VISFATIN AND NESFATIN EXPRESSION IN BROILER CHICKENS

by

RAMATA SISSOKO CISSE

(Under the Direction of Adam J. Davis)

ABSTRACT

Research in mammals indicates that nesfatin (or NUCB2) and visfatin are protein hormones involved in regulating energy balance. Broiler breeder hens are feed restricted during rearing and production to control body weight. Ad libitum feeding during either the rearing or production phase decreases reproductive performance. Implementation of feed restriction programs typically results in the birds being fed a limited amount once every other day during rearing and once a day during production. This feeding schedule results in significant fast periods between feedings which can be detrimental to follicular development. Therefore, in the current research, the ovarian mRNA expression of nesfatin and visfatin was examined in hens that had been fed daily or fasted for 72 hours. Theca and granulosa tissue was isolated from individual F1, F2, F3 and F4 follicles, a pool of small yellow follicles (SYF) and a pool of large white follicles (LWF). The isolated theca and granulosa tissue from each follicle size was combined from 2 birds, and 3 replicate samples of each tissue type for each follicle size within each feeding treatment were obtained. Total RNA was isolated from each sample for real time PCR that utilized specific Taqman MGB probes and primers for detecting nesfatin, visfatin and GAPDH (endogenous control). The mRNA for nesfatin and visfatin was detected in granulosa and theca tissues from all follicle sizes examined. The expression of nesfatin and visfatin mRNA

was greater in granulosa tissue than in theca tissue in hierarchical follicles. Fasting had no effect on granulosa visfatin or nesfatin mRNA expression, but decreased theca visfatin mRNA expression. The results indicate that local production of visfatin and nesfatin could influence follicular development. The decrease in theca expression of visfatin during fasting should be further investigated to determine its possible role in follicular atresia induced by the lack of nutrient intake.

INDEX WORDS: Theca, Granulosa, NUCB2

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DEDICATION

This dissertation is dedicated to all the children in the world; to the hopeless and to the hopeful; to the ones with parents and to the orphans; to the ones to whom breakfast, lunch and dinner are guaranteed and to the ones who go to bed hungry and to who breakfast in not guaranteed the next morning. It is dedicated to the fortunate children who go to school every day and to the ones who do not have access to education; to the homeless children and to the ones under a roof; to the abused kids and to the protected ones; to the happy and to the sad kids; to the healthy ones and to the sick ones; to the girls and to the boys; to the rich and to the poor; to the anorexic and to the obese; to the physically fit and to the physically challenged ones; to the weak and to the strong.

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CHAPTER 1

NUTRITION AND REPRODUCTION

1.1. Energy demand of reproduction

Reproduction can be considered a luxury for an individual animal because the animal's survival does not depend on successfully completing it. In fact, reproduction typically puts tremendous energy demands on the pregnant or laying animal. Reproduction is sensitive to nutrition and in mammals it has been suggested that the availability of food is the most important factor that influences reproduction (Bronson, 1989). Inadequate nutrition can delay sexual maturation, inhibit the ovulatory cycle and adversely affects reproductive behavior. Specifically, low energy levels inhibit pituitary luteinizing hormone (LH) release through a decreased level of hypothalamic gonadotropin releasing hormone (GnRH) secretion via the hypothalamic-pituitary-gonadal axis (Foster and Nagatani, 1999). Because reproduction imposes additional nutrient needs during pregnancy or egg production, females store fat prior to the onset of reproduction in order to be able to cover additional energy demand that is not met by daily energy intake. However, a chronic imbalance of high energy intake exceeding energy expenditure causes the accumulation of excess adipose tissue which in turn hinders fertility (Ahima, 2006; Friedman and Halaas, 1998).

1.2. Hormones potentially involved in the nutrition/reproduction axis

Given that chronic under-nutrition and over-nutrition are both detrimental to the health and well being of animals and compromise reproductive capability, it is not surprising that the central nervous system regulates body energy homeostasis through a variety of hormones that have complex orexigenic and anorexigenic effects (Berthoud and Morrison, 2008; Crowley, 2008). Many of the key hormones that affect energy balance are produced by the gastrointestinal tract or by adipose tissue. Adipose tissue is not simply a passive storage depot for excess energy in the form of triglycerides. Fat, especially visceral fat, is a highly active endocrine organ (Ahima and Flier, 2000) secreting biologically active molecules, such as leptin, resistin, adiponectin (Ahima and Lazar, 2008), tumor necrosis factor alpha, angiotensinogen, interleukin 6, agouti protein and adipophilin (Ahima and Flier, 2000).

