 LH 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).

Research supports the idea that the fasting periods created as a result of current poultry industry feed restriction practices depress total egg production in broiler breeder hens. Often in commercial settings, a skip-a-day feeding program is continued until the broiler breeder flock reaches 5% egg production. This is done to control flock body weight uniformity and to help control body weight gain, since even a very slight excess in 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 when the birds are photostimulated for reproduction at 21 weeks of age 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.

The research reported by Gibson et al. (2008) suggested the significant fasting period that 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 et al. (2008) except when the pullets were photostimulated for reproduction they were fed either once a day (equivalent to the everyday treatment) 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 improved the total number of eggs produced per hen through 41 weeks of age by 5 eggs and improved the overall percent hen day egg production through 59 weeks of age by 2%. 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 to 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 being in the twice a day feeding treatment while being in lay. 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 with broiler breeder roosters, the onset of testosterone production is delayed as the degree of feed restriction is increased during rearing (Stevens, 2010). Stevens' 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 suggesting 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; Sexton et al., 1989b; Cerolini et al., 1995; Bramwell et al., 1996; Romero-Sanchez et al., 2008).

In addition to the role fasting may have on reproduction through modification of gonadotropin secretion, birds that are subjected to longer fasting durations may be more stressed than those with shorter fasting times. Unlike mammals, corticosterone is the primary stress related glucocorticoid produced by the adrenal gland in avian species (Carsia and Harvey, 2000). Elevated plasma corticosterone levels have been detected in skip-a-day fed broilers during rearing (de Beer et al., 2008) and at the onset of lay (Ekmay et al., 2010). Although 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 et al., 2007). 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. Corticosterone and its ovarian receptor in avian species will be the focus of the next chapter.

Summary

Follicular development and maturation have been well studied in avian species due to the economic importance of egg production in commercial poultry operations and because the avian

ovary structure is ideal for such studies. The histological structure of avian preovulatory follicles which allow for manual and enzymatic separation of the theca and granulosa tissue layers, as well as the arrangement of the preovulatory follicles in a size hierarchy relative to ovulation, makes it an ideal model to determine how factors such as nutrition and stress may affect ovarian development and egg production. In broiler breeders, the role of nutrition and stress in reproduction is further complicated by the need to feed restrict broiler breeder hens to prevent excessive weight gain which is detrimental to the reproductive performance of the flock. However, the restrictive feeding programs, such as the skip-a-day feeding regimen, that have been implemented by the industry may themselves limit reproductive performance by creating lengthy fasting periods and stress.

CHAPTER 2

THE AVIAN GLUCOCORTICOID SYSTEM

Glucocorticoids

Corticosteroids are steroid hormones containing a 21 carbon cholesterol ring structure and are primarily produced in the cortex of the adrenal gland. Their synthesis is under the control of the hypothalamic-pituitary-adrenal (HPA) 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). Although recent 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). Glucocorticoids affect almost all tissues in the body, regulating a wide range of biologically essential functions such as metabolism, reproduction, immunity and behavior (Carsia and Harvey, 2000). However, despite having countless effects upon multiple systems the primary role of glucocorticoids is to facilitate glucose release for utilization throughout diverse 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 known as 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 hydroxlase 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 HPA 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 [(CRH) Harvey and Hall, 1990].

Released CRH stimulates corticotrophic cells of the anterior pituitary gland to produce and secrete adrenocorticotropic hormone (ACTH). ACTH is a 39 amino acid peptide that activates adrenocortical functions resulting in synthesis and secretion of corticosterone (Beuving and Vonder, 1978, 1986; Radke et al., 1985a,b) and aldosterone (Radke et al., 1985a, b). Both CRH (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 ACTH secretion from the pituitary gland in response to changes in the physiological status of the bird.

Circulating levels of ACTH 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 ACTH binds to G-protein coupled receptors which produce cAMP and activate protein kinases that stimulate mitochondrial steroidogenesis of glucocorticoids. Etches and Cunningham (1976) recorded an increase and decrease in plasma corticosterone concentration after administration of ACTH and dexamethasone, respectively, in laying hens. Secreted corticosterone provides a

negative feedback to the hypothalamus and the anterior pituitary gland to dampen the production and secretion of CRH and ACTH, respectively.

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 hold 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 (hsp90), hsp70, hsp90-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)

Glucocorticoid Receptor Characterization

The complete glucocorticoid receptor cDNA has been characterized and cloned in several species such as mouse (Francke, 1980), rat (Miesfeld et al., 1986), rabbit (James, 2003), trout (Gao, et al., 1994) human (Hollenberg et al., 1985) and chicken (Kwok et al., 2007). In chickens, glucocorticoid receptor mRNA has been detected in several avian tissues such as breast muscle, brain, testis, pituitary, lung, spleen, liver, intestine, ovary, heart, kidney and pancreas with the highest expression found in pituitary, ovary, kidney and muscle tissue (Kwok et al., 2007). More recently, Lattin et al. (2012) provided evidence in house sparrows supporting two corticosterone binding sites similar to mammalian systems in which there is the low-affinity glucocorticoid receptor and the high-affinity mineralocorticoid receptor. In brain, liver, kidney and testes both receptor types were present to bind corticosterone, but only glucocorticoid receptor binding was found in muscle, spleen, fat, ovary and skin.

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). Moreover, increased levels of glucocorticoids are known to suppress anabolic processes such as growth in birds (Mueller 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 higher plasma corticosterone concentrations than non-fasted controls. Similarly, 7 to 8 week old cockerels that were the progeny from Light Sussex crossed with Rhode 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, but feed was not. 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% of the ad libitum feed consumption of controls from hatching to 8 weeks of age had plasma corticosterone concentrations that were 73% 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.

Like food deprivation in Leghorn type breeds of poultry, 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 fed restricted to gain 50% 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 (Mench, 1991). 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. In a subsequent report from this research group (Ekmay et al., 2010), 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 et al., 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 LH and FSH concentrations as seen in laying hens (Johnson, 1981; Etches et al., 1984a) and turkey hens (Rozenboim et al., 2004). Furthermore, ovarian regression, decreased LH 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 VLDL 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.

Summary

Corticosterone, the primary avian glucocorticoid hormone, is a steroid hormone produced by the adrenal gland under the control of the HPA axis. 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 especially since the metabolic role of corticosterone is to shift nutrients to meet the needs of the prevailing stress (in this case fasting) state at the expense of anabolic activities such as yolk/egg formation.

CHAPTER 3

THE EFFECT OF TRYPTOPHAN ON AVIAN BEHAVIOUR

Introduction

Tryptophan is an aromatic amino acid (Figure 3.1) characterized by containing an indole functional group that consists of a benzene and pyrrole ring. It is an indispensable amino acid for all vertebrate species studied. Among the 20 amino acids that commonly constitute proteins, tryptophan is one of those found in the lowest proportion in proteins and plasma as reviewed by Le Floc'h et al. (2011). Despite its relatively low concentration in tissues, tryptophan is essential for protein synthesis and it is the metabolic precursor to several important biological compounds such as serotonin, melatonin and niacin.

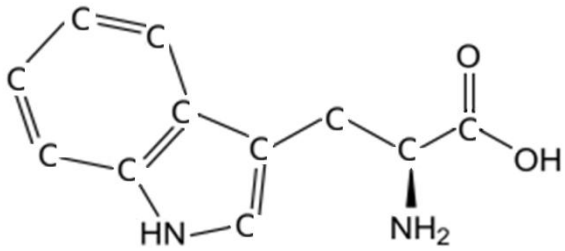

Figure 3.1. The chemical structure of L-tryptophan.

Metabolism

Niacin

The term "niacin" is often defined as nicotinic acid (Bourgeois et al., 2006) but it can be named as nicotinamide (nicotinic acid amide) and pyridine-3-carboxylic acid, which are water-soluble vitamins of the vitamin B complex and derivatives that exhibit the biological activity of nicotinamide (MacKay et al., 2012). The most important bioactive sources of niacin in feeds are

tryptophan and the pyridine nucleotides such as nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate [(NADP+), Shin et al., 1991; Markel, 2011].

To obtain niacin, tryptophan has to be degraded through the kynurenine pathway (Figure 3.2). The kynurenine pathway is the primary pathway for the catabolism of tryptophan. Tryptophan can be converted to N'-formyl-kynurenine in hepatic tissue by tryptophan 2,3-dioxygenase and in other tissues by indoleamine 2,3-dioxygenase. N'-formyl-kynurenine can then be converted to kynurenine by formamidase (Shin et al., 1991). Kynurenine is in turn converted to 3-hydroxykynurenine by kynurenine hydroxylase or kynurenine can also be converted to kynurenic acid by kynurenine aminotransferase. 3-hydroxykynurenine can be converted to 3-hydroxyanthranilic acid by kynureninase or it can be converted to xanthurenic acid or quinolinic acid. Kynurenic, xanthurenic and quinaldic acids can all be found in the plasma of vertebrates and they are excreted by the kidney via the urine, but they also have been implicated as mediators of immune function and cytotoxicity (Schwarcz and Pellicciari, 2002; as reviewed by Le Floc'h et al., 2011)

The niacin production pathway continues with 3-hydroxyanthranilic acid being converted by 3-hydroxyanthranilic acid oxidase to 2-amino-3-carboxymuconic-6-semialdehyde (ACMS), also known as 2-amino-3-(3-oxoprop-1-enyl)-fumaric acid. A spontaneous non-enzymatic cyclization changes ACMS into quinolinic acid (quinolinate) which is ultimately converted to NAD which can be converted to nicotinamide and nicotinic acid. However, ACMS can also be catabolized to 2-aminomuconic semi-aldehyde by ACMS decarboxylase (ACMSD) also known as picolinate carboxylase and the amount of niacin formed from tryptophan varies among species depending on the activity of hepatic picolinate carboxylase (Ikeda, 1965; Kirkland, 2007).

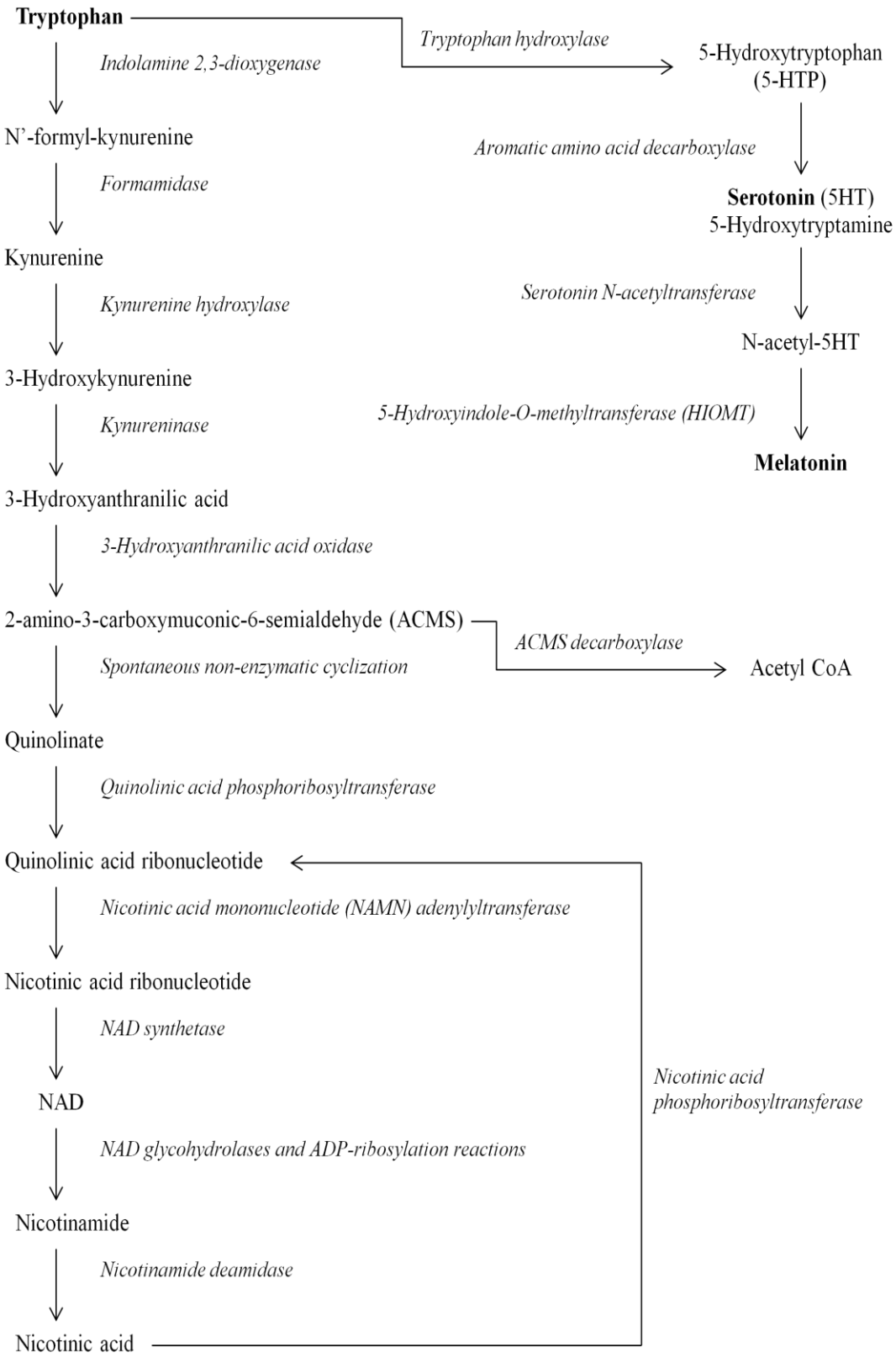

Figure 3.2. Tryptophan metabolism to nicotinic acid and serotonin.

For example, cats have very high levels of hepatic picolinate carboxylase and thus tryptophan cannot replace dietary niacin, but in other animals such as humans, if adequate dietary tryptophan is available niacin is not required.

The metabolic relationship between tryptophan and niacin was revealed by Krehl et al. (1945) in which dietary tryptophan levels above the requirement for protein accretion in rats led to the conversion of compounds that held niacin-like activity such as nicotinic acid mononucleotide. Subsequent research, reviewed by Combs (1992), confirmed that only a small fraction of tryptophan is converted to nicotinic acid mononucleotide. Estimates on the conversion efficiency (wt:wt) of tryptophan as a niacin precursor in both humans and rats are settled around 50:1 (Krehl et al., 1946; Goldsmith et al., 1961). Moreover, experiments in chicks confirmed a conversion efficiency (wt:wt) of 50:1 (Allen et al., 1971; Baker et al., 1973; Chen and Austic, 1989). Diets mainly composed of cereal grains without supplementation of tryptophan and niacin may cause a niacin deficiency as the levels of bioavailable niacin and tryptophan are low (Kodicek and Wilson, 1959; Goldsmith et al., 1961; Yen et al., 1971; Darby et al., 1975; Yen et al., 1977).

Serotonin

As reviewed by Chen and Miller (2012), the synthesis of melatonin begins with the hydroxylation of tryptophan by tryptophan hydroxylase (TPH), to 5-hydroxytryptophan (5-HTP) which is then decarboxylated to serotonin (5-hydroxytrptamine) by an aromatic amino acid decarboxylase also known as dopa decarboxylase enzyme (DDC) or 5-hydroxytryptophan decarboxylase. TPH is the rate limiting step of serotonin synthesis and there are two isoforms of THP. TPH1 is mostly expressed in enterochromaffin cells of the gut and the pineal gland, TPH2 is mainly expressed in the brain and enteric neurons (Walther and Bader, 2003; Walther et al.,

2003; Zhang et al., 2004; Zill et al., 2004; McKinney et al., 2005). In humans, both TPH protein isoforms share 71% identity in overall amino-acid sequence differing only in their solubility and kinetic properties (Walther and Bader, 2003; McKinney et al., 2005). About 95% of serotonin body content is present in the gut, found in mucosa cells of the gastrointestinal tract (Peroutka and Howell, 1994), where it acts as a major neuromediator involved in motility and secretory mechanisms as reviewed by Le Floc'h et al. (2011). Plasma serotonin from gut tissue is also taken up by blood platelets and thrombocytes (MacLean et al., 2000). Serotonin cannot cross the blood brain barrier but it is broadly synthesized in the central nervous system (CNS) where it acts as a neurotransmitter that regulates the central neuroendocrine system and is involved in such things as cognitive function, mood, behavior and food intake (Van de Kar, 1991; Peroutka and Howell, 1994; Le Floc'h et al., 2011). In the CNS serotonin is synthesized in the neuron's perikarya (Hamon et al., 1982) and maintained in neuronal vesicles to avoid immediate enzymatic oxidation to 5-hydroxy-indol acetic acid (5-HIAA) by monoamine oxidase (Jorgensen, 2007) until it is released into the synaptic cleft. Released serotonin activates post- and pre-synaptic receptors, is oxidized by monoamine oxidase or reabsorbed via the 5-HT transporter molecule into the neuron (Hamon et al., 1982).

Melatonin

Melatonin is synthesized from serotonin. The rate-limiting step is the conversion of serotonin to N-acetyl serotonin by the cytoplasmic enzyme serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase), which displays a circadian rhythm with increased activity during the dark phase (Klein and Weller, 1970; Klein et al., 1997). The last step in the biosynthesis of melatonin is catalyzed by 5-hydroxyindole-O-methyltransferase), which does not

exhibit circadian variations in its activity (Klein et al., 1997) and converts N-acetyl serotonin to melatonin.

Melatonin is mainly synthesized by the pineal gland. In the pineal gland, the synthesis of melatonin changes considerably based on the circadian rhythm of serotonin N-acetyltransferase such that the highest level of synthesis and secretion of melatonin occurs throughout the dark phase of the day. Thus, it is not surprising that serotonin functions as an important neuroendocrine signal that conveys both circadian and seasonal information to numerous organ systems, such as the brain as reviewed by Ruddick et al. (2006). Melatonin has an effect in many physiological and behavioural responses including sexual maturation, reproductive behavior, thermoregulation, sleep patterns, metabolism, hematopoiesis, immune responses, and reduction of oxidative stress as reviewed by Shedpure and Pati (1995) and Waldhauser et al. (1993).

Plasma Tryptophan

Unlike other amino acids, tryptophan circulates in blood primarily bound to albumin existing in an equilibrium between albumin-bound and free forms (McMenamy, 1965). Typically about 90% of total plasma tryptophan is bound to albumin, and this complex is not able to cross the blood brain barrier while unbound tryptophan is available for transport across the blood brain barrier into the brain (Madras et al., 1974). Factors such as non-esterified fatty acids or some drugs are able to alter the binding efficiency of tryptophan to albumin, however, whether or not the ability of tryptophan to bind albumin changes the availability of tryptophan for tissue metabolism is still controversial (Smith and Pogson, 1980; Pardridge, 1983).

Increasing levels of dietary tryptophan result in an elevation of plasma tryptophan as well as increased brain levels of tryptophan and serotonin in mammals (Culley et al., 1962; Wurtman

et al., 1981; Meunier-Salaün et al., 1991) and in fish (Johnston et al., 1990). Transport of tryptophan across the blood brain barrier is accomplished by a transporter that also transports large neutral amino acids (LNAA) such as leucine, valine, isoleucine, and to a lesser extent tyrosine, phenylalanine and methionine. Thus, an excess of these amino acids relative to tryptophan, can lead to lower brain tryptophan and serotonin concentrations.

Tryptophan and Poultry Production

Growth and production

As an essential amino acid tryptophan plays an important role in protein nutrition. In laying hen diets tryptophan is often considered to be the third-limiting amino acid, behind methionine and lysine (Peganova et al., 2003). The dietary requirement for Leghorn-type laying hens consuming 100 grams of feed per day is 0.16% (NRC, 1994) which closely agrees with subsequent research. Russell et al. (1999) indicated for Leghorns consuming 100 g of feed per day, that egg production, egg mass and egg content was maximized when feeding 157 mg per hen per day and similarly Deponti et al. (2007) reported that 161 mg per hen per day of tryptophan maximized egg production. Antar et al. (2004) reported that dietary tryptophan inclusion rates of 0.166, 0.176 and 0.193% in laying hens from 32 to 40 weeks of age did not differ in egg production and egg mass even though egg weight and feed intake were higher for 0.176% of dietary tryptophan. In addition, 31 week-old laying hens fed diets with 0.175, 0.195, 0.392 and 0.591% tryptophan until 47 weeks of age did not show differences in feed intake, egg weight, egg mass, feed conversion and mortality (Rech et al., 2010). However, other previous research reports on the tryptophan requirement for laying hens indicated a wide range of tryptophan intake up to 239 mg/hen per day is required to optimize or maximize performance in laying hens consuming 100 g of feed per day (Bray, 1969; Wethli and Morris, 1978; Ishibashi,

1985; Othani et al., 1989; Jensen et al., 1990). Given the wide range of physiological functions that tryptophan can have such as niacin production and immune function, it may not be surprising that tryptophan supplementation well above the NRC requirement may at times result in improved production. However, very high levels of dietary tryptophan supplementation 0.5 to 1% can decrease feed intake and egg production (Usami et al., 1992). In addition, oral administration of water containing tryptophan that pushes consumption well above the requirement also reduced feed intake (Lacy et al., 1982, 1986).

Research on the requirement for dietary tryptophan in broiler breeders is very limited. For broiler breeder hens, the dietary requirement was set at 0.19% (NRC, 1994) based on mathematical models and limited research. Wilson and Harms (1984) indicated that egg production was normal when daily intake of tryptophan was 223mg. Powell and Gehle, (1977) did not see a difference in egg production when broiler breeder hens were fed 0.230 or 0.199% dietary tryptophan. The dietary tryptophan requirement for male broiler breeders has not been determined.

In broilers, the dietary tryptophan requirement is 0.20% from 0 to 21 days of age, 0.18% from 22 to 41 days of age, and 0.16% from 42 to 56 days of age (NRC, 1994). Similar to the variation among research reports on the requirement of dietary tryptophan for laying hens, the dietary requirement for tryptophan in broilers tends to vary among research reports. Almquist (1947) suggested a 0.25% requirement of tryptophan in the diet of broiler males from 14 to 24 days of age while Griminger et al. (1956) reported a 0.20% dietary requirement for tryptophan in chicks from 10 to 20 days of age. Dean and Scott (1965) indicated the dietary tryptophan requirement for birds from 7 to 14 days of age was 0.225% while Sasse and Baker (1973), indicated a requirement of 0.15% in birds from 8 to 14 days of age. Subsequently, Freeman

(1979) suggested a requirement value of total tryptophan in the diet of 0.24% in chicks from 0 to 7 days of age, and 0.17% on the same birds from 7 to 35 and 36 to 56 days of age. Smith and Waldroup (1988) reported a 0.16% dietary tryptophan requirement in broiler males from 0 to 21 days of age.

More recently, dietary tryptophan has been found to be optimum for male chickens at a total concentration of 0.22% from 8 to 22 days of age (Han et al., 1991), 0.18-0.19% from 21 to 42 days of age (YanLing and YuMing, 2000), 0.16-0.17% until 21 days of age (Rosa et al., 2001) and 0.14-0.15% from 7 to 21 days of age (Shan et al., 2003). Using Ross 508 male broiler chicks from 1 to 20 days of age, Corzo et al. (2005b) determined the total dietary tryptophan requirement was 0.20, 0.21 and 0.22 % for feed intake, body weight gain and feed conversion, respectively. In another experiment with Ross308 broiler males, Corzo et al. (2005a) determined a dietary tryptophan requirement of approximately 0.17% between 42 and 56 days of age. Finally, Emadi et al. (2010) fed diets containing 0.23, 0.34 and 0.46% tryptophan during the starter period (1-21 days of age), 0.18, 0.27 and 0.36% tryptophan during the grower period (21-42 days of age), and 0.17, 0.25 and 0.34% tryptophan during the finisher period (42-49 days of age), to Cobb500 male broiler chickens. The highest inclusion level of dietary tryptophan at each time period led to a significant enhancement in feed intake, body weight gain and feed conversion ratio (Emadi et al., 2010).

The variation in the determined dietary tryptophan requirement no doubt stems from differences in bird strain, age, genetic improvements for growth, differences in dietary niacin levels and differences in immune and stress challenges in which the catabolism of tryptophan can be increased. However, when analyzing the tryptophan requirement data from 29 experiments in

the literature for broiler male chickens until 21 days of age, Rosa and Pesti (2001) suggested that the estimate of 0.20% of dietary tryptophan requirement by the NRC (1994) was probably low.

Serotonin and behavior

Because of serotonin's role in neurotransmission, dietary tryptophan content has been linked to alterations in behavior in poultry by a few research reports. Laycock and Ball (1990) reported that when a commercial layer breeder flock suffering from daily bouts of hysteria was fed a basal diet containing 2.6 g/kg supplemented with a further 5 g/kg tryptophan the incidence of hysteria decreased from 5 episodes per hour to 0 episodes per hour within 8 days. Once the birds were returned to the unsupplemented diet the incidence of hysteria returned to previous levels until the birds were again given the tryptophan supplemented diet and the bouts of hysteria disappeared in a few days. In the supplemented birds relative to unsupplemented controls, plasma concentrations of tryptophan doubled and serotonin and related metabolites increased in both the hypothalamic region and the remainder of the brain (Laycock and Ball, 1990).

Feed restricted broiler breeder males are more aggressive than males fed ad libitum (Mench, 1991). Shea et al. (1990) reported that supplemental dietary tryptophan depressed aggression in broiler breeder males fed on a skip a day basis during rearing, once the peck-order was established at about 10 weeks of age. The decrease in aggression was seen in males fed 0.38, 0.75 and 1.5% dietary tryptophan relative to control birds fed a diet at the recommended level of dietary tryptophan which is 0.19%. In subsequent work reported by this research group it was demonstrated that the decrease in aggression was not related to increases in blood niacin levels as niacin can be synthesized from tryptophan (Shea et al., 1996) and that the depressed aggression is more pronounced in dominate males than subordinate males (Shea et al., 1991).

Savory et al. (1999) reported that growing bantams fed diets containing 22.6 g/kg tryptophan had suppressed pecking damage of feathers caused by aggression related pecking and non-aggressive pecking relative to control birds fed a standard diet containing 2.6 g/kg tryptophan. Similarly van Hierdan et al. (2004) reported that White Leghorn chicks fed a diet containing 2% tryptophan resulted in an overall decrease in the frequency of gentle feather pecking relative to chicks fed a standard rearing diet containing 0.16% tryptophan.

Melatonin and reproduction

It is well known that the annual changes in the secretion of pineal melatonin influence the reproductive activity of photoperiodic mammals (Bronson, 1989). However, previous studies strongly suggest that melatonin is not responsible for changes in seasonal reproduction in birds (Wilson, 1991; Juss et al., 1993). Furthermore, in pigeons and Japanese quails, pinealectomy has little to no effects on free-running circadian activity rhythms (Simpson and Follett, 1981; Ebihara et al., 1987; Chabot and Menaker, 1992). However, some research reports indicate the regulation of seasonal processes by melatonin, including gonadal activity and gonadotropin secretion (Ohta et al., 1989; Bentley et al., 1999; Bentley and Ball, 2000; Bentley, 2001; Guyomarc'h et al., 2001; Rozenboim et al., 2002). Kang et al. (2007) reported *de novo* production of melatonin by the avian hypothalamus and confirmed the existence of melatonin in the hypothalamus of turkeys as well as tryptophan hydroxylase-1 and 5-hydroxytryptamine N-acetyl-transferase, the crucial enzymes in the biosynthesis of melatonin. This discovery, if applicable to all bird species, could potentially explain the absence of effect of pinealectomy on the avian reproductive system. The fact that the avian hypothalamus can produce melatonin fits with the fact that the avian hypothalamus has photoreceptors that are stimulated by light passing through the thin skull of the bird, which directly stimulates the production of GnRH.

Gonadotropin-inhibitory hormone (GnIH) was discovered in quail and is produced in the hypothalamus. GnIH inhibits gonadotropin release and synthesis in several species of birds (Tsutsui et al., 2000; Ciccone et al., 2004; Osugi et al., 2004; Ubuka et al., 2006). Melatonin stimulates GnIH synthesis and release in avian species (Chowdhury et al., 2010, 2012). Therefore, the stimulatory action of melatonin on GnIH release and synthesis may be one of the main causes for decreasing plasma LH and thereby gonadal regression in birds under short-day photoperiods (Chowdhury et al., 2012).

Summary

Tryptophan is an indispensible amino acid that has to be supplemented in poultry feed to allow maximum growth and production. Tryptophan not only has a significant role in protein biosynthesis, but it is also a precursor to serotonin, melatonin and niacin. Because of serotonin's role in neurotransmission, dietary tryptophan content has been linked to alterations in behavior and in particular suppressing aggression in poultry. Finally, the importance of melatonin, especially as it relates to reproduction, is just becoming clear in avian species after decades of acceptance of the general dogma that melatonin played no role in reproduction. Further research will be needed to determine if dietary tryptophan levels above the requirement for growth and egg production influence reproduction efficiency.

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CHAPTER 4

STATEMENT OF PURPOSE

In broiler breeders the role of nutrition and stress in reproduction is further complicated by the need to feed restrict broiler breeder pullets and hens to prevent excessive weight gain which is detrimental to the reproductive performance of the flock. However, the restrictive feeding programs, such as the skip-a-day feed regimen utilized during rearing, that have been implemented by the industry may themselves limit reproductive performance by creating lengthy fasting periods that lead to metabolic and or general stress.

Tryptophan is an indispensible amino acid that is obtained from the diets consumed by avian species. It is not only needed as a constituent for protein synthesis, but as a precursor for serotonin and melatonin production. In addition, it can be converted to niacin if the dietary supply of niacin is not adequate. Because of serotonin's role in neurotransmission, low levels of brain tryptophan have been linked to an enhancement of negative mood while elevated levels of brain tryptophan and subsequent production of serotonin are correlated with stress reduction and in poultry a decrease in aggressive behavior.

The avian glucocorticoid hormone, corticosterone is a steroid hormone produced by the adrenal gland in response to stress. Corticosterone is the key negative feedback regulatory mechanism controlling the hypothalamus-pituitary-adrenal axis. The plasma concentration of corticosterone in avian species is typically elevated under fasting conditions such as those associated with feed restriction of broiler breeders. While the avian ovary does not produce corticosterone, this hormone can still potentially impact ovarian maturation and function because

the glucocorticoid receptor is present on the ovary. However its expression level in follicular tissue and relative to follicular development has not been determined.

Therefore, the goals of the present research are to determine if supplementation of dietary tryptophan above the recommended level (1) modulates plasma corticosterone concentration in feed restricted broiler breeders or (2) improves the reproductive efficiency of these hens, and to determine the influence of feeding regimens during rearing on (3) plasma corticosterone concentration and (4) on glucocorticoid receptor mRNA expression in follicular tissues from broiler breeder hens.

CHAPTER 5

DIETARY TRYPTOPHAN SUPPLEMENTATION LOWERS PLASMA CORTICOSTERONE LEVELS IN BROILER BREEDER HENS[1]

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ABSTRACT

Limited previous research in chickens indicates that providing dietary tryptophan above the requirement for maintenance and growth or production may alleviate behavioral stress. It was hypothesized that the elevated intake of tryptophan increased the production of serotonin which positively modulated behavior. Broiler breeder hens are feed restricted during rearing and breeding which may cause stress. In the current research, broiler breeder hens were fed a diet supplemented with 0.05% crystalline tryptophan to determine if it would enhance reproductive performance and reduce stress as indicated by plasma corticosterone levels. At 29 weeks of age, 96 birds were selected from 600 Cobb 500 fast feathering pullets, split into 2 equal groups and placed into individual cages. The body weight profile of the 48 hens in each group was equivalent and reflected the body weight profile of the entire cohort of pullets from which they were selected. At 35 weeks of age, after allowing acclimation to the cages and ensuring that the original groups were still equal in weight profile and production parameters, one group of hens was maintained on a broiler breeder diet containing 0.20% tryptophan while the other group was fed this diet supplemented with 0.05% tryptophan. All birds were fed individually the same amount of total feed once per day until 62 weeks of age. Hens were artificially inseminated. Body weight and egg production, weight, specific gravity, fertility and hatchability were monitored. Blood samples were taken at 39, 48 and 58 weeks of age for plasma corticosterone assessment by EIA. While the tryptophan supplement had no overall effect on any reproductive parameter, plasma corticosterone was decreased ($P<0.05$) by tryptophan supplementation at each time point examined. The results indicate that tryptophan supplementation may alleviate stress associated with restrictive feeding broiler breeder hens which might have a more profound effect on reproductive performance in an industry setting.

KEYWORDS: broiler breeder, production, tryptophan, stress

INTRODUCTION

As an essential amino acid tryptophan plays an important role in protein nutrition. In laying hen diets tryptophan is often considered to be the third-limiting amino acid, behind methionine and lysine (Peganova et al., 2003). The dietary tryptophan requirement for Leghorn-type laying hens consuming 100 grams of feed per day is 0.16% (NRC, 1994) which agrees fairly closely with subsequent research (Russell and Harms, 1999; Antar et al., 2004; Deponti et al., 2007; Rech et al., 2010). However, other research reports on the tryptophan requirement for laying hens have indicated a tryptophan requirement that varies significantly from the NRC recommendation (Bray, 1969; Morris and Wethli, 1978; Ishibashi, 1985; Othani et al., 1989; Jensen et al., 1990). Given that tryptophan is a precursor molecule for the synthesis for a wide range of molecules such as, niacin, serotonin, melatonin and kynurenine which have a host of physiological functions from energy metabolism, immune function to stress as recently reviewed by Le Floc'h et al. (2011), it is not be surprising that the tryptophan requirement would vary depending on such experimental factors as dietary niacin content, immune challenge and stress.