The study of hormones that regulate energy and balance influence the reproductive axis is an evolving science. Delayed puberty, ovulatory cycle irregularities, annovulation or reduced fertility experienced by anorexics, gymnasts, and marathon runners on one extreme and the extremely obese on the other extreme are all indications that the energy status of an individual regulates his or her reproductive capability. How hormones involved in food intake and energy balance, such as leptin, ghrelin, thyroid hormone, insulin, adiponectin and resistin, directly influence reproductive capability is just starting to be elucidated.

Leptin

Leptin is an adipocyte-derived peptide hormone that regulates food intake, body weight, and energy balance and influences the immune system (Zieba, et al., 2005). It acts at the level of the hypothalamus to signal that adipose stores are sufficient to begin puberty, maintain normal sexual maturation and initiate fertility (Friedman and Halaas, 1998; Baldelli, et al., 2002). An

early indication that leptin might have an influence on reproduction came from observations that ob/ob mice, which lack functional leptin, and db/db mice, which lack functional leptin receptors, did not reach sexual maturity and were infertile (Zhang, et al., 1994; Tartaglia, et al., 1995;). Fertility was restored in ob/ob mice when leptin was injected (Barash, et al., 1996; Chehab, et al., 1996).

The plasma concentration of leptin decreases during food restriction and starvation and is associated with a concomitant decrease in GnRH and LH secretion from the hypothalamus and anterior pituitary, respectively. However, GnRH and LH levels can be restored even during fasting by both intracerebroventricular (*icv*) and intraperitoneal (*ip)* injections of leptin (Farooqi, et al., 2002). In addition, *icv* injection of leptin antibodies reduces pulsatile LH release. Leptin receptors, which are responsible for signal transduction, appear to be localized in the gonads, the pituitary and within the hypothalamus in proximity to neuropeptides also thought to be important for controlling both the food intake and GnRH release (Donato, et al., 2011). Recent studies indicate that leptin acts through the hypothalamic kisspeptin regulatory system to increase GnRH production (Smith, et al., 2006).

In avian species, a putative leptin protein is produced in the liver, the source of *de novo* fat synthesis in birds, in response to feeding (Ashwell, et al., 1999; Taouis, et al., 1998; Kochan, et al., 2006). The biology of the chicken leptin receptor has also been fairly well characterized (Horev, et al., 2000; Ohkubo, et al., 2000). Leptin's role in avian reproduction is not well investigated, but based on preliminary reports in hens, it may provide an endocrine mechanism that allows nutritional status to influence reproduction (Ohkubo, et al., 2000; Paczoska-Eliasiewicz, et al., 2003).

Ghrelin

Ghrelin is a 28 amino acid hormone that was isolated from rat stomach as the endogenous ligand of the growth hormone (GH) secretogogue receptor (GHS-R) (Kojima, et al., 1999). Ghrelin is a potent stimulator of growth hormone secretion and a peripheral signal for energy insufficiency (Elmquist and Zigman, 2003) that also has central and peripheral effects upon metabolism and reproduction in mammalian species (van der Lely, et al., 2004). Specifically, ghrelin production is increased when there is a negative energy balance, and many of its physiological actions involve increasing feed intake and influencing metabolism as reviewed by Korbonits and Grossman (2004), Van der Lely *et al*. (2004), and Ueno *et al*. (2004). Ghrelin participates in the regulation of gonadotropin secretion as the central administration of ghrelin suppressed pulsatile LH secretion in ovariectomized female rats (Furuta, et al., 2001b; Martini, et al., 2006). Furthermore, hypothalamic secretion of GnRH from ovariectomized females was significantly inhibited by ghrelin (Fernandez-Fernandez, et al., 2005). In female and male rats, female monkeys, ewes, and human males, icv or peripheral injection of ghrelin decreases the pulse frequency of LH release from the pituitary and plasma LH levels regardless of whether or not the animal is gonadally intact (Furuta, et al., 2001a; Vulliemoz, et al., 2004; Fernandez-Fernandez, et al., 2005; Iqbal, et al., 2006; Garcia, et al., 2007; Kluge, et al., 2007). The effects of LH secretion appear to be mediated by GnRH, because GnRH secretion by hypothalamic fragments from ovariectomized female rats is inhibited by ghrelin (Fernandez-Fernandez, et al., 2004).