Research on the requirement for dietary tryptophan in broiler breeders is limited with the requirement for broiler breeder hens set at 0.19% based on mathematical models and limited research (NRC, 1994). Powell and Gehle, (1977) did not see a difference in egg production when broiler breeder hens were fed 0.230 or 0.199% dietary tryptophan. A dietary tryptophan requirement has not been established for broiler breeder males.

Broiler breeders have to be severely feed restricted to prevent them from growing too quickly and becoming too large and obese by the photosensitivity-based sexual maturity that occurs at 20 to 21 weeks of age. Typically, feed allocations are 60-80 percent less during the rearing period and 25-50 percent less during the laying period than what the breeder pullets/hens

would consume ad libitum (Renema and Robinson, 2004). 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., 1999; Hocking and Robertson, 2005; Onagbesan et al., 2006), promotes flock body weight uniformity (Bennett and Leeson, 1989; de Beer and Coon, 2007) and decreases mortality (Robbins et al., 1986; Katanbaf et al., 1989; Heck et al., 2004; Bruggeman et al., 1999). 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 1987; Hocking et al., 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., 1999; Leeson and Summers, 1985; Onagbesan et al., 2006) because they lay longer sequences (Robinson et al., 1991), 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. Overweight broiler breeders have compromised fertility due to reduced locomotion and their physical difficulty in successfully copulating (Duff and Hocking, 1986). Fertility is reduced in overweight hens even when artificial insemination is used (Brake and McDaniel, 1981) and this may be due to the fact that the excess adipose tissue may actually make the insemination more difficult or may block the sperm storage tubules or inhibit sperm movement (Hocking, 1987).

Increasing dietary tryptophan content and thus brain serotonin, has been linked to alterations in behavior in poultry. Laycock and Ball (1990) reported that when a commercial layer breeder flock suffering from daily bouts of hysteria was fed a basal diet containing 2.6 g/kg

supplemented with a further 5 g/kg tryptophan the incidence of hysteria decreased from 5 episodes per hour to 0 episodes per hour within 8 days. Once the birds were returned to the unsupplemented diet the incidence of hysteria returned to previous levels until the birds were again given the tryptophan supplemented diet. In the supplemented birds relative to unsupplemented controls, plasma concentrations of tryptophan doubled and serotonin and related metabolites increased in both the hypothalamic region and the remainder of the brain (Laycock and Ball, 1990).

Feed restricted broiler breeder males are more aggressive than males feed ad libitum (Mench, 1991). Shea et al. (1990) reported that supplemental dietary tryptophan depressed aggression in broiler breeder males fed on a skip a day basis during rearing, once the peck-order was established at about 10 weeks of age. The decrease in aggression was seen in males fed 0.38, 0.75 and 1.5% dietary tryptophan relative to control birds fed a diet at the recommended level of dietary tryptophan which is 0.19%. In subsequent work reported by this research group, it was demonstrated that the decrease in aggression was not related to increases in blood niacin levels as niacin can be synthesized from tryptophan (Shea et al., 1996) and that the depressed aggression is more pronounced in dominate males than subordinate males (Shea et al., 1991).

Savory et al. (1999) reported that growing bantams fed diets containing 22.6 g/kg tryptophan had suppressed pecking damage of feathers caused by aggression related pecking and non-aggressive pecking relative to control birds fed a standard diet containing 2.6 g/kg tryptophan. Similarly van Hierdan et al. (2004) reported that White Leghorn chicks fed a diet containing 2% tryptophan resulted in an overall decrease in the frequency of gentle feather pecking relative to chicks fed a standard rearing diet containing 0.16% tryptophan.

Given that broiler breeder hens are fed once a day a restricted amount of feed that they consume very quickly, they experience a significant fasting period each day. The lack of food and resulting fast potentially stresses these birds on daily basis which could be alleviated by an increase in dietary tryptophan. Therefore, the goal of the current research was to determine if broiler breeder hens fed a diet containing 0.25% versus 0.20% tryptophan would have improved reproductive performance and lower plasma corticosterone levels.

MATERIALS AND METHODS

Rearing Management of Birds

All animal procedures were approved by the Animal Care and Use Committee at the University of Georgia. At 1 day of age, 600 Cobb 500 fast-feathering pullets were randomly divided among 2 rooms. The rooms measured 7.32 × 9.14 m and had pine shavings for litter. The rooms were environmentally controlled, with the temperature maintained at 32.2°C for the first week and then decreased by approximately 2.8°C every week thereafter until the target temperature of 21°C was reached. From 1 to 3 day of age, the chicks were given 24 hours of light per day, and then from 4 to 14 day of age, the amount of light was decreased from 24 to 8 hours per day. The 8 hour per day lighting schedule was then maintained until the birds reached 29 weeks of age. All birds were fed a standard corn-soybean meal starter diet ad libitum from 0 to 2 weeks of age and a developer diet from 2 to 29 weeks of age (Table 5.1). From 2 to 29 weeks of age, the birds were feed restricted and fed on a skip a day (SAD) basis. Feed was distributed by automatic chain feeders, and the birds were given ad libitum access to water from nipple drinkers. Every week, the birds in each room were penned off and 50 birds per room were randomly selected and individually weighed. The weights were then used to determine feed

allocations so that body weight gain of the pullets matched the recommended guidelines of the primary breeder. All pullets were wing-banded at 8 weeks of age for identification purposes.

Breeding Management of Birds

At 29 weeks of age all birds were weighed and 96 birds were selected and placed in individual cages and assigned to one of the two dietary treatments that would be initiated at 35 weeks of age. The body weight profile of the 48 hens in each dietary treatment was equivalent and reflected the body weight profile of the entire cohort of pullets from which they were selected. The birds were photostimulated at 29 weeks of age by providing 14 hours of light (lights on at 0600 hours), and this photoperiod was maintained until the hens reached 62 weeks of age when the experiment ended. The hens were also switched to a layer diet (Table 5.1) and to once a day feeding at 29 weeks of age.

Each cage measured 38.1 cm (h) x 50.8cm (d) x 40.3 cm (l) had an inclined floor, trough feeder providing 40.3 cm of feeding space, 2 nipple drinkers and an individual egg collection area underneath the feeder. Droppings from each cage fell to an automated manure belt, which was cleaned every other day. Egg production and body weight was monitored until the start of the experiment at week 35 to ensure that there was no difference between the treatment groups.

At 35 weeks of age the dietary experiment started and one of the groups of 48 hens was fed the control diet which was a standard broiler breeder laying hen diet (Table 5.2) containing 0.20% tryptophan (digestible tryptophan content, 0.18%) while the other group of 48 hens was fed the control diet supplemented to contain 0.25% dietary tryptophan. For the experimental diets, a proximate analysis and amino acid analyses (both total and digestible) were obtained for all major ingredients prior to formulating and mixing the diets. Diets were prepared monthly throughout the experiment and after each diet was mixed and pelleted, a representative sample

was taken for proximate and amino acid analyses. The daily feed allotment during the pre-experimental period (29-34 weeks of age) and the experimental period (35-62 weeks of age) was based upon body weight, egg production and following the recommended guidelines of the primary breeder. Each hen was fed individually and the birds in both treatments received the same total amount of daily feed.

All hens were weighed every other week from 30 to 62 wks of age. Eggs were manually collected 2 times per day and the number of hatchable, abnormally shaped, cracked, double-yolked, soft-shell, and membrane eggs for each hen was recorded. Hen-day egg production was calculated weekly from daily egg counts.

Every other week beginning when the birds were 36 weeks of age, all eggs from a 5 day period of production were weighed to obtain an average egg weight for each hen. Beginning at 42 weeks of age, specific gravities were also determined on these eggs using the saline flotation method (Phillips and Williams, 1943).

Hens were artificially inseminated at 3 week intervals starting at 33 weeks of age. The hens were artificially inseminated using pooled semen which had been collected from Cobb 500 fast feathering males that were the same age as the hens. Hens were inseminated in the early afternoon and 42 hours after insemination the next 3 to 4 hatching eggs produced from each hen were saved and stored at 18.3 to 19.9°C for no more than 7 days. Eggs were incubated (Natureform Hatchery Systems, Jacksonville, FL) at 37.8°C with 53% RH from 0 to 18 days, and then at 37.2°C with 70% RH from 19 to 21 days. Eggs were candled and transferred for hatching on the 19th day of incubation. During candling, transfer, and after hatching, eggs were characterized as being infertile, cracked, contaminated, or containing early dead embryos (less than 7 d), mid dead embryos (7 to 14 d), or late dead embryos (15 to 21 d). Eggs cracked during

transfer to the hatchery were removed from the data set as lost eggs. After hatching, the numbers of live and dead pips and live and dead chicks were recorded.

Blood Sample Collection

At 39, 48 and 58 weeks of age, blood samples were collected from each hen just prior to their 08:00 hour daily feeding. Blood was collected from the brachial vein and immediately placed into individual glass vacutainers (Becton, Dickinson, and Co., Franklin Lakes, NJ) containing EDTA as an anticoagulant and stored on ice. Blood samples were collected within one minute of physical contact with each hen to avoid corticosterone levels being influenced by handling stress (Romero and Reed, 2005). Blood samples were centrifuged at 1,000 x *g* at 4º C for 10 min. Plasma was collected from each sample and frozen at -80º C.

Plasma corticosterone determination

Plasma corticosterone concentrations were determined using a corticosterone specific enzyme immunoassay (EIA) kit (Enzo Life Sciences, Plymouth Meeting, PA). For each sample an aliquot of thawed plasma was mixed with the steroid displacement reagent provided in the kit following the manufacturer's protocol. After displacement steroid extraction was completed by taking 25 µL of sample and mixing it with 3 mL of anhydrous ethyl ether in a 16 x 125 mm glass tube. The tube was allowed to incubate at room temperature for 30 minutes. The tube containing the sample was then placed in a -80˚C freezer for 1 minute. The sample tube was then removed and the supernatant was quickly poured into another glass tube and then allowed to completely dry at room temperature overnight. The dried samples were resuspended in AB15 diluent provided in the corticosterone EIA kit. Corticosterone content was then determined following the manufacturer's protocol. Duplicate corticosterone determinations were made for each sample. The optical density for each duplicate sample was detected with a Victor 3

Multilabel Plate Reader (Perkin Elmer, Waltham, MA). The mean interassay and intraassay CV for both assays were less than 20% and 10%, respectively.

Statistical Analyses

Analysis of variance was completed using the GLM to detect significant weekly or overall experimental period differences between the birds fed the two dietary treatments (Neter et al., 1990). The overall experimental period values are means of the 48 birds per treatment with the value for each bird obtained by averaging the values collected over the duration of the experiment for that bird. 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

Dietary tryptophan supplementation did not affect average body weight in broiler breeder hens at any time period (Figure 5.1) and the final mean ± SEM body weight was 3,471 ± 97.4 and 3,504.0 ± 104 grams for the control and the tryptophan supplemented birds, respectively.

Dietary tryptophan supplementation did not significantly affect egg production in broiler breeder hens from 35 to 62 weeks of age (Figure 5.2). Hen-housed egg production (mean ± SEM) from 35 through 62 weeks of age was 129 ± 4 and 128 ± 4 eggs per hen for the control and tryptophan supplemented hens, respectively. Total egg production (mean ± SEM) from 35 through 62 weeks of age adjusted for mortality was 132 ± 3 and 133 ± 3 eggs per hen for the control and tryptophan supplemented hens, respectively.

Egg weight did not vary between the two dietary treatments throughout the experiment (Figure 5.3). Overall mean ± SEM egg weight was 67.3 ± 3.70 grams for the control group and

66.8 ± 4.00 grams for the tryptophan supplemented treatment. Likewise, egg specific gravity was not significantly influenced by dietary tryptophan supplementation throughout the experiment (data not shown). The overall mean ± SEM specific gravity for the control and tryptophan supplemented birds was 1.078 ± 0.003 and 1.079 ± 0.004, respectively.

The overall infertility rate and hatch of fertile eggs was not different between the two dietary treatments (Table 5.3). Similarly, there were no differences in the overall incidence of early, mid or late dead embryos as well in the incidence of piped eggs between the two dietary treatments (Table 5.4).

Plasma corticosterone concentrations were lower for the tryptophan supplement birds than the control birds when measured near the beginning, middle and end of the experiment (Table 5.5). In both the control birds and the tryptophan supplemented birds there was a decrease in plasma corticosterone from 39 weeks of age to 48 weeks of age (Table 5.5).

DISCUSSION

Previous research indicates that feed restriction increases plasma corticosterone levels in Leghorn type (Nir et al., 1975; Scanes et al., 1980; Freeman et al., 1981; Harvey and Klandorf 1983; Weber et al., 1990) and broiler (Mench 1991; Hocking et al., 1996; de Jong et al., 2002; de Beer et al., 2008; Ekmay et al., 2010) chickens. In addition, dietary tryptophan supplementation has been associated with a decrease in aggressive behavior, feather picking and abnormal behaviors (Laycock and Ball, 1990; Shea et al., 1990, Shea et al., 1991; Shea et al., 1996; Savory et al., 1999; van Hierdan et al., 2004). Thus, the guiding hypothesis of this research was that increased dietary intake of tryptophan would lower stress as measured by corticosterone in feed restricted broiler breeder hens and this is what was found.

The decrease in plasma corticosterone in feed restricted broiler breeders supplemented with dietary tryptophan agrees with research obtained in mammalian species, but not with prior research in avian species. Although as indicated earlier, dietary tryptophan has been utilized successfully to alter behavior in poultry, only two research reports have reported plasma corticosterone levels. van Hierden et al. (2004) indicated that plasma corticosterone was actually increased in Leghorn chicks fed a diet from 35 to 50 days of age containing 2% dietary tryptophan compared to controls fed 0.16% dietary tryptophan. Pecking behavior was decreased by this large dietary tryptophan supplement. In another report, overall plasma corticosterone levels did not differ in broiler breeder males fed on a skip a day basis from 4 weeks of age to 14 weeks of age a control diet containing 0.20 (control), 0.82, or 1.58% dietary tryptophan (Mench, 1991). The reason(s) for the difference in the current results and the previous results are not clear, the levels of dietary tryptophan supplementation were very high and in the case of van Hierdan et al. (2004) the experimental duration was also very short. In pigs, (Koopmans et al., 2005; Guzik et al., 2006; Koopmans et al., 2006; Shen et al., 2012a; Shen et al., 2012b) and humans (Markus et al., 2000; Firk and Markus, 2009) plasma and or salivary cortisol (the adrenal produced stress hormone in these animals) concentrations are decreased by dietary supplementation of tryptophan and in humans ingestion of tryptophan-rich protein sources dampens the cortisol response to acute stress (Markus et al., 2000; Firk and Markus, 2009).

The mechanisms by which increases in dietary tryptophan alter behavior and reduce stress are not fully elucidated. However tryptophan's role as a precursor to serotonin seems to be the key factor. Serotonin cannot cross the blood brain barrier for entry into the brain and thus the rate limiting factor in serotonin production in the brain becomes tryptophan availability. Tryptophan transport across the blood brain barrier is carrier mediated, but all of the large neutral

amino acids (LNAA) compete for this transporter. Therefore, increasing plasma tryptophan levels through dietary supplementation, and more specifically the tryptophan/LNAA ratio, increases serotonin production. Laycock and Ball (1990) reported that laying hens given 5 g of tryptophan rather than 2.6 g per kg of diet had plasma tryptophan concentrations that were double and more importantly serotonin and related metabolites also increased in both the hypothalamic region and the remainder of the brain in tryptophan supplemented hens (Laycock and Ball, 1990).

Brain produced serotonin is well recognized for its role in regulating behaviors such as mood, appetite and aggression as recently reviewed by Le Floc'h et al. (2011). On the other hand, the interaction between central serotonin production and stress is very complex with conflicting findings on whether it enhances or depresses the hypothalamus-pituitary-adrenal stress response. Based on research with humans, Markus et al. (2000) and Firk and Markus (2009) suggest that the differences in results can be explained by whether the stress is chronic or acute. In chronic stress, serotonin stimulates cortisol secretion while in acute stress serotonin may improve the ability to cope with stress and may contribute to reducing the cortisol response. In the current research, although the duration is over a several month period, the feed restricted broiler breeder hens are likely suffering from daily acute stress. Thus the reduction of cortisol seen in these birds in response to dietary tryptophan supplementation would be in line with the hypothesis that in acute stress serotonin dampens the cortisol response to stress. The reason the birds are likely suffering from daily acute stress is although they are feed restricted, every 24 hours they are fed their entire feed allotment. After quickly consuming this feed the birds are likely satiated and with relatively low stress levels. However, as time passes and the birds enter into a fasting state their stress levels likely progressively increase until they receive their next

meal and the process starts all over again. In the current research, the blood samples were collected just before feeding when stress levels were likely to be their highest. Future research will need to focus on obtaining plasma corticosterone throughout the 24 hour cycle between feedings to prove that the levels return to a baseline level each day after feeding. But already research reported by de Beer et al. (2008) with skip a day feed restricted birds indicates that the feed restricted birds return to a baseline level after feeding. This daily cycle of developing acute stress followed by returning to an unstressed state with feeding probably prevents the adaptation normally seen with chronic stress.

In the present research, plasma corticosterone levels were higher regardless of dietary treatment in the hens at 39 weeks of age than at 48 and 58 weeks of age. This is probably related to age, as plasma corticosterone levels tend to decrease with age to some degree (de Jong et al., 2002). Finally, the current results indicate that the NRC (1994) recommendation for dietary tryptophan being 0.19% in broiler breeder hens is not too low as increasing the dietary level of dietary tryptophan from 0.20% to 0.25% in the current experiment did not improve reproductive performance. However, if further research indicates that higher levels of dietary tryptophan can alleviate the stress of feeding broiler breeders a restricted feed amount once a day especially in a floor pen commercial breeding environment, then this would have to be considered in setting the dietary tryptophan requirement.

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Table 5.1. Composition of the starter, developer and layer diets.

	Diets[1]		
Ingredient	Starter	Developer	Layer
	----------(%)----------		
Corn	62.95	65.88	70.80
Soybean meal	22.24	15	18.11
Poultry fat	0	0	0.91
Wheat middlings	10.53	14.83	0
Limestone	1.16	1.28	7.33
Dicalcium phosphate	1.75	1.57	1.49
Sodium chloride	0.54	0.60	0.51
Vitamin premix[2]	0.50	0.50	0.50
DL-Methionine	0.15	0.13	0.15
L-Lys·HCl	0.10	0.13	0.12
Trace mineral premix[3]	0.08	0.08	0.08
Calculated analysis[4]			
ME (kcal/kg)	2,865.00	2,920.00	2,920.00
CP (%)	18	15	15
Lys (%)	1.00	0.83	0.83
Calcium (%)	0.91	0.92	3.22
Met and Cys (%)	0.73	0.64	0.64
Available phosphorus (%)	0.45	0.42	0.38

[1]Starter diet was fed from 0 to 2 weeks of age, developer diet was fed from 2 to 29 weeks of age and the layer diet was fed from 29 to 35 weeks of age.
[2]Vitamin premix provided the following per kilogram of diet: vitamin A, 5,510 IU; vitamin D3, 1,100 IU; vitamin E, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin, 4.4 mg; niacin, 44.1 mg; D-pantothenic acid, 11.2 mg; choline, 191.3 mg; menadione sodium bisulfate, 3.3 mg; folic acid, 5.5 mg; pyridoxine HCl, 4.7 mg; thiamin, 2.2 mg; D-biotin, 0.11 mg; and ethoxyquin, 125 mg.
[3]Trace mineral premix provided the following in milligrams per kilogram of diet: Mn, 60; Zn, 50; Fe, 30; I, 1.5; and Se, 0.5.
[4]Calculated analysis was based on Dale (2001).

Table 5.2. Composition of the experimental diets.

	Dietary treatments[1]	
Ingredient	Control	Tryptophan
	----------(%)----------	
Corn	64.165	64.115
Soybean Meal	13.595	13.595
Limestone	7.292	7.292
Corn DDGS	7.000	7.000
Meat and Bone Meal	4.308	4.308
Poultry Fat	1.934	1.934
Vitamin Premix[2]	0.500	0.500
Salt	0.330	0.330
Sodium Carbonate	0.306	0.306
DL-Methionine	0.191	0.191
L-Lysine HCl	0.100	0.100
UGA Trace Mineral Premix[3]	0.080	0.080
L-Threonine	0.052	0.052
L-Trytophan	-	0.050
Choline Cl (60%)	0.043	0.043
Potassium Sulfate	0.030	0.030
L-Isoleucine	0.029	0.029
Tribasic Copper Chloride	0.025	0.025
Quantum Phytase 2500X	0.020	0.020
Calculated Analysis[4]		
ME (kcal/kg)	2921.00	2921.00
CP (%)	15.34	15.34
Lys (%)	0.79	0.79
Calcium (%)	3.36	3.36
Met and Cys (%)	0.66	0.66
Available phosphorus (%)	0.50	0.50

[1]The experimental diets were fed from 35 wks of age to 62 wks of age.
[2]Vitamin premix provided the following per kilogram of diet: vitamin A, 5,510 IU; vitamin D3, 1,100 IU; vitamin E, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin, 4.4 mg; niacin, 44.1 mg; D-pantothenic acid, 11.2 mg; choline, 191.3 mg; menadione sodium bisulfate, 3.3 mg; folic acid, 5.5 mg; pyridoxine HCl, 4.7 mg; thiamin, 2.2 mg; D-biotin, 0.11 mg; and ethoxyquin, 125 mg.
[3]Trace mineral premix provided the following in milligrams per kilogram of diet: Mn, 60; Zn, 50; Fe, 30; I, 1.5; and Se, 0.5.
[4]Calculated Analysis was based on Dale (2001).

Table 5.3. Infertile egg production and hatchability of fertile eggs from broiler breeder hens fed either a control diet or this diet supplemented with 0.05% tryptophan from 35 through 62 weeks of age.[1]

	Infertile		Hatch of Fertile	
Age (wks)	Control	TRP	Control	TRP
	---------- (%) ----------			
34	5.77 ± 3.24	5.71 ± 3.13	89.10 ± 3.45	97.30 ± 2.03
37	3.79 ± 2.23	3.10 ± 1.86	93.94 ± 2.71	89.53 ± 2.72
40	2.17 ± 1.05	3.44 ± 1.34	97.10 ± 1.25	97.64 ± 1.15
43	2.91 ± 1.41	5.36 ± 2.47	92.33 ± 2.40	97.74 ± 1.09*
46	1.36 ± 0.96	8.17 ± 3.00*	96.46 ± 1.54	94.83 ± 2.04
49	2.52 ± 1.51	6.38 ± 2.48	91.46 ± 2.58	89.63 ± 3.45
52	7.20 ± 2.24	7.21 ± 2.82	91.74 ± 2.98	91.92 ± 2.21
55	9.79 ± 2.79	5.48 ± 1.83	93.85 ± 2.33	93.83 ± 2.10
58	15.09 ± 3.38	14.56 ± 3.65	91.24 ± 3.18	91.10 ± 3.88
Overall	5.26 ± 0.83	7.36 ± 1.25	93.67 ± 0.88	91.34 ± 1.81

*Significantly different from the corresponding value for the hens fed the control diet ($P \leq 0.05$).
[1]Values are the mean ± SEM for eggs set from each (n = 48) individually caged hen for both dietary treatments

Table 5.4. The incidence of early, mid and late embryo mortality, as well as the incidence of pips in eggs incubated from broiler breeder hens fed either a control diet or this diet supplemented with 0.05% tryptophan from 35 through 62 weeks of age.[1]

	Early dead		Mid dead		Late dead		Pips	
Age	Control	Tryptophan	Control	Tryptophan	Control	Tryptophan	Control	Tryptophan
(wk)	---(%)---							
34	5.00 ± 2.30	1.75 ± 1.21	1.54 ± 1.07	0.95 ± 0.95	2.05 ± 1.47	0	2.31 ± 1.69	0
37	1.52 ± 1.06	8.14 ± 2.56*	0.76 ± 0.76	0	3.03 ± 2.38	2.33 ± 1.31	0.76 ± 0.76	0
40	1.27 ± 0.90	0.73 ± 0.73	0	0.54 ± 0.54	1.09 ± 0.76	1.09 ± 0.76	0.54 ± 0.54	0
43	2.21 ± 1.07	0.60 ± 0.60	2.02 ± 1.61	0	3.45 ± 1.58	1.67 ± 0.94	0	0
46	2.02 ± 1.16	0	0	0.83 ± 0.83	0.78 ± 0.78	3.21 ± 1.65	0	0.50 ± 0.50
49	3.66 ± 1.57	4.47 ± 1.80	1.42 ± 1.00	2.24 ± 1.27	1.22 ± 1.22	1.22 ± 0.85	0.81 ± 0.81	0
52	3.30 ± 2.36	2.08 ± 1.48	0	0	2.50 ± 1.35	4.04 ± 1.57	2.46 ± 1.45	1.96 ± 1.13
55	2.91 ± 1.71	1.58 ± 1.11	0	1.12 ± 0.80	2.66 ± 1.65	2.25 ± 1.28	0.58 ± 0.58	1.22 ± 0.85
58	0.86 ± 0.86	0.53 ± 0.53	3.85 ± 1.96	0	1.28 ± 0.95	5.75 ± 2.98	1.50 ± 1.05	2.63 ± 2.63
Overall	2.22 ± 0.47	2.18 ± 0.52	0.89 ± 0.34	0.56 ± 0.21	1.56 ± 0.41	2.55 ± 0.61	0.85 ± 0.30	0.64 ± 0.24

*Significantly different from the corresponding value for the hens fed the control diet ($P \leq 0.05$).

[1]Values are the mean ± SEM for eggs set from each (n = 48) individually caged hen for both dietary treatments. Embryo mortality was classified as early dead (less than 7 d), mid dead (7-14 d), or late dead (15-21 d of incubation). Pips included both live and dead pips at time of hatch.

Table 5.5. Plasma corticosterone concentration in broiler breeder hens fed either a control diet or this diet supplemented with 0.05% tryptophan.[1]

Age	Control	Tryptophan
(wks)	---------- (ng/ml) ----------	
39	1.09 ± 0.06^{a}	$0.89 \pm 0.07^{*a}$
48	0.41 ± 0.03^{b}	$0.31 \pm 0.03^{*b}$
58	0.41 ± 0.02^{b}	$0.34 \pm 0.02^{*b}$
Overall	0.64 ± 0.02	$0.52 \pm 0.03^{*}$

[*]Significantly different from the corresponding value for the hens fed the control diet ($P \leq 0.05$).
[a-b]Corticosterone values within a dietary treatment without a common superscript differ; $P < 0.05$.
[1]Values are the mean ± SEM, n = 48 individually caged hens for both dietary treatments.

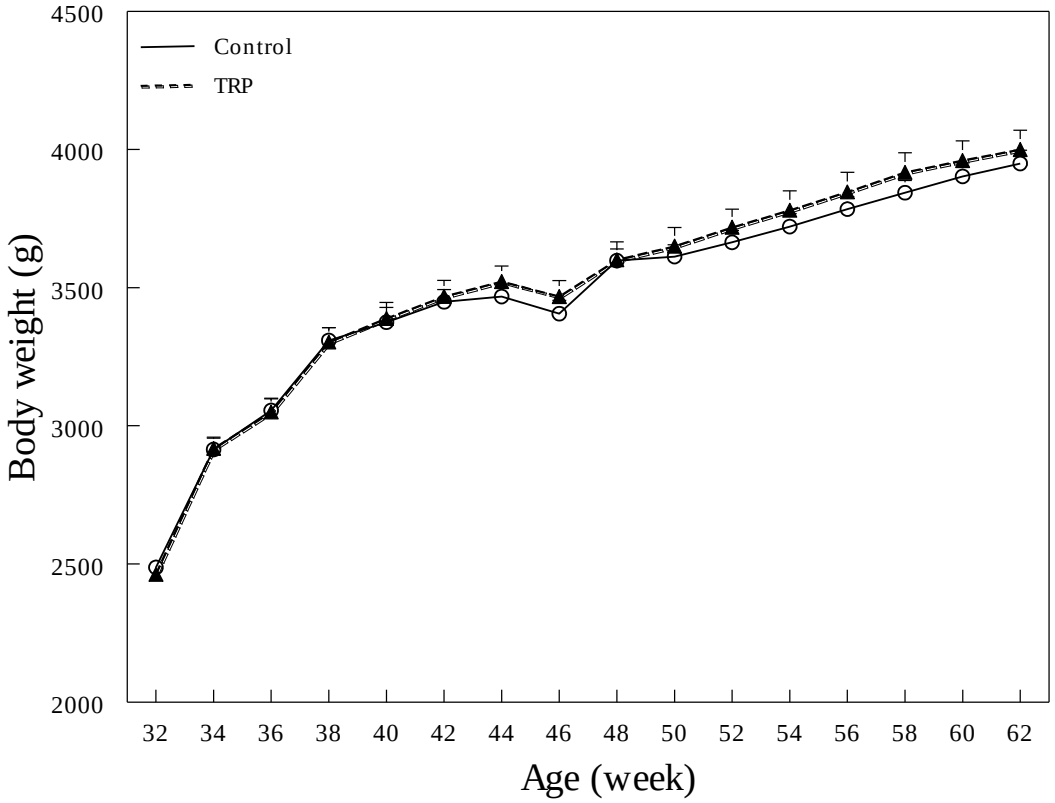

Figure 5.1. Body weight of broiler breeder hens fed a control diet or this diet supplemented with 0.05% tryptophan (TRP) from 35 through 62 weeks of age. Body weight values from week 32 through week 34 of age are included to indicate that the experimental groups did not differ prior to the onset of the experiment. Values are means ± SEM, n = 48 hens per treatment.

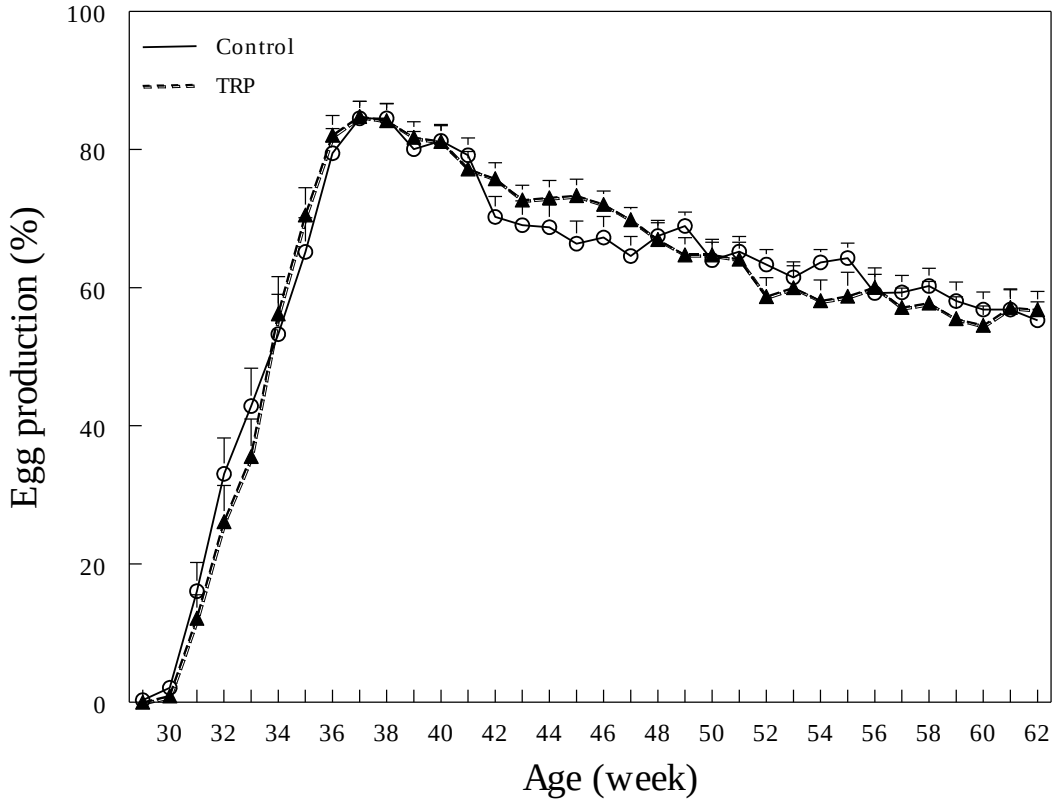

Figure 5.2. Weekly hen day egg production of broiler breeder hens fed a control diet or this diet supplemented with 0.05% tryptophan (TRP) from 35 through 62 weeks of age. Egg production from week 29 through week 34 of age is included to indicate that the experimental groups did not differ prior to the onset of the experiment. Values are means ± SEM, n = 48 hens per treatment.