In chickens, ghrelin mRNA expression is highest in the proventriculus (Kaiya, et al., 2002; Richards, et al., 2006). Plasma ghrelin levels increase when chicks are fasted and after refeeding return to baseline levels (Kaiya, et al., 2007). Plasma ghrelin levels also increase in

broiler breeder hens that are fasted (Freeman and Davis, 2008). The mRNA for the ghrelin receptor has been detected in the theca and granulosa cells from hierarchical and nonhierarchical follicles and the mRNA expression of the ghrelin receptor is down-regulated by follicle stimulating hormone (FSH) and LH in cultured granulosa cells (Freeman and Davis, 2008).

Thyroid Hormone

In mammalian species, thyroid hormones (T3 and T4) are well established as regulators of metabolism, but there is emerging evidence they may be involved in regulating reproduction as well. Elevated levels of thyroid hormone can delay sexual maturity, alter gonadotropin release, and increase sex hormone binding globulin production such that steroid hormone activity is altered (Doufas and Mastorakos, 2000; Fitko and Szlezyngier, 1994). Low levels of thyroid hormones are also associated with decreased androgen production (Doufas and Mastorakos, 2000).

In avian species, thyroid hormones help regulate body temperature (Danforth and Burger, 1984), and growth and maturation (Bouvet, et al., 1987). The role of thyroid hormones in regulating reproduction in avian species has not been examined extensively. Exogenous thyroid hormone will stimulate testicular growth in quail (Follett and Nicholls, 1985; Yoshimura, et al., 2003). In addition, T4 concentrations are elevated and T3 concentrations are depressed during molting (Brake, et al., 1979; Lien and Siopes, 1989; Davis, et al., 2000b). Elevated T4 levels observed during molting are interesting, given that feed restricted broiler breeder hens (Bruggeman, et al., 1997), male chicks food deprived for about one day (Buyse, et al., 2000), and male quail food deprived for three days (Kobayashi and Ishii, 2002) all have reduced plasma concentrations of T3.

Insulin

Insulin is a peptide hormone involved in the regulation of both growth and reproduction. It is secreted by the beta cells of the islets of Langerhans in the pancreas, although its synthesis has been detected in the chick embryo even before the detection of beta cells (De Pablo, et al., 1982). Insulin production begins early in chick embryogenesis and influences the development of the gonads in both male and female chicks (Velazquez, et al., 2006). The addition of exogenous insulin accelerates while the addition of insulin antibodies delays the growth and differentiation of early chicks embryo cells (de Pablo, et al., 1985). The mRNA for insulin receptors, immunoglobuline F (IGF) and IGF receptors is expressed in the ovaries and follicular tissues of laying and broiler breeder hens (Heck, et al., 2003). Insulin increases basal androgen production in cultured cells from the testis of newly hatched chicks and actually enhances the proliferation of chick's embryo testis cells (Bobes, et al., 2001). These findings suggest that it is likely that insulin plays an important role in the control of ovarian follicular development and in linking energy status with fertility in birds as it does in mammalian species (Monget and Martin, 1997; Diskin, et al., 2003).

Resistin and adiponectin

Resistin and adiponectin are both adipocyte-secreted protein hormones that have received a great deal of attention in the last few years for their respective roles in insulin sensitivity and recently for their potential roles in reproduction as reviewed by Mitchell, et al., 2005; Budak, et al., 2006; Campos, et al., 2008; Bohler, et al., 2010; Michalakis and Segars, 2010. Plasma resistin concentrations are elevated in obese individuals (Steppan, et al., 2001). Resistin mediates insulin resistance and impairs glucose uptake by tissues and thus is associated with type II diabetes (Dargova, et al., 2011). Plasma adiponectin concentrations, on the other hand, are

negatively correlated with body weight, body fat mass and insulin levels. Adiponectin circulates in the blood at very high levels (µg/ml), with females having significantly higher levels than males (Geyikli and Akan, 2011). Adiponectin increases glucose uptake and fat oxidation in muscle cells and inhibits hepatic glucose production while increasing tissue sensitivity to insulin (Michalakis and Segars, 2010).