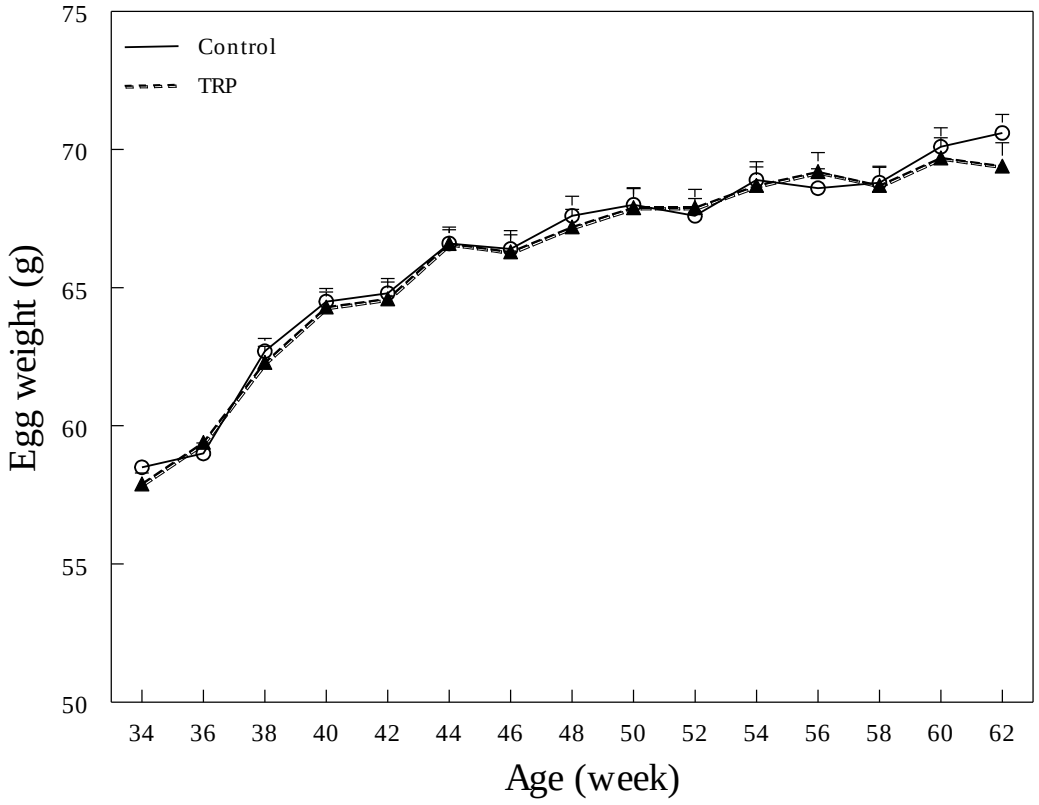

Figure 5.3. Weight of eggs produced by broiler breeder hens fed a control diet or this diet supplemented with 0.05% tryptophan (TRP) from 35 through 62 weeks of age. Egg weight values from week 34 of age are included to indicate that the experimental groups did not differ prior to the onset of the experiment at week 35. Values are means ± SEM, n = 48 hens per treatment.

CHAPTER 6

THE INFLUENCE OF FEEDING REGIMEN DURING REARING ON PLASMA CORTICOSTERONE CONCENTRATION AND GLUCOCORTICOID mRNA RECEPTOR EXPRESSION IN BROILER BREEDER PULLETS

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ABSTRACT

During rearing, feed restriction is typically accomplished by utilizing a skip-a-day (SAD) feeding regimen to control body weight gain to maximize future reproductive performance in broiler breeder hens. However, the fasting period associated with SAD feeding may cause stress. In this study, plasma corticosterone was measured as a potential indicator of stress in broiler breeder pullets that were fed during rearing using an automatic chain feeder on a SAD or everyday (ED) basis and in pullets that were fed ED with the feed broadcast (EDB) on top of their litter. All treatments received an equal total amount of feed over every 48 hour period. At 21 weeks of age the pullets were distributed into laying pens, placed on an ED feeding schedule and photostimulated. Blood was collected for plasma corticosterone assessment and follicular tissue was collected for glucocorticoid receptor mRNA measurement. At 11 weeks of age plasma corticosterone concentrations were not different between any treatments when measured 7 hours after feeding. However, the corticosterone concentration in plasma obtained from the SAD birds 31 hours after feeding was elevated relative to the values obtained at 7 hours. Similarly, at 17 weeks of age plasma corticosterone was not different between the SAD and ED birds when measured 24 hours after feeding, but the level of corticosterone was elevated in blood obtained from SAD birds after 48 hours without feed. At 28 weeks of age, the overall glucocorticoid receptor mRNA expression in the F1 through F4 follicles was not different between the ED, EDB and SAD hens, but the expression of the glucocorticoid receptor mRNA was greater in granulosa tissue compared to theca tissue in the ED and EDB hens, but not in the SAD hens. The results indicate that SAD feeding, during rearing, led to elevation of plasma corticosterone levels during their extended fast which may lead to altered future follicular glucocorticoid receptor mRNA expression and thus altered follicular development.

KEYWORDS: skip-a-day, everyday, stress, granulosa, theca

INTRODUCTION

To prevent broiler breeder pullets from growing too quickly and becoming too large and obese by the photosensitivity-based sexual maturity that occurs at 20 to 21 weeks of age, their feed intake is severely restricted. Typically, feed allocations are 60-80 percent less during the rearing period and 25-50 percent less during the laying period than what the breeder pullets/hens would consume ad libitum (Renema and Robinson, 2004). 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), promotes flock body weight uniformity (Bennett and Leeson, 1989; de Beer and Coon, 2007) 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 1987; Hocking et al., 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; Leeson and Summers, 1985; Onagbesan et al., 2006) because they lay longer sequences (Robinson et al., 1991), 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.

In the United States broiler breeder pullets are typically provided their restricted feed amount once every other day during rearing [skip-a-day (SAD) feed regimen] and then fed once everyday (ED) starting at photostimulation for reproduction, when the flock produces its first egg or reaches 5% egg production. This feed is quickly consumed by the birds and as a result they will fast for a significant portion of each day. Research indicates this fasting may be detrimental

to total egg production in broiler breeder hens. Often in commercial settings, a SAD feeding program used to be continued until the broiler breeder flock reached 5% egg production. Gibson et al. (2008) reported that initiating an ED feeding regimen when the birds were photostimulated for reproduction at 21 weeks of age increased total egg production by about 19 eggs per bird by the end of 65 weeks of age compared to continuing the SAD 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 SAD basis until 5 percent egg production compared to the hens that were fed ED after being photostimulated. The research reported by Gibson et al. (2008) suggested the significant fasting period that the broiler breeder pullets experienced between meals on a SAD feeding program after photostimulation for reproduction might be detrimental to normal ovarian development. Spradley et al. (2008) completed research that was very similar to Gibson et al. (2008) except when the pullets were photostimulated for reproduction they were fed either once a day (equivalent to the ED treatment) 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 improved the total number of eggs produced per hen through 41 weeks of age by 5 eggs per bird.

Corticosterone is the primary stress related glucocorticoid produced by the adrenal gland in avian species (Carsia and Harvey, 2000), and fasting is associated with increased plasma levels of corticosterone in young, light-breeds of chicks such as Leghorns (Nir et al., 1975; Scanes et al., 1980; Freeman et al., 1981; Harvey and Klandorf, 1983; Weber et al., 1990). In broiler breeder pullets or cockerels feed restriction during rearing has also been associated with increased plasma concentrations of corticosterone (Mench, 1991; Hocking et al., 1996; de Jong

et al., 2002). However, the effect of feed restriction on corticosterone appears to vary depending on the time during the day when the birds were sampled relative to feeding (Savory et al., 1993; Savory and Mann, 1997; de Beer et al., 2008) and age of the birds (Hocking et al., 1993).

de Beer et al. (2008) examined plasma corticosterone concentrations in broiler breeder pullets at 16 weeks of age that had been reared with an ED or SAD feed restriction system in which the birds received the same total amount of feed over every 48 hour period. Overall, the SAD birds had a higher plasma corticosterone levels than the ED fed birds with levels being consistently elevated in the period from 20 to 48 hours after the last feeding for the SAD pullets. In a subsequent report from this research group (Ekmay et al., 2010), the overall plasma concentration of corticosterone over a 24 hour period was still greater in the SAD hens versus the ED hens at 26.4 weeks of age, even though all of the hens had started to be fed on an ED basis at 24 weeks of age. The Ekmay report, like a report in Leghorns (Weber et al., 1990), suggests that alterations in corticosterone production can persist weeks after the original feed restriction stressor has been removed.

Although ovarian cells lack 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 the ovary (Kwok et al., 2007). The role of glucocorticoids in the direct regulation of ovarian function is poorly understood, but it is plausible that higher levels of corticosterone caused by feed restriction regimens may have a negative influence on ovarian development in broiler breeders. Therefore, the primary objectives of the current research was to compare plasma corticosterone levels during rearing in broiler breeder pullets fed either on an ED or SAD basis and to determine if glucocorticoid receptor mRNA expression varied in the theca and granulosa tissue of the 4 largest preovulatory follicles

of 28 week old hens that had been fed on an ED or SAD basis until photostimulation at 21 weeks of age.

MATERIALS AND METHODS

Rearing management of birds

All animal procedures were approved by the Animal Care and Use Committee at the University of Georgia. At 1 day of age 1,800 Cobb 500 fast-feathering pullets were randomly divided among 3 different treatments in 9 pens. Each pen measured 3.66 × 9.14 m and had pine shavings for litter. The pens were in environmentally controlled rooms, with the temperature maintained at 32.2°C for the first week and then decreased by approximately 2.8°C every week thereafter until the target temperature of 21°C was reached. From 1 to 3 days of age, the chicks were given 24 hours of light per day, and then from 4 to 14 days of age, the amount of light was decreased from 24 to 8 hours per day. The 8 hour per day lighting schedule was then maintained until the birds reached 20 weeks of age. All birds were fed a standard corn-soybean meal broiler breeder starter diet ad libitum from 0 to 2 weeks of age and then they were feed restricted using a standard broiler breeder developer diet from 2 to 23 weeks of age and thereafter a standard broiler breeder layer diet. The composition of the diets were previously described (Table 5.1). Water was provided ad libitum from nipple drinkers.

When the chicks were 2 weeks of age, the 3 different feeding regimens were initiated. For the ED treatment, the birds were fed once every 24 hours by an automatic chain feeder. For the everyday broadcast treatment (EDB) the birds were fed everyday but their daily feed allotment was scattered by hand on top of the litter. Finally, for the SAD treatment the birds were fed two days worth of feed every other day and this feed was distributed by an automatic

chain feeder. Over every 48 hour period all of pens of birds received the same amount of total feed.

From 2 to 20 weeks of age a 10% random sample of birds from each pen were individually weighed every week and on week 11, 15 and 19 of age all birds were weighed. The weights were then used to determine feed allocations so that the body weight gain of the pullets matched the recommended guidelines of the primary breeder (Cobb-Vantress 2008a, Cobb-Vantress 2008b). All pullets were wing-banded at 8 weeks of age for identification purposes.

Breeding management of birds

Based on the weights obtained at 19 weeks of age, the pullets were selected within each feeding treatment and assigned to laying pens that each contained 46 pullets and 4 roosters at the end of their 20th week of age. The pullets from each feeding treatment were assigned to breeding pens such that the body weight profile was similar across pens and reflected the overall body weight profile of the feeding treatment obtained at 19 weeks of age. Each laying pen measured 2.44 × 3.66 m with the floor space of each pen consisting of one-third pine shavings litter and two-thirds elevated slats. Each pen had one 6-hole nest section located on the raised slats along with a nipple drinker line that provided 10 nipples. In the laying pens, the hens and roosters were hand-fed using plastic feeder pans. Each pen contained 3 hen feeder pans, which were fitted with rooster exclusion grills. The feeding system provided 7 cm of feeder space per hen. Males were given their own feeder pan, which was elevated in height to prevent females from consuming their feed. Each rooster had 25.9 cm of feeder space.

Once the birds were moved to the laying pens, all of the birds were placed on a once a day feeding schedule and they were fed at 06:30 hours. Photostimulation occurred at the

beginning of the birds 21st week of age by providing 14.5 hours of light (lights on at 06:00 hours), and this photoperiod was maintained until the end of the experiment.

Roosters and hens were weighed weekly from 25 to 29 weeks of age. Eggs were manually collected 3 to 4 times per day. Hen-housed and hen-day egg production were calculated weekly from daily egg counts and the numbers of hatchable, abnormally shaped, cracked, double-yolk, dirty, membrane, and total eggs were recorded daily for each pen. The daily amount of feed provided to the hens was determined using the guidelines of the primary breeder (Cobb-Vantress 2008a, Cobb-Vantress 2008b) based on the weekly body weight measurements and the egg production rate of the hens.

Blood sampling

At 11, 17, and 25 weeks of age blood samples were obtained from 25 randomly selected birds from each feeding treatment. At 11 weeks of age the samples were obtained from the ED, EDB and SAD birds 7 hours after feeding. In addition, for the SAD treatment, blood samples were also taken when the birds were on their subsequent off-feed day or 31 hours after feeding. At 17 weeks of age the samples were obtained from the ED, EDB and SAD at 24 hours since last feeding (just prior to feeding for the ED and EDB birds) and for the SAD birds another set of samples was collected just prior to feeding at 48 hours since the last feeding. At 25 weeks of age once all the birds had been placed on a once a day feeding schedule the blood samples were obtained 7 hours after feeding.

Blood was collected from the brachial vein and immediately placed into individual glass vacutainers (Becton, Dickinson, and Co., Franklin Lakes, NJ) containing EDTA as an anticoagulant and stored on ice. Samples were then centrifuged at 1,000 x *g* at 4º C for 10 minutes and plasma was collected from each sample and frozen at -80º C. Blood samples were

obtained in less than one minute from initial physical contact with the bird to eliminate handling stress from altering plasma corticosterone concentrations (Romero and Reed, 2005).

Follicle tissue collection

Leghorns

To determine if the glucocorticoid receptor is expressed in the granulosa and theca tissue of chicken preovulatory follicles, an initial characterization was completed using Hy-line W-36 laying hens. Six 45 to 55 week old hens were killed by cervical dislocation 2 to 4 hours prior to ovulation and the ovary was collected from each hen. The theca and granulosa layers from each of the F1, F2, F3, and F4 follicles were manually separated from one another (Huang and Nalbandov, 1979) while the theca and granulosa layers from the small yellow follicles (SYF, 5 to 10 mm in diameter) and large white follicles (LWF, 2 to 5 mm in diameter) were separated enzymatically (Davis et al., 2000). The individual theca and granulosa tissue for each follicle size obtained from one hen were combined with corresponding samples from another hen for RNA extraction and subsequent RT-PCR detection of the glucocorticoid receptor mRNA. Thus, a total of 3 sets (n = 3) of samples for each follicle size was obtained. The theca layers collected from each individual F1 through F4 follicle and from the individual pools of SYF and LWF were placed in 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA). The single cell layer of granulosa tissue from each hierarchical follicle and granulosa cells isolated from the individual pools of SYF and LWF was placed in 3 mL of guanidinium isothiocyanate solution and vortexed for 20 seconds. The tissue solutions were stored at -80ºC for subsequent RNA extraction.

Broiler breeders

At 28 weeks of age, 12 birds were killed by cervical dislocation from each rearing feeding regimen. Each bird was killed 2 to 4 hours prior to ovulation and the ovary was collected from each hen. The 4 largest hierarchical follicles F1, F2, F3 and F4 were separated from each collected ovary and the granulosa cell layer was manually separated from the theca cell layers of each follicle and processed as described for the laying hen follicles.

Corticosterone determination

Plasma corticosterone concentrations were determined using a corticosterone specific enzyme immunoassay (EIA) kit (Enzo Life Sciences, Plymouth Meeting, PA). For each sample an aliquot of thawed plasma was mixed with the steroid displacement reagent provided in the kit following the manufacturer's protocol. After displacement steroid extraction was completed by taking 25µL of sample and mixing it with 3 mL of anhydrous ethyl ether in a 16 x 125 mm glass tube. The tube was allowed to incubate at room temperature for 30 minutes. The tube containing the sample was then placed in a -80˚C freezer for 1 minute. The sample tube was then removed and the supernatant was quickly poured into another glass tube and then allowed to completely dry at room temperature overnight. The dried samples were resuspended in AB15 diluent provided in the corticosterone EIA kit. Corticosterone content was then determined following the manufacturer's protocol. Duplicate corticosterone determinations were made for each sample. The optical density for each duplicate sample was detected with a Victor 3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA). The mean interassay and intraassay CV for the assay were less than 20% and 10%, respectively.

Thyroid hormone assays

To determine if broiler breeder feed restriction feeding regimens changed plasma total 3, 5, 3′- triiodothyronine (T_3) and free T_3 concentrations, they were determined on the collected plasma samples by RIA using the Coat-A-Count Total T_3, and Free T_3 Kits (Seimens, Los Angeles, CA). The T_3 and free T_3 concentrations were determined following the manufacturer's protocol except that an additional standard (0.250 pg/ml) was included to increase sensitivity at the lower end of the standard curve for the free T_3 assay. RIA samples were counted with a Wallace Wizard 1470 gamma counter (Perkin Elmer, Waltham, MA). The mean interassay and intraassay CV for both assays were less than 10%.

RNA extraction

Total RNA was extracted from tissue samples using the guanidinium thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987). 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. RNA samples were stored at -80ºC. For the broiler breeder experiment, based on the quality of the RNA, the best 6 replicate samples were chosen out of the 12 replicate samples for the determination of the mRNA expression of the glucocorticoid receptor.

Real time RT-PCR

Primers and TaqMan MGB probes (Table 6.1) specific for chicken glucocorticoid receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the endogenous control were generated using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) and were synthesized by Applied Biosytems. Primers and probes were designed based on reported chicken cDNA sequences for the glucocorticoid receptor (GenBank accession # DQ227738) and GAPDH (GenBank accession # M11213). 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, Foster City, CA).

Extracted RNA samples were DNase treated using the TURBO-DNA-free kit (Ambion, Austin, TX) to remove any potential genomic DNA contamination. Reverse-transcription cDNA synthesis reactions were completed utilizing the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol. For real-time PCR, 25µL reactions were prepared with 100ng cDNA, 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of either the glucocorticoids receptor or GAPDH primer pairs, and 25 nM of probe. The reactions for each tissue sample were completed in duplicate for both glucocorticoid receptor and GAPDH detection. Reactions were completed in an ABI 7500 Thermocycler (Applied Biosystems) with the cycling program set for 95°C for 10 min followed by 40 cycles each consisting of 95°C for 15 seconds and 60°C for one minute. Sequence detection software (version 1.2.2, Applied Biosystems) was used to determine the cycle threshold (C_T) for each reaction. Relative quantification was completed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the glucocorticoid receptor Ct was determined for each sample and then normalized to the GAPDH Ct from the same sample (GAPDH Ct subtracted from the glucocorticoid receptor Ct yields the ΔCt). After all the ΔCt values were obtained for a replicate experiment, the ΔCt values were all compared relative to the sample with the highest mRNA expression of glucocorticoid receptor using the $2^{-\Delta\Delta Ct}$ method. Thus, the glucocorticoid receptor

mRNA expression data for each individual sample is expressed as the fold-difference relative to sample with the highest glucocorticoid receptor mRNA expression.

Statistical analyses

Data were subjected to ANOVA according to the General Linear Model (GLM). Tukey's multiple-comparison procedure (Neter et al., 1990) was used to detect significant differences in plasma steroid and thyroid levels between the feeding regimens and to detect differences in glucocorticoid receptor mRNA expression among tissues and different follicular sizes. Differences were considered significant when *P*-values were < 0.05. All statistical procedures were done with the Minitab Statistical Software Package (Release 16.2.2, State College, PA).

RESULTS

All of the pullets were weighed at 11, 15 and 19 weeks of age during the rearing period and at 25 weeks of age during the breeding period. The mean pullet weight (g) ± SEM at 11 weeks was 1173 ± 48, 1078 ± 18 and 1172 ± 10; at 15 weeks it was 1532 ± 15, 1477 ± 22 and 1484 ± 14; at 19 weeks it was 2199 ± 7, 2063 ± 11 and 2048 ± 18; and at 25 weeks it was 3132 ± 13, 3151 ± 48 and 3020 ± 17 for the ED, SAD and EDB birds, respectively. Some hens from all of the feeding treatments utilized in rearing had initiated lay by 25 weeks of age and had reached 70% weekly hen day egg production at the conclusion of their 28th week of age (Figure 6.1). There were no differences in egg production between the hens raised on different rearing feeding programs.

Plasma corticosterone was elevated in the SAD pullets at 11 and 17 weeks of age when the samples were collected at 31 hours and 48 hours after feeding, but did not differ from the levels found in ED pullets when measured at 7 and 24 hours after feeding (Table 6.2). Four

weeks after all the pullets had started to be fed on an ED basis, plasma corticosterone concentrations did not differ at 25 weeks of age between the ED, SAD and EDB hens.

At 11 weeks of age the EDB pullets had lower total and free plasma T_3 concentrations than the ED and SAD pullets when measured 7 hours after feeding (Table 6.3). Plasma from the SAD pullets collected 31 hours after feeding at 11 weeks of age had the lowest level of total and free T_3 (Table 6.3). At 17 weeks of age, there were no differences in the concentration of total T_3 in plasma samples obtained from the ED and EDB pullets 24 hours after their last feeding and the plasma samples obtained from SAD pullets 48 hours after their last feeding (Table 6.3). Total plasma T_3 in the SAD pullets obtained 24 hours after their last feeding was significantly greater than what was found in the SAD pullets fasted for 48 hours or the ED or EDB pullets fasted for 24 hours (Table 6.3). At 25 weeks of age, the hens that had been fed on an ED, EDB or SAD basis during rearing had no differences in their plasma total and free T_3 levels (Table 6.3).

The overall expression of the mRNA for the glucocorticoid receptor in Leghorn hens tended ($P = 0.060$) to be higher in granulosa tissue than theca tissue (Figure 6.2). The expression of glucocorticoid receptor mRNA in the theca and granulosa samples (Figure 6.3) from the largest two follicles (F1 and F2) was lower ($P < 0.05$) in comparison to the expression in these tissues in the prehierarchical follicles (SYF and LWF).

In the broiler breeder hens that had been reared using an ED, SAD or EDB feeding regimen, the overall expression of the glucocorticoid receptor mRNA in follicular tissue from the F1 through F4 follicles was greater in the EDB hens than in the SAD hens (Figure 6.4). For the hens reared on the ED treatment, the overall expression of the mRNA for the glucocorticoid receptor in the F1 through F4 follicles was greater in granulosa tissue than in theca tissue (Figure

6.5). In addition, mRNA expression of the glucocorticoid receptor in F4 granulosa tissue was greater than the expression in theca tissue from the F1, F2, F3, or F4 follicle (Figure 6.6). In contrast, the mRNA expression of the glucocorticoid receptor in the hens that had been reared on a SAD basis did not differ between granulosa and theca tissue (Figure 6.7) or between follicle sizes (Figure 6.8). Finally, for the hens that had been reared using an EDB regimen, the overall mRNA expression for the glucocorticoid receptor mRNA was greater in granulosa tissue than theca tissue (Figure 6.9). However, there were no differences in the mRNA expression of the glucocorticoid receptor between the granulosa and theca tissues of the individual follicles (Figure 6.10).

DISCUSSION

In the current research, broiler breeders were feed restricted during rearing and production which itself can cause stress, but what was being tested was if the feeding method (ED, SAD and EDB) by which this restricted feed amount was being delivered during rearing led to even further differences in stress. The results indicate that a SAD feeding regimen is more stressful than an ED feeding regimen based on plasma corticosterone levels as well as alterations in plasma T_3 concentrations and ovarian glucocorticoid receptor mRNA expression.

Baseline plasma corticosterone levels are important in regulating metabolism to ensure fuel is available through energy mobilization, while higher concentrations of corticosterone play a key role in the vertebrate stress response. It is well established that caloric restriction and fasting elevate plasma corticosterone levels in poultry (Nir et al., 1975; Scanes et al., 1980; Freeman et al., 1981; Harvey and Klandorf, 1983; Weber et al., 1990; Mench, 1991; Hocking et al., 1996; de Jong et al., 2002; de Beer et al., 2008). Mench (1991) indicated that male broiler breeders that were feed restricted utilizing a SAD feeding program had higher plasma

concentrations of corticosterone than ad libitum fed controls and that the plasma corticosterone levels were also greater in the SAD cockerels on off-feed days relative to fed days. The current research takes this prior research a step further by comparing the plasma corticosterone levels in SAD and ED feed restricted broiler breeder pullets during rearing. This allowed for the determination that SAD pullets are further stressed based on plasma corticosterone levels than broiler breeders fed on an ED basis. Plasma corticosterone levels did not differ between SAD and ED pullets 7 and 24 hours after feeding. But at some point after 24 hours and before 31 hours from the last feeding, plasma corticosterone levels increase beyond the levels seen in ED birds being fed every 24 hours. Once the SAD birds are fed, corticosterone levels return to ED baseline levels again.

The SAD birds are likely suffering from acute stress because the elevated corticosterone levels are returning to a baseline level every 48 hours. Admittedly, the duration of this every other day increase in corticosterone is long term as it lasts throughout the rearing period. However, because the stress is a repeated acute stress event and not chronic stress, this may not allow the birds to adjust to the stress as has been seen previously in birds (Freeman et al., 1981). In addition, the current research along with that of de Beer et al. (2008) which indicates that plasma corticosterone levels return from their elevated state to a baseline level after feeding may help explain why based on timing of blood sampling relative to feeding, others may have not seen an increase in plasma corticosterone concentrations in feed restricted broiler breeders (Savory et al., 1993; Savory and Mann, 1997). Plasma corticosterone decreases with age [de Jong et al., 2002; Chapter 5 (Neves et al., 2012)] and likely explains or contributes to the decrease in plasma corticosterone seen in all of the hens at 25 weeks of age.

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). In addition, Ekmay et al. (2010) reported that the overall plasma concentration of corticosterone over a 24 hour period was greater in SAD reared hens versus ED reared hens at 26.4 weeks of age, even though all of the hens had started to be fed on an ED basis at 24 weeks of age. The Ekmay report like the Weber report, suggests that alterations in corticosterone production can persist 2 to 4 weeks after the original feed restriction stressor has been removed. In the current research, there were no differences in the plasma corticosterone levels between SAD and ED reared birds at the end of their 25^{th} week of age which was almost 5 weeks after all of the birds had been switched to ED feeding schedule. Thus, feed induced alterations in plasma corticosterone production may not persist beyond 2 to 4 weeks once the feed stress is removed.

The EDB pullets had elevated plasma corticosterone levels at 17 weeks of age relative to the ED birds. The blood samples were taken at 17 weeks of age just before the ED and EDB pullets were to be fed, so it had been 24 hours since their last feeding. We believe that the elevated level of corticosterone may have been inadvertently caused by our experimental protocol. The EDB pullets were accustomed to a human arriving at feeding time each day to scatter their feed on top of the litter and this would have been the bird's expectation when we entered the room to collect blood samples. Because we were collecting blood samples, the birds did not receive the expected feed associated with human contact at that time of day and as result they likely became behaviorally stressed. The ED birds on the other hand were fed by an

automatic chain feeder, so human contact was not associated with food and this would have left them unaffected by our presence to collect blood just before feeding at 17 weeks of age.

T_3

The current research suggests based on plasma corticosterone concentrations that the extra activity of scratching for food in the litter did not reduce stress in the EDB pullets relative to the ED pullets. However, the extra activity and associated longer feeding times probably account for the slower rise in plasma total and free T_3 seen at 11 weeks of age when blood was collected 7 hours after feeding.

It is generally accepted that acute or chronic administration of corticosterone decreases the plasma T_3 concentration in chickens (Kuhn et al., 1985; Buyse et al., 1987; Mitchell et al., 1986; Williamson and Davidson, 1987). However, in laying hens injected daily with corticosterone for a week to 4 weeks, the plasma concentrations of both T_3 and corticosterone increased (John et al., 1987; Liu et al., 2012). Despite these two reports, fasting as already discussed is associated with an increase in corticosterone and fasting and feed restriction are also associated with a decrease in plasma T_3 concentrations in chickens (May, 1978; Moss and Balnave, 1978; Decuypere and Kuhn, 1984; Harvey and Klandorf, 1983; Buyse et al., 2000; 2002). The decrease in T_3 concentrations during fasting reduce metabolism and preserve nutritional resources as well as support a decrease in lipogenesis as T_3 is a well known stimulator of the biosynthetic capacity for de novo lipid metabolism.

Food intake by fasted chickens results in a return of plasma T_3 levels to pre-fasting levels within hours (Buyse et al., 2002; de Beer et al., 2008). de Beer (2008) indicated that after re-feeding, both ED and SAD feed restricted pullets at 16 weeks of age reached maximum plasma T_3 concentrations 8 hours after feeding and the maximum level did not differ between ED and

SAD pullets. However, once the maximum was reached, plasma T_3 levels decreased much quicker in ED pullets. Pre-feeding levels were reached in 20 hours in the ED pullets while it took 32 hours in the SAD pullets (de Beer et al., 2008). This slower decline in plasma T_3 levels was also found in the current results. At 17 weeks of age the plasma T_3 concentration in the SAD birds 24 hours after feeding was over 2 times greater than the level found in ED and EDB pullets while at 48 hours after feeding the plasma level of T_3 in the SAD birds was lowered to the level found at 24 hours in the ED and EDB birds. The T_3 results combined with the corticosterone results would indicate that the SAD birds are responding to the stress of feed restriction differently than the ED pullets despite receiving the same total amount of feed over a 48 hour period.

Glucocorticoid receptor

The response of the SAD pullets to their restriction feeding program may also influence their future reproductive success, as Gibson et al. (2008) reported that broiler breeder pullets fed on a SAD basis until flock egg production reached 5% produced 19 less eggs than their counter parts that initiated ED feeding about 5 weeks earlier with photostimulation. Kwok et al. (2007) cloned the chicken glucocorticoid receptor and reported that the mRNA for the receptor was detected in a preparation from the whole ovary (excluding the F1 through F3 follicles) of 25 week old birds. More recently, Lattin et al. (2012) provided evidence in house sparrows which supported that avian species like mammalian species have a two tiered corticosterone receptor system in which corticosterone binds with high affinity (more than 10 fold greater) to the mineralocorticoid receptor and with less affinity to the glucocorticoid receptor. In the ovary, only the glucocorticoid receptor was detected (Lattin et al., 2012). This suggests that ovarian

tissue might be primarily responsive to stress-induced levels of corticosterone rather than baseline levels of corticosterone that utilize the mineralocorticoid receptor.

The pullets reared on a SAD feed restriction program had less overall glucocorticoid receptor mRNA in the theca and granulosa tissue of the four largest hierarchical follicles than the EDB hens. But more importantly, the expression pattern of the glucocorticoid receptor mRNA in the SAD hens did not mirror that of the ED and EDB hens where F1 through F4 follicles had higher overall expression of the glucocorticoid receptor mRNA in granulosa tissue compared to theca tissue. In the SAD hens the expression of the mRNA for the glucocorticoid receptor was equal between the theca and granulosa tissue. Although there is not enough research on the glucocorticoid receptor in avian species to know its potential role in follicular development, it is noteworthy that in the highly efficient Leghorn laying hen, the clear trend based on only 3 replicates was that the theca tissue expressed less glucocorticoid receptor mRNA than the granulosa tissue.

Additional research is needed to examine the role of corticosterone on the development of the avian ovary as exposure of the developing ovary to high levels of corticosterone for several hours every other day during rearing may affect its development as seen in the present research with regard to glucocorticoid receptor mRNA expression. As reviewed by Tetsuka (2007) in other species, corticosterone/cortisol has been implicated in antagonizing oocyte maturation and potentially follicular maturation.

In summary, based on plasma corticosterone concentrations, a SAD feed restriction program is more stressful to broiler breeders than an ED feed restriction program. In addition, relative to an ED feeding program there are significant perturbations in T_3 hormone dynamics and ovarian glucocorticoid receptor mRNA expression in pullets reared on a SAD feeding

program. The EDB feeding program did not differ substantially from the ED feeding program. Further research is needed, but based on the current results rearing broiler breeder pullets on an ED feeding basis rather than the standard industry practice of rearing them on a SAD feeding basis is recommended.

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Table 6.1. Real-time RT-PCR primer and probe designs for the glucocorticoid receptor (GR) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Gene	Primer	Oligonucleotide Sequence	Product Size
	Forward	5'- AGACCAAGACGAAACCAGGAAAA-3'	(base pairs)
GR	Reverse	5'- AAGGGCGGATGGAAGTC-3'	75
	Probe	5'-CATCACGATCCCTCCTTTG-3'	
	Forward	5'-TTGGCATTGTGGAGGGTCTTC-3'	
GAPDH	Reverse	5'-GGGCCATCCACCGTCTTC-3'	70
	Probe	5'-TGACCACTGTCCATGCCAT-3'	

Table 6.2. Plasma concentration of corticosterone in broiler breeder pullets fed during rearing (from 2 through 20 weeks of age) on an everyday (ED), everyday broadcast (EDB) or skip a day (SAD) basis[1].