The roles of adiponectin and resistin in reproduction are still being determined. Both resistin and adiponectin appear to be expressed in the gonads, and receptors for both are found on the gonads. Plasma resistin levels are abnormally high in women suffering from polycystic ovary syndrome (PCOS), and adipose mRNA expression for resistin decreases in surgical catheterization of ovarian section treated PCOS women (Seow et al., 2007). Adiponectin levels are abnormally low in women suffering from PCOS (Michalakis and Segars, 2010). Adiponectin's role in reproduction also seems to vary based on its concentration. At normal physiological levels, it is supportive of ovarian development and steroidogenesis (Campos et al, 2008), but at very high levels, similar to those found in women suffering from anorexia or those who are marathon runners, adiponectin has a negative effect on GnRH production. In the chicken ovary, adiponectin is expressed by the ovary and may influence ovarian steroidogenesis (Chabrolle et al., 2006).

1.3. Summary

Although the correlation between nutrient intake and reproduction in animals is well established, the mechanisms by which energy status influences the hypothalamus-pituitary-gonadal axis are not well defined. Hormones produced by adipose tissue and the gastrointestinal tract seem to be the key players in linking the nutritional axis with the reproductive axis. In

particular, leptin produced by adipose tissue plays a key role in initiating the production of GnRH in females when body energy stores are sufficient to support the energy demands of reproduction. On the other hand, ghrelin produced by the stomach during times of caloric insufficiency, can inhibit GnRH production and thus prevent reproductive success when extra nutrients are not available to support reproduction. Further research may determine if other hormones produced by adipose tissue, such as resistin and adiponectin, also play key roles in linking energy/nutrient status with reproductive capability.

CHAPTER 2

VISFATIN AND NESFATIN

The search for hormones that can link energy intake and body stores with reproductive function is ongoing, and several hormones that are involved in nutrition and reproduction are discussed in the last chapter. Two other hormones, visfatin and nesfatin, which are secreted by adipose tissue, also have potential to be modulators of reproductive status, and both are receiving research attention due to their potential roles in obesity, inflammation and type II diabetes mellitus.

2.1. Visfatin

Visfatin (visceral fat-derived hormone) is a 52-kDa peptide hormone secreted by visceral fat tissues in mammals. It was identified by Fukuhara et al. (2005) as an insulin mimetic factor. However, visfatin had been previously identified and characterized based on other functions. The gene encoding visfatin was first isolated from a human blood lymphocyte cDNA library in 1994 (Samal, et al.1994) and was characterized as a cytokine, called pre B cell colony enhancing factor (PBEF), which enhanced the maturation of B-lymphocyte precursors. But, the visfatin peptide was originally identified in 1966 (Dietrich, et al., 1966; Powanda, et al., 1969; Streffer and Benes, 1971) as an intracellular enzyme called nicotinamide phosphoribosyltransferase (NAMPT), which catalyzes the rate-limiting conversion of nicotinamide with 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide (NMN), a substrate in the biosynthesis of

nicotinamide dinucleotide (NAD) (Samal, et al., 1994). All three names, visfatin, PBEF and NAMPT are used in the literature for the same protein with different biological functions. In this review, the term "visfatin" will be used exclusively for all of the functions of this unique protein that is both an intracellular enzyme and a secreted hormone.

Visfatin enzymatic effects

Visfatin is synthesized as an intra-cellular enzyme (Rongvaux, et al., 2002) and is involved in energy metabolism, reductive biosynthesis and antioxidant functions in many cellular processes (Belenky, et al., 2007). The predominantly nuclear location of visfatin and its increased cytoplasmic expression in dividing cells suggests a role in cell cycle regulation (Kitani, et al., 2003). Specifically, cell cycle analysis indicated that visfatin increased S-stage percentage and proliferation rate in a dose- and time-dependent manner (Yu, et al., 2010). As the rate limiting enzymatic component in the biosynthesis of NAD+, a classic coenzyme in cellular redox reactions, it is not surprising that visfatin is a key component in cell growth. Additionally, NAD+ is utilized by the enzyme SIRT1 (NAD-dependent deacetylase sirtuin-1) which deacetylates proteins that contribute to cellular regulation of metabolism, differentiation, stress resistance (Yang and Sauve, 2006), apoptosis, and inflammation (Revollo, et al., 2004). In addition, the production of SIRT1 extends the lifespan of smooth muscle cells by restraining the accumulation of p53 (van der Veer, et al., 2007), thus protecting the cells against apoptosis (Hasmann and Schemainda, 2003). A major cause of cell death is thought to be due to the depletion of NAD+ from the nucleus and the cytoplasm. Furthermore, reducing visfatin activity with the antagonist FK866, induced premature senescence in smooth muscle cells. In contrast, introducing the visfatin gene into aging human smooth muscle cells delayed senescence and

substantially lengthened cell lifespan, together with enhanced resistance to oxidative stress (van der Veer, et al., 2007).

Homozygous mice carrying a mutant visfatin allele die at day 10.5 of embryonic development (Revollo, et al., 2007b), indicating that visfatin may be essential for successful development. Visfatin-deficient heterozygous (visfatin+/−) female mice show moderately impaired glucose tolerance and reduced glucose-stimulated insulin secretion. A haploid deficiency of the visfatin gene in female mice resulted in deficiencies in the biosynthesis of NAD+ and glucose stimulated insulin secretion from pancreatic islets due to a decrease in both intracellular and plasma visfatin levels (Revollo, et al., 2007b). Interestingly, these results were sex-specific because a decrease in visfatin levels was only observed in the female mice (Revollo, et al., 2007b). It is not clear whether the visfatin synthesized inside the cell is the exact same form as the one secreted as an extra cellular cytokine (Revollo, et al., 2007b).

Secretion

Given visfatin's role in NAD+ production, it is not surprising that visfatin has been identified in multiple tissues of humans (Samal, et al., 1994), dogs (McGlothlin, et al., 2005), laboratory rodents (Kloting and Kloting, 2005), and pigs (Chen, et al., 2007). The mRNA for visfatin is expressed by many organs, including heart (Chang, et al., 2011), brain, lung, spleen, testis (Rongvaux, et al., 2002) and kidney, with the highest expressions in the fat depots (Fukuhara, et al., 2005), liver and bone marrow (Chen, et al., 2007; Revollo, et al., 2007a). Although visfatin has no signal peptide sequence for its secretion, it is secreted from lymphocytes (Samal, et al., 1994), neutrophils (Jia, et al., 2004), adipocytes (Fukuhara, et al., 2005) and human amniotic epithelial cells (Ognjanovic, et al., 2005) in an endoplasmic

reticulum-golgi or micro vesicle independent pathway (Tanaka, et al., 2007). Visfatin has been detected in both milk and blood (Yonezawa, et al., 2006).

Visfatin is synthesized as a dimer protein composed of two monomers each containing 491 amino acids in humans (Wang, et al., 2006). Each monomer contains two structural domains of 19 beta strands and 13 alpha helices forming two interfaces as potential active sites. To date no specific receptors have been identified for visfatin.

Visfatin as a hormone

Despite not having an identified receptor, visfatin has functions that classify it as a hormone. Visfatin secretion is regulated by inflammation and metabolic syndromes as well as obesity (Dahl, et al., 2010). Visfatin is synthesized and released by neutrophils in response to inflammatory stimuli and can be regulated by various cytokines. The pro-inflammatory stimuli TNF-α and interleukin-1beta (IL-1β) up-regulate the expression of visfatin expression in monocytes (Dahl, et al., 2007) and in amniotic epithelial cells (Ognjanovic, et al., 2001), respectively. Expression of visfatin is activated by IL-6 (Krzysik-Walker, et al., 2011), an essential cytokine for postnatal muscle growth in mammals. In contrast, IL-6 is a negative regulator of visfatin gene expression in 3T3-L1 cell line adipocytes (Kralisch, et al., 2005). Visfatin up-regulates the production of the inflammatory cytokines IL-1β, IL-1Ra, IL-6, IL-10, and TNF-α in human monocytes in a dose-dependent manner and enhances the surface expression of lymphocyte co-stimulatory molecules such as CD40 and CD80 (Moschen, et al., 2007).

Plasma visfatin levels correlate with white blood counts (Lajunen, et al., 2012) and increase during HIV infection (Van den Bergh, et al., 2012) indicating its proinflammatory effects. Furthermore, visfatin levels were shown to be high in the plasma of patients with

inflammatory bowel disease (Neubauer and Krzystek-Korpacka, 2010), were increased in active acromegaly, and may be an inflammatory mediator that causes monocyte infiltration in adipose tissue (Olarescu, et al., 2012).