Treatments	Week of age		
	11	17	25
	----------ng/mL----------		
ED	2.16 ± 0.20[b]	2.23 ± 0.17[b]	0.58 ± 0.04
EDB	2.43 ± 0.30[b]	3.62 ± 0.28[a]	0.59 ± 0.05
SAD (day 1)	2.28 ± 0.49[b]	3.73 ± 0.43[a]	0.69 ± 0.04
SAD (day 2)	5.06 ± 0.51[a]	1.82 ± 0.16[b]	

[a-b] Means within each column with different superscripts are statistically different, $P < 0.01$.
[1]Values are means ± SEM, n=25. At 11 weeks of age the samples were obtained from the ED, EDB and SAD (day 1) birds 7 hours after feeding. In addition, for the SAD treatment, blood samples were also taken when the birds were on their subsequent off-feed day (day 2) or 31 hours after feeding. At 17 weeks of age the samples were obtained from the ED and EDB birds at 24 hours since last feeding (just prior to feeding for the ED and EDB birds) and they were obtained from the SAD (day 1) just prior to their feeding or 48 hours since their last feeding. In addition, for the SAD birds another set of samples (day 2) was collected 24 hours after their last feeding. At 25 weeks of age once all the birds had been placed on a once a day feeding schedule the blood samples were obtained 7 hours after feeding.

Table 6.3. Plasma concentration of total 3, 5, 3′- triiodothyronine (T_3) and free T_3 in broiler breeder pullets fed during rearing from 2 through 20 weeks of age on an everyday (ED), everyday broadcast (EDB) or skip a day (SAD) basis[1].

Treatment	Week of age					
	11		17		25	
	Total T_3	Free T_3	Total T_3	Free T_3	Total T_3	Free T_3
	----------pg/ml----------					
ED	177.7 ± 11.2[a]	1.46± 0.07[a]	35.9 ± 2.2[b]	ND	64.7 ± 3.7	0.48 ± 0.05
EDB	130.9 ± 8.7[b]	1.05 ± 0.06[b]	24.4 ± 1.4[b]	ND	69.1 ± 4.5	0.49 ± 0.04
SAD (day 1)	176.0 ± 8.4[a]	1.54± 0.08[a]	27.5 ± 1.0[b]	ND	69.4 ± 6.2	0.50 ± 0.07
SAD (day 2)	81.0 ± 7.9[c]	0.31± 0.04[c]	87.6 ± 3.0[a]	0.89 ± 0.05		-

[a-c] Means within each column with different superscripts are statistically different ($P \leq 0.01$).

[1]Values are means ± SEM, n=25. At 11 weeks of age the samples were obtained from the ED, EDB and SAD (day 1) birds 7 hours after feeding. In addition, for the SAD treatment, blood samples were also taken when the birds were on their subsequent off-feed day (day 2) or 31 hours after feeding. At 17 weeks of age the samples were obtained from the ED and EDB birds at 24 hours since last feeding (just prior to feeding for the ED and EDB birds) and they were obtained from the SAD (day 1) just prior to their feeding or 48 hours since their last feeding. In addition, for the SAD birds another set of samples (day 2) was collected 24 hours after their last feeding. At 25 weeks of age once all the birds had been placed on a once a day feeding schedule the blood samples were obtained 7 hours after feeding. ND = not detectable.

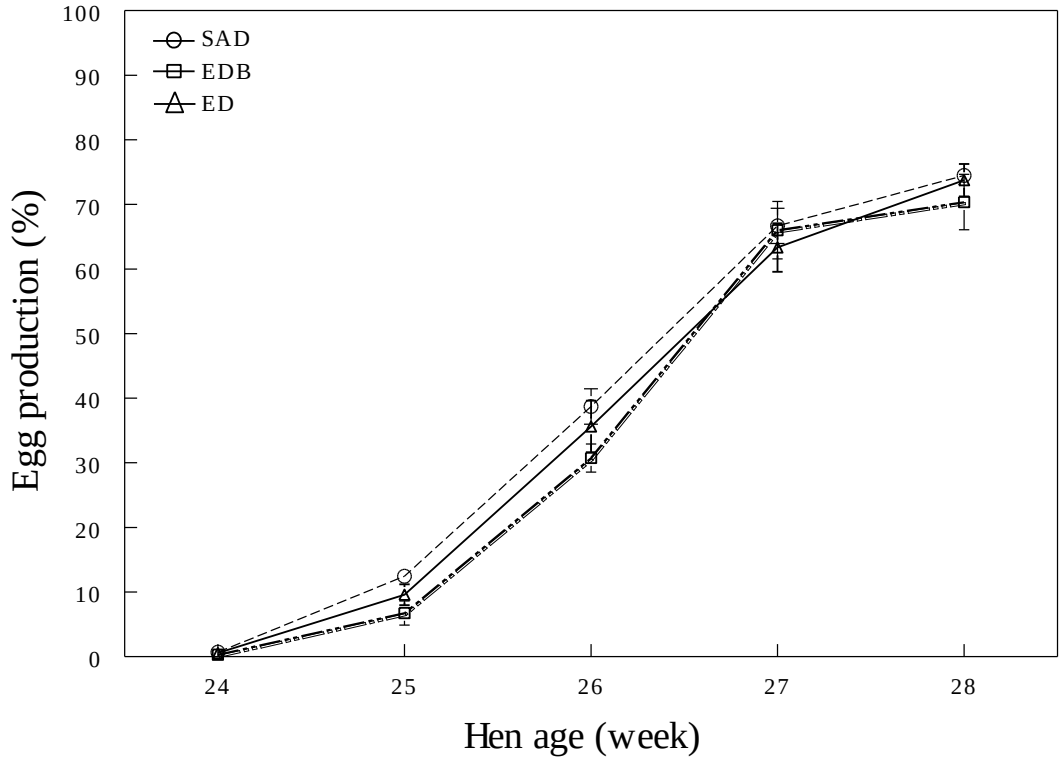

Figure 6.1. Weekly hen day egg production of broiler breeder hens that had been fed during rearing (from 2 through 20 weeks of age) on an everyday (ED), everyday broadcast (EDB) or skip a day (SAD) basis. Values are means ± SEM.

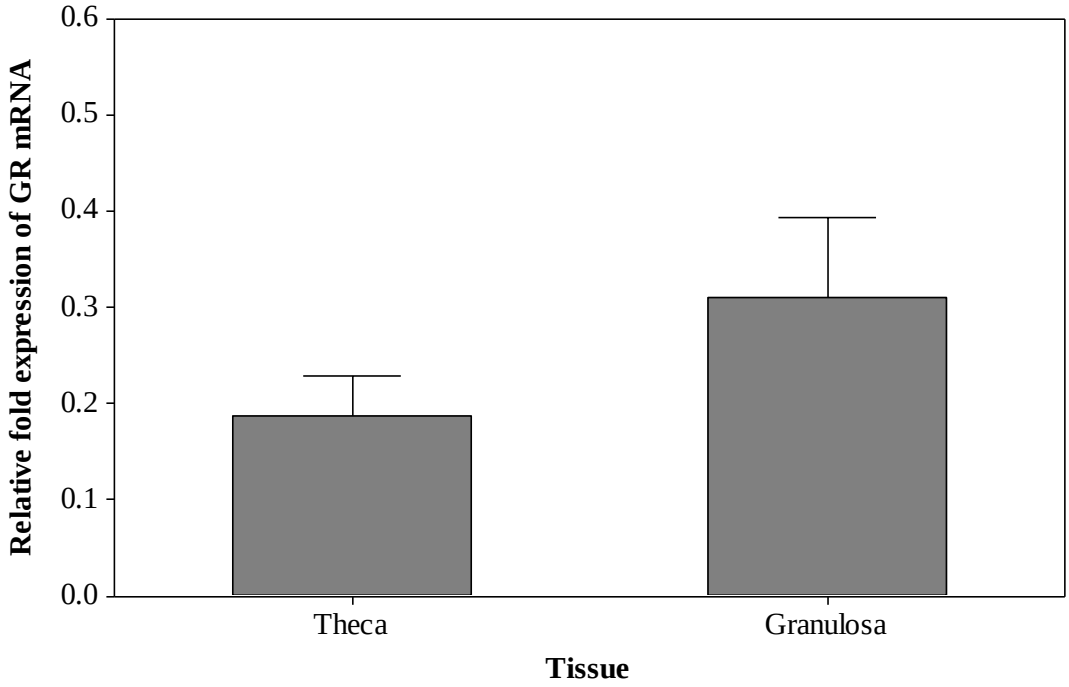

Figure 6.2. The overall relative fold expression of glucocorticoid receptor (GR) mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles, the small yellow and large white follicles from 45-55 week old Leghorn hens. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 18 (3 samples from each of the 6 follicle sizes).

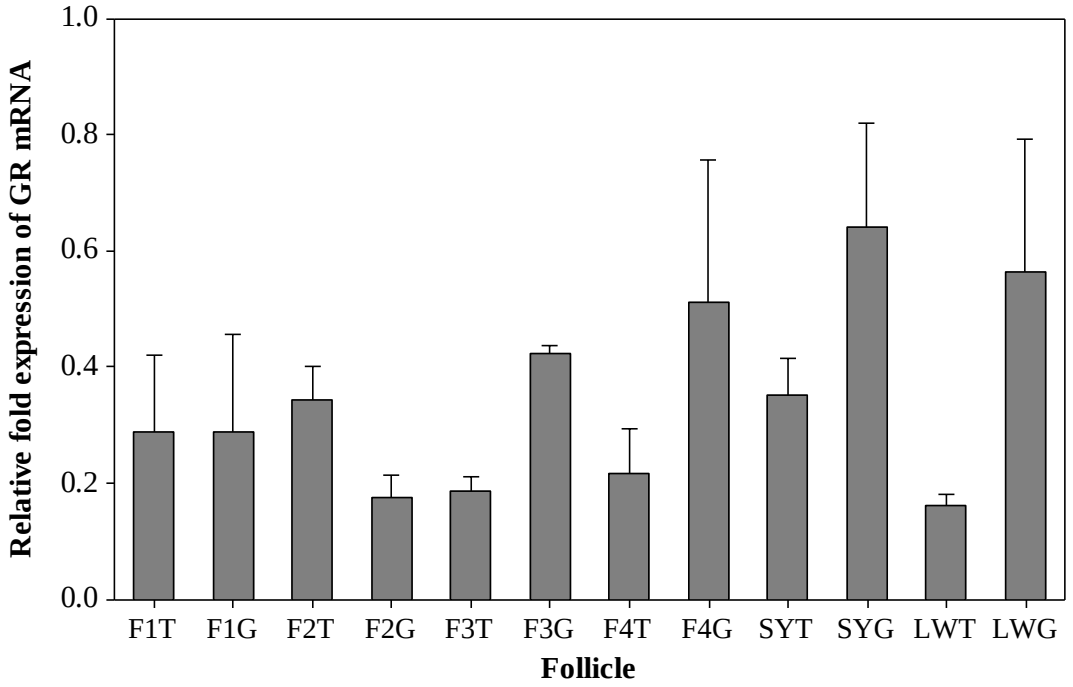

Figure 6.3. The relative fold expression of glucocorticoid receptor (GR) mRNA in theca (T) and granulosa (G) tissue collected from the four largest hierarchical (F1 through F4) follicles and from small yellow (SY) and large white (LW) prehierarchical follicles from 45 to 55 week old Leghorn hens. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 3.

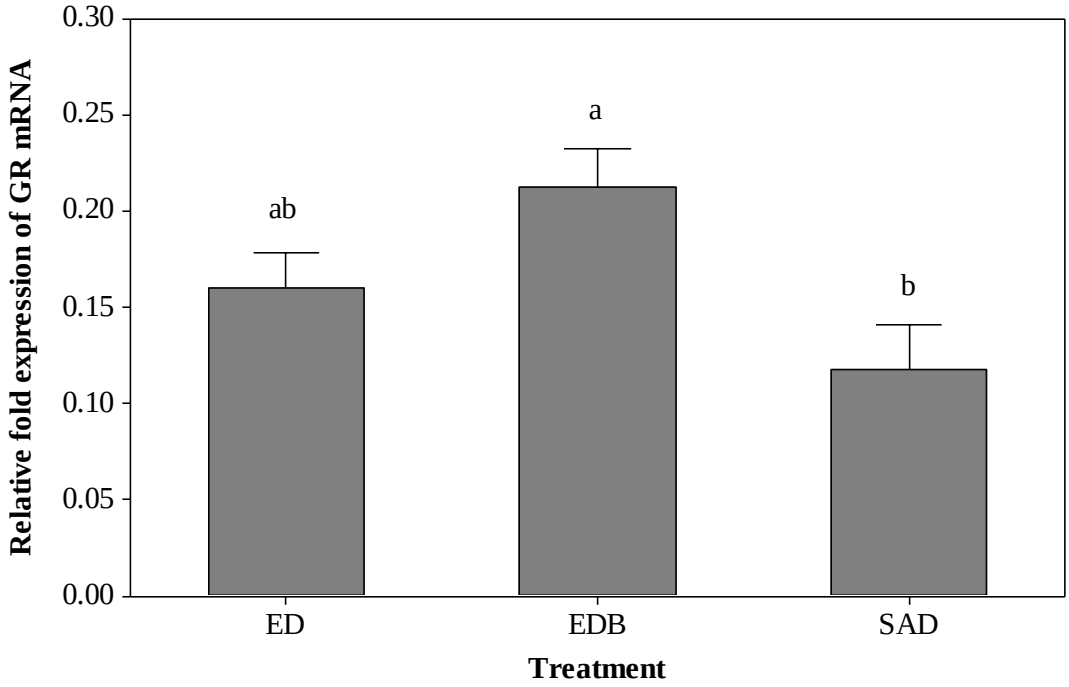

Figure 6.4. The relative fold expression of glucocorticoid receptor (GR) mRNA in theca and granulosa tissue collected from the F1 through F4 hierarchical follicles in 28 week old broiler breeder hens that had been fed during rearing (from 2 through 20 weeks of age) either on an everyday (ED), everyday broadcast (EDB) or skip a day (SAD) basis. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 48 (2 tissue types for 4 follicle sizes with 6 replicates per treatment). $^{a-b}$Means with different letters differ, $P < 0.05$.

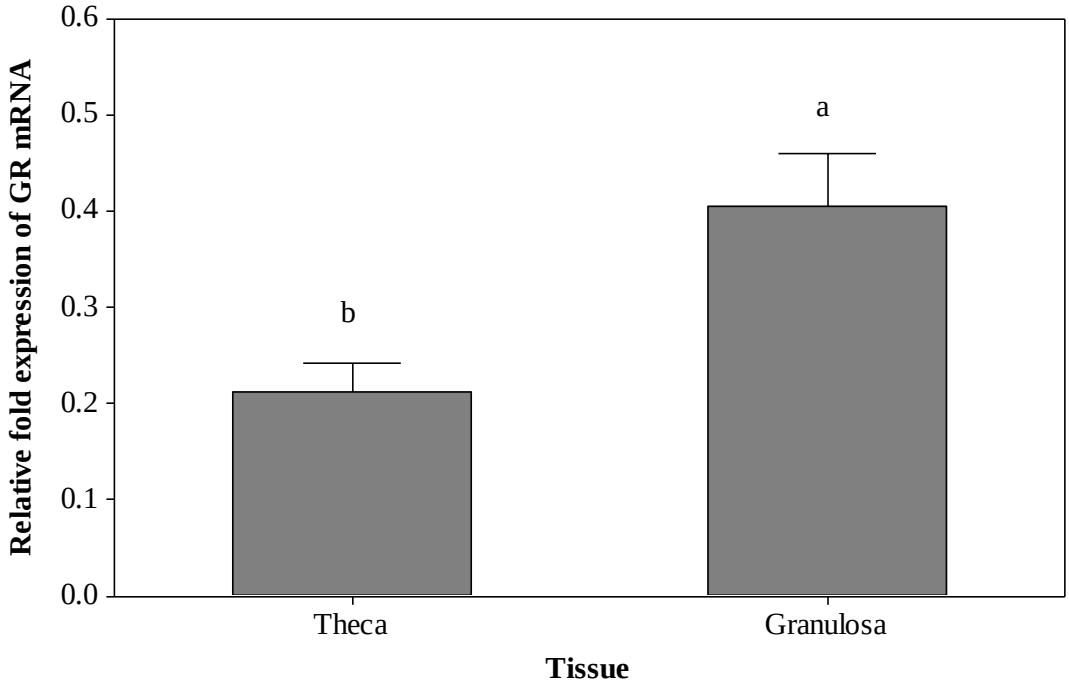

Figure 6.5. The overall relative fold expression of glucocorticoid receptor (GR) mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens fed on an everyday (ED) basis from 2 through 20 weeks of age. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 24 (6 replicate samples from each of the 4 follicle sizes). $^{a-b}$Means with different letters differ, $P < 0.05$.