Visfatin expression may also be involved in the persistence of inflammation through its capacity to inhibit neutrophil apoptosis (Jia, et al., 2004; Luk, et al., 2008), and visfatin is up-regulated in a variety of pathophysiological conditions of the immune system, including rheumatoid arthritis (Nowell, et al., 2006), psoriasis (Koczan, et al., 2005) and acute lung injury (Garcia and Moreno Vinasco, 2006). Taken together, it appears plausible that adipocyte and macrophage-derived visfatin might be an important pro-inflammatory and immunomodulating regulatory hormone.

Glucose metabolism is another area where visfatin acts as a hormone which has insulin-mimetic effects in various tissues in humans and mice. Specifically, visfatin has insulin-like glucose-lowering functions and even appears to bind to the insulin receptors, although this remains controversial (Fukuhara, et al., 2005; Fukuhara, et al., 2007; Xie, et al., 2007). Visfatin was found to stimulate tyrosine phosphorylation of insulin receptors and increase glucose uptake in cultured human osteoblasts (Xie, et al., 2007). Fukuhara et al. (2005) reported that heterozygous visfatin+/− mice have lower plasma visfatin levels compared with wild-type mice and have moderately higher plasma glucose levels under fasting as well as feeding conditions. Furthermore, during glucose tolerance tests, plasma glucose levels in visfatin+/− mice were significantly higher compared with controls. Visfatin also stimulates the phosphorylation of insulin signaling molecules at much lower molar concentration compared with insulin and stimulates the accumulation of triglycerides in pre-adipocytes in the same way as cells treated with insulin (Fukuhara, et al., 2005; Xie, et al., 2007).

In patients with type II diabetes mellitus, a condition characterized by insulin resistance and chronic hyperglycemia, plasma visfatin concentrations are significantly increased (Chen, et al., 2006). Interestingly, longstanding type I diabetes mellitus patients have higher plasma visfatin concentration compared with subjects with type II diabetes mellitus or non-diabetic subjects (Lopez-Bermejo, et al., 2006).

The correlation between circulating levels of visfatin and obesity in humans is controversial. One study found plasma visfatin levels to be significantly lower in obese subjects (Pagano, et al., 2006), but other studies found higher visfatin concentrations in obese humans (Haider, et al., 2006c; Filippatos, et al., 2007). Obese children have two-fold higher plasma visfatin levels compared to lean ones (Haider, et al., 2006a). Some studies have found a positive correlation between percent body fat and plasma visfatin levels (Berndt, et al., 2005; Hallschmid, et al., 2009), but another study found no association between plasma visfatin levels and waist circumference or fat mass for both lean or obese subjects (Sun, et al., 2007). Circulating visfatin concentrations were found to be correlated with the amount of visceral fat in healthy non-obese humans (Fukuhara, et al., 2005). Finally, glucose has been found to stimulate visfatin release from cultured adipocytes and to increase circulating plasma visfatin levels in healthy adult men (Haider, et al., 2006b).

Visfatin and reproduction

There has been limited research about the potential role visfatin might have on reproduction in mammals. Ovarian visfatin expression is low in aged mice compared with young mice, and visfatin administration accelerates embryo developmental rate in aged mice and increases the number of delivered pups from old females compared to untreated controls (Choi, et al., 2012). This study suggests that visfatin administration during superovulation can improve

oocyte quality and fertility of aged female mice. Although Shen et al. (2010) found no correlation between the plasma and follicular fluid levels of visfatin in humans; they did find that visfatin levels in both follicular fluid and plasma are positively correlated to the number of oocytes retrieved from women undergoing controlled ovarian stimulation. In human females the luteal granulosa cells produce visfatin and visfatin synthesis is increased by human chorionic gonadotropin and prostaglandin (PGE2) treatment (Shen, et al., 2010).

In humans elevated plasma visfatin levels have been associated with conditions such as PCOS (Chan, et al., 2007) and gestational diabetes mellitus (GDM), (Krzyzanowska, et al., 2006) where blood glucose levels are elevated. However, Chan et al. (2007) concluded that visfatin levels in women with GDM were lower compared with controls (Chan, et al., 2007). Visfatin has been linked to the events initiating labor in human as it is up-regulated by mechanical force (Ognjanovic, et al., 2003), in particular by distension of human amniotic epithelial cells (Nemeth, et al., 2000). Finally, serum visfatin levels are increased in women who develop high blood pressure condition during the last trimester of pregnancy called preeclampsia (Ferreira, et al., 2012).