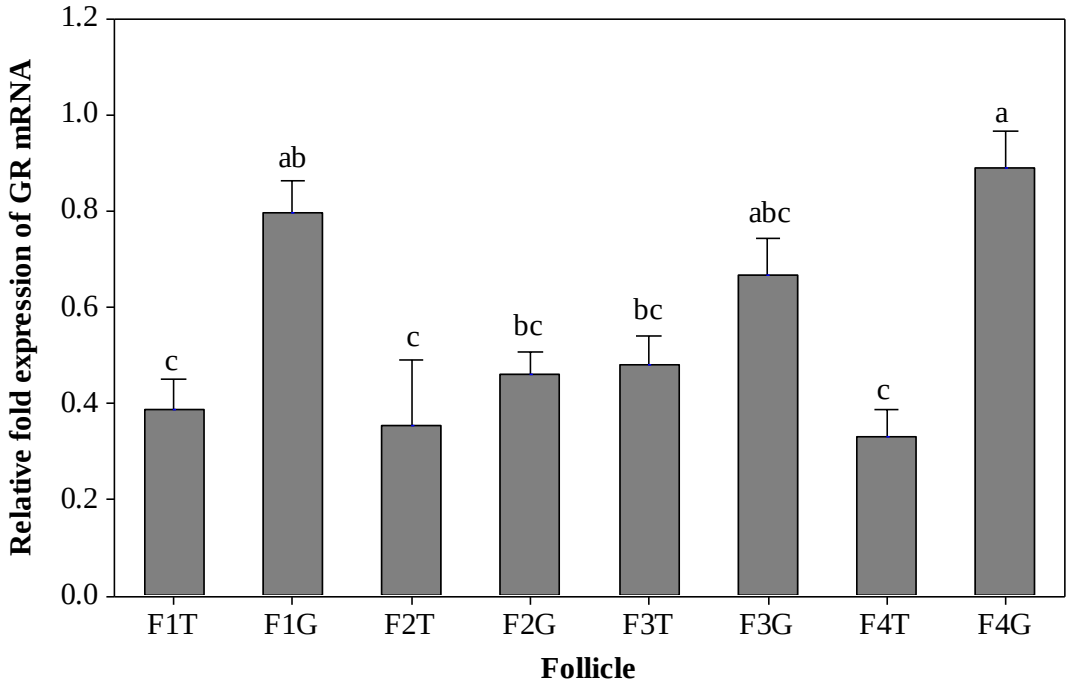

Figure 6.6. The relative fold expression of glucocorticoid receptor (GR) mRNA in theca (T) and granulosa (G) tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens fed on an everyday (ED) basis from 2 through 20 weeks of age. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. $^{a\text{-}c}$Means with different letters differ, $P < 0.05$.

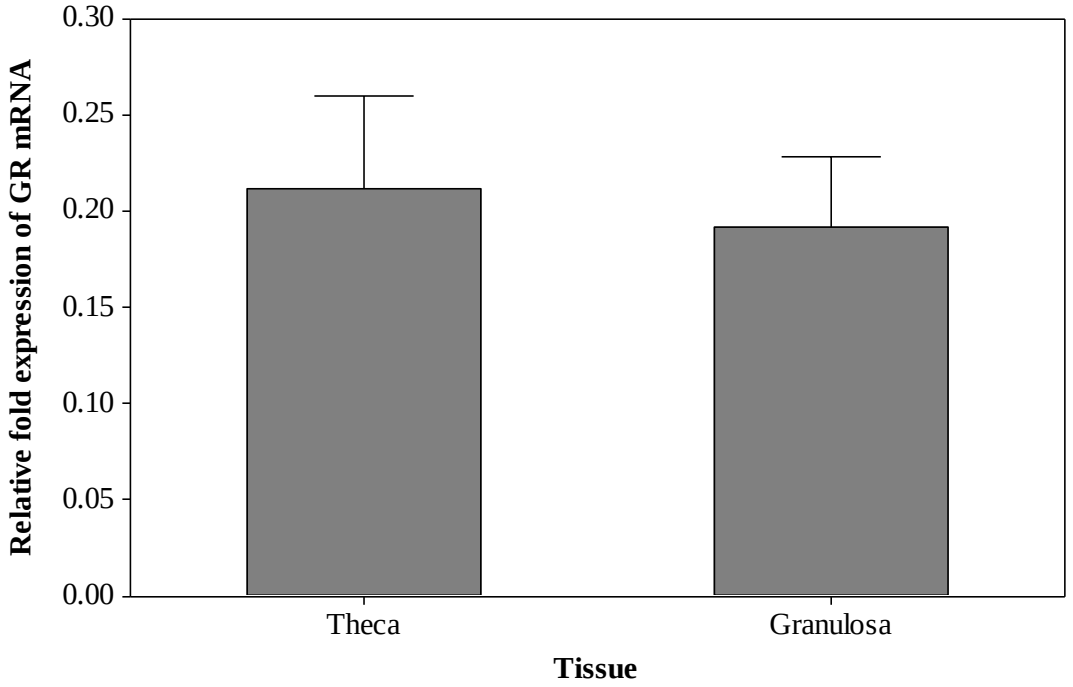

Figure 6.7. The overall relative fold expression of glucocorticoid receptor (GR) mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens fed on a skip a day (SAD) basis from 2 through 20 weeks of age. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 24 (6 replicate samples from each of the 4 follicle sizes).

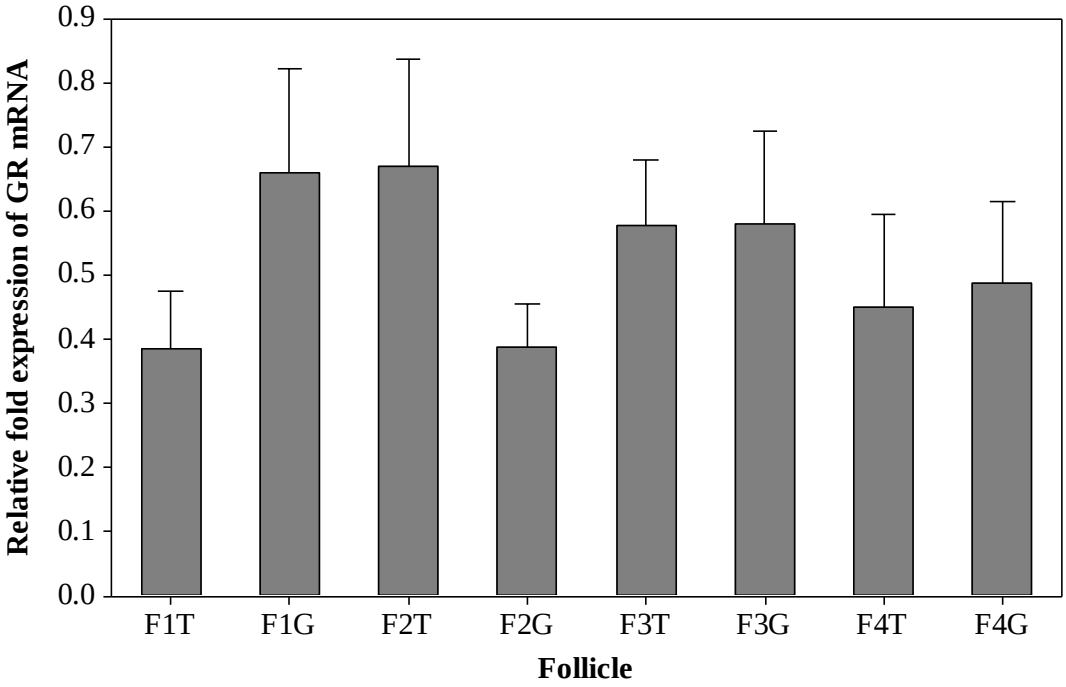

Figure 6.8. The relative fold expression of glucocorticoid receptor (GR) mRNA in theca (T) and granulosa (G) tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens fed on a skip a day (SAD) basis from 2 through 20 weeks of age. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6.

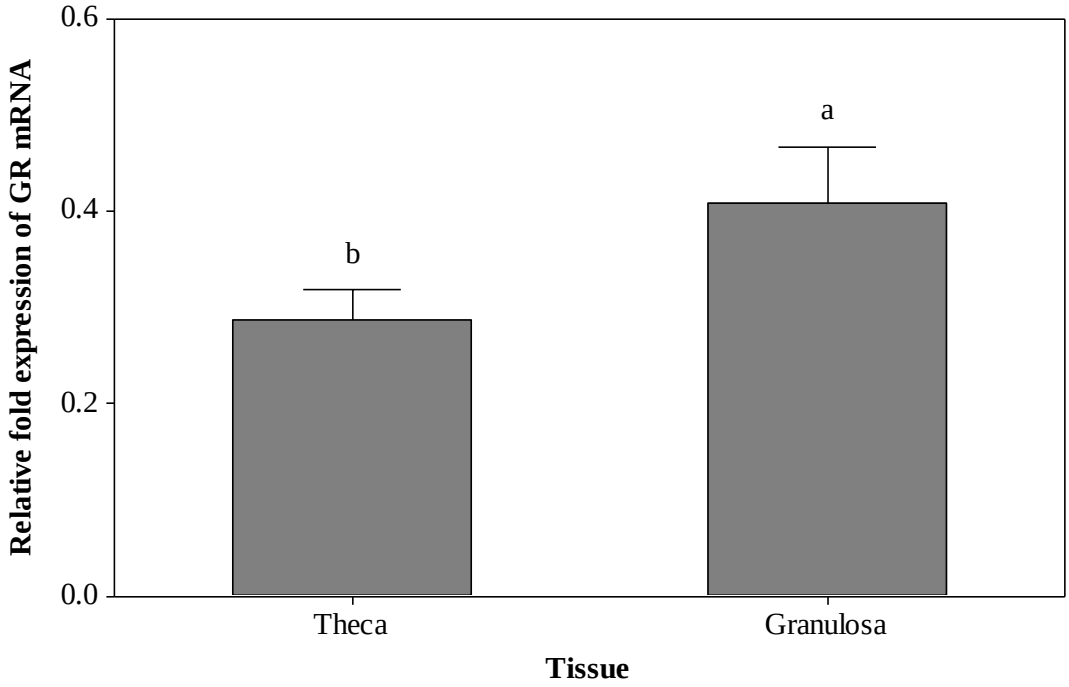

Figure 6.9. The overall relative fold expression of glucocorticoid receptor (GR) mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens fed on a everyday broadcast (EDB) basis with the feed broadcast on the litter from 2 through 20 weeks of age. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 24 (6 replicate samples from each of the 4 follicle sizes).

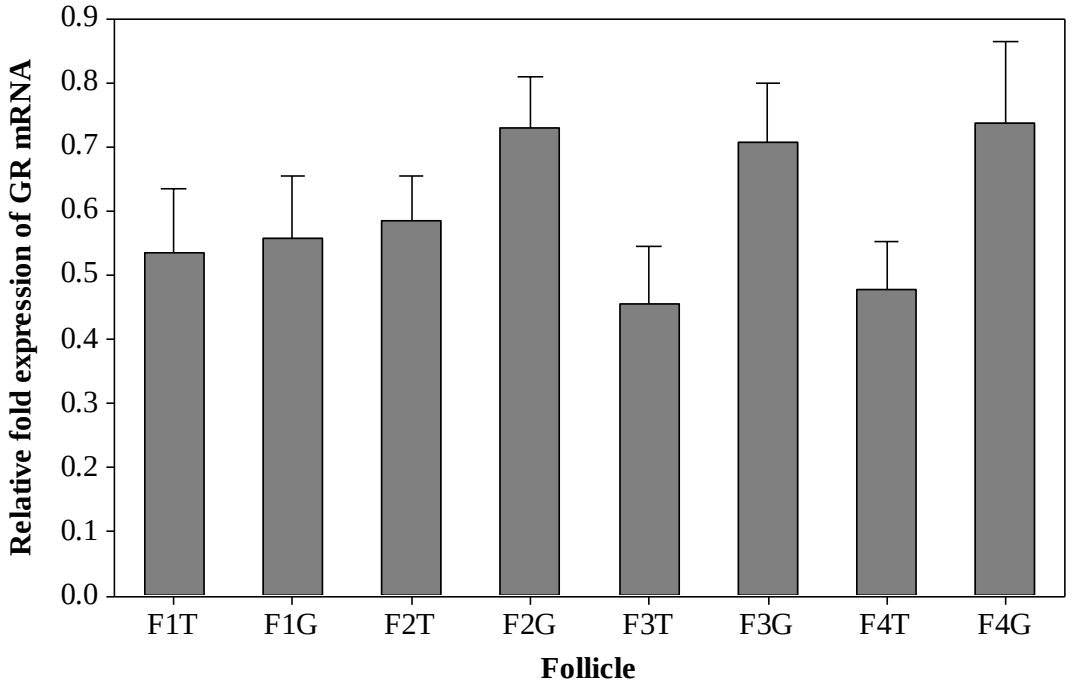

Figure 6.10. The relative fold expression of glucocorticoid receptor (GR) mRNA in theca (T) and granulosa (G) tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens fed on an everyday broadcast (EDB) basis with the feed broadcast on the litter from 2 through 20 weeks of age. The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6.

GENERAL OVERVIEW

The necessity for feed restriction in broiler breeders is readily evident given that their progeny reach the ideal body weight for reproduction by 5 to 6 weeks of age if allowed to eat ad libitum. However, the hypothalamus-pituitary-gonad axis does not mature for optimal reproduction until about 20 weeks of age. Therefore, if broiler breeders were allowed to consume food freely until the hypothalamus-pituitary-gonad axis matured, they would be too large in body size for optimal natural mating, their livability and mobility would be decreased and they could have accumulated too much body fat which decreases reproductive capability.

To ensure broiler breeders are at optimal body weight for reproduction at the time of photostimulation, the broiler industry severely restricts feed intake during rearing and during the breeding period. The benefits of these feed restriction practices have been tremendous as feed restricted broiler breeders produce significantly more fertile eggs than their ad libitum fed counterparts. However, despite the success of feed restriction in improving production, that does not mean that current feed restriction practices could not be improved to realize even greater production gains as broiler breeder hens still are not meeting their reproductive capacity based on the preovulatory follicles available to produce eggs.

The broiler industry in the United States typically utilizes an energy dense, highly digestible corn and soybean meal based diet to feed broiler breeders. Because only a small portion of this type of diet can be offered on a daily basis while preventing excessive body weight gain, it is common to combine the feed allotment from two days and then to feed this amount of feed every other day – the SAD feeding regimen. As the broiler breeders age and start producing eggs their nutrient requirements increase allowing more feed to be fed which facilitates an ED feeding schedule. The advantage of the SAD feeding regimen is that it ensures

that there is enough feed to distribute to all available feeder space in large broiler breeder rearing complexes. By utilizing all of the available feeder space there is less competition between birds which ensures all birds, not just the most dominate receive an allotment of feed which helps to maintain flock body weight uniformity. Maintaining flock body weight uniformity assists in the successful management of the birds as their nutritional requirements remain equal, they reach management milestones such as first egg produced at a uniform time and they produce eggs which are uniform in size which ensures uniform incubation conditions and uniformity in chick size. Uniform chicks in turn are also easier to manage and they reach market size at the same time and more uniform body weights which are ideal for automated processing.

The potential downfall of a SAD feeding program and for that matter, to lesser extent an ED feeding program is the significant fasting period that results from birds being fed once every other day or even once a day. In a commercial setting, once food is delivered (almost universally in the morning once the lights come on in industry settings) to the broiler breeders it is typically completely consumed within 30 to 60 minutes. Although each bird will have stored ingested food in its crop during this quick intake of all available food, the crop will empty within a few hours leaving the bird to fast until its next feeding. In the wild, birds including jungle fowl the original source for modern day domestic chicken breeds, forage throughout the day and have a significant evening foraging period in which they consume food to help sustain their metabolic needs throughout the night.

A focus of our laboratory's research has been to establish that the fasting associated with SAD or ED feeding is ultimately detrimental to reproductive performance. The current research indicates that rearing broiler breeder pullets on a SAD basis adds further stress to these birds as measured by plasma corticosterone levels when compared to feeding them on an ED basis. This

adds to previous research that indicated feeding broiler breeder pullets after photostimulation for reproduction decreases egg production and promotes the formation of a polycystic ovary syndrome in the developing ovary.

The current research also indicates that the stress response associated with the fasting of feed restriction might be dampened by providing supplementary dietary tryptophan above the requirement for growth and production as it lessens plasma corticosterone production. This potential management tool adds to our previous research which provided other management tools such as the utilization of feed ingredients with less available nutrients so that more feed can be delivered and allows for twice a day feeding.

In conclusion, feed restriction is necessary for broiler breeders to have optimal livability and production. However, the methods to accomplish this feed restriction to minimize stress and thus reproductive performance and livability can be and should be further refined for the welfare of the birds and the profitability and sustainability of the industry.