Avian visfatin synthesis and distribution

A full-length 1482-base pair chicken visfatin cDNA was cloned in 2008 by Krzysik-Walker et al. using skeletal muscle RNA. The 493 deduced amino acid chicken visfatin sequence has a high degree of sequence homology with mammalian visfatin (Krzysik-Walker, et al., 2008). Avian visfatin mRNA is expressed in the breast and leg muscles, brain, hypothalamus, pituitary, myocardium, kidney, spleen, abdominal fat, liver, gizzard, pancreas, proventriculus, ovary, testis, lung, intestine, adipose tissue, and heart (Ons, et al., 2010). However, unlike mammalian species, where the highest expression level is in the adipose tissue,

breast muscle, skeletal muscle and the liver, have the highest expression of visfatin in the chicken. No differences have been found in the expression level between the subcutaneous, gizzard and abdominal fat depots (Ons, et al., 2010). Lipogenesis occurs primarily in the liver in the chicken, rather than in the adipose tissues as in rodents (Leveille, et al., 1975), and this might explain the high expression level in the liver versus adipose tissue in the chicken. Visfatin expression is sex dependent in the chicken, with females exhibiting a greater abundance of visfatin mRNA in abdominal adipose tissue compared to males (Ons, et al., 2010).

In addition to expression being gender dependent, visfatin expression is also age-dependent in the chicken. Skeletal muscle visfatin mRNA and protein quantities, as well as plasma visfatin levels, are significantly higher in 8-wk-old compared with 4-wk-old chickens (Krzysik-Walker, et al., 2008). In addition, testicular mRNA expression and plasma visfatin concentration were fourfold and 28 fold greater, respectively, in adult roosters versus prepubertal cockerels (Ocon-Grove, et al., 2010).

The complete functional significance of visfatin with regard to food intake in the chicken remains undetermined with research reports contradicting one another. Unlike mammalian research which indicates visfatin reduces feed intake (Park et al, 2011), visfatin has been found to be a potent *in vivo* stimulator of feed intake in broiler chicks when injected either peripherally or intracerebroventricularly (Cline, et al., 2008). However, Krzysik-Walker et al. (2008) reported that broilers fasted for 48 hours had plasma visfatin levels which were unchanged and remained unchanged 12 hours after the birds had been re-fed. Ons et al. (2010) reported that food deprivation for 24 hours increased visfatin mRNA abundance in the livers, but not in the muscle tissue or the hypothalamus of chickens from both obese and lean broiler genetic lines. Additionally, cerulenin, a feeding inhibitor, increased visfatin gene expression in chicken liver

and muscle while leptin treatment reduced visfatin gene expression in chicken liver and hypothalamus, but had no effect on the muscle mRNA level (Ons, et al., 2010).

2.2. Nesfatin

In a search for new appetite–controlling signals, Oh et al. (2006) identified a target that corresponded to the gene encoding nucleobindin 2 (NUCB2), which is also called NEFA (for DNA binding/EF-hand/acidic protein). NUCB2 had been previously cloned and its protein product was identified as novel but unnamed DNA binding protein (Miura, et al., 1992). The NUCB2 gene codes for a 396 amino acid precursor protein that is preceded by a 24 amino acid signal peptide for secretion. Based on structural analyses of the protein and the presence of several conserved cleavage sites, Oh et al., (2006) described putative post-translational processing of NUCB2 by pro-hormone convertases (PC 1/3 and PC2) to yield 3 distinct fragments designated nesfatin-1, nesfatin-2 and nesfatin-3. Nesfatin-1 corresponds to the N-terminal region (amino acids 1 to 82), nesfatin-2 is comprised of amino acids 85 to 163 and nesfatin-3 the C-terminal region consisting of amino acids 166 to 396. Nesfatin-1 in turn has been suggested (Shimizu, et al., 2009a) to be composed of 3 distinct fragments corresponding to the N-terminal amino acid segment spanning from amino acids 1 to 23 (N23), the middle fragment corresponding to amino acids 23 to 53 (M30) and the c-terminal fragment corresponding to amino acid 53 to 82 (C29), but to date there is no evidence to suggest that nesfatin 1 is further processed in vivo. The study by Oh et al., (2006) is the only one to date to detect the presence of nesfatin-1 in the cerebrospinal fluid of rats. However, Oh et al. (2006) were unable to detect nesfatin-1 in other tissues. Subsequent studies (Foo et al, 2008; Stengel et

al, 2009; Gonzalez, et al., 2010; Hausman, et al., 2012) have only detected full length NUCB2 in tissues and plasma. Thus, NUCB2 is now typically referred to as nesfatin.

Secretion and tissues distribution

Nesfatin is expressed in the brain and peripheral tissues in rodents and humans. It is expressed in appetite-controlling hypothalamic nuclei, such as the arcuate nucleus, paraventricular nucleus, supraoptic nucleus, the lateral hypothalamic area (Oh, et al., 2006), as well as, in the solitary tract (NTS) and dorsal nucleus of vagus (Brailoiu, et al., 2007). Several peripheral tissues express nesfatin mRNA, including adipose tissues (Oh et al., 2006; Hausman, et al., 2012), serum, gastric mucosa (Stengel, et al., 2009) and pancreatic beta cells (Gonzalez, et al., 2010).

Physiological effects

The main physiological function of nesfatin is its pivotal role in regulating feeding by reducing feed intake (Oh et al., 2006; Brailoiu, et al., 2007; Maejima, et al., 2009). Nesfatin inhibits food intake by modulating the glucose sensing neurons controlling food intake in the hypothalamus (Chen, et al., 2012). Nesfatin reduces body weight gain in rodents (Stengel, et al., 2011) and its levels are elevated in obese subjects (Tan, et al., 2011). While icv injection of nesfatin decreases food intake in rats, providing antibodies that bind nesfatin stimulates feeding and increases body weight (Oh, et al., 2006) suggesting its role as an anorexigenic factor and modulator of energy balance. Interestingly, icv injection of nesfatin-1 decreases food intake (Colmers, 2007; Myers, 2006) but similar injections of nesfatin-2 or nesfatin-3 do not (Oh, et al., 2006; Shimizu, et al., 2009a). Injection of nesfatin-1 by icv also induces anxiety and fear related behavior in rats (Merali, et al., 2008).

Nesfatin appears to play an important role in metabolic control through its anorexigenic and anti-hyperglycemic effects and protects against the development of obesity (Zegers, et al., 2011). Like leptin, intranasal application of nesfatin-1 reduces food intake in male Wistar rats (Shimizu, et al., 2009b). Nesfatin level in the plasma is elevated in subjects with impaired glucose tolerance and type II diabetes (Zhang, et al., 2012). Nesfatin-1 levels are negatively correlated with protein intake, but the significance of this observation is unclear at this point (Saldanha, et al., 2012).

Nesfatin and reproduction

Very recent research from one laboratory indicates that nesfatin might be involved in regulating the reproductive axis in rats. Conditions of negative energy balance, such as short term fasting and sustained under-nutrition, reduced NUCB2 mRNA and protein expression in the hypothalamus of pubertal female rats (Garcia-Galiano, et al., 2010). Furthermore, a functional knockdown of endogenous nesfatin tone in the hypothalamus of female pubertal rats delayed vaginal opening and reduced the weight of ovaries as well as the levels of LH (Garcia-Galiano, et al., 2010). In prepubertal rats, but not adult female rats, the icv infusion of nesfatin stimulates an increase in LH and FSH secretion (Garcia-Galiano, et al., 2010). NUCB2 mRNA expression has been detected in human, rat and mouse testis, as well as, rat ovary (Garcia-Galiano, et al., 2010).

Avian nesfatin

No research has been published on avian nesfatin. However, the NUCB2 gene was identified from the sequenced chicken genome and has the accession number NM_001006468 XM_421004.

2.3. Summary

Visfatin and nesfatin are two hormones that have recently been shown to regulate energy balance in mammalian species. Both hormones appear to influence reproductive capability, but the nature of these effects needs much more investigation, especially to determine if either of these hormones has direct effects on reproduction or simply appear to modulate reproduction based on the correlation between energy status and reproduction. Visfatin has been identified in avian species, but its functional receptor has not been identified in avian or mammalian species. Although nesfatin has been identified in the chicken genome, no research has been published on the possible tissue expression or function of this molecule in avian species.

CHAPTER 3

AVIAN REPRODUCTIVE PHYSIOLOGY

3.1. The avian ovary

The functional left ovary of the sexually mature laying hen typically contains a visually evident hierarchy of follicles based on size and time until ovulation. In the laying hen, there are commonly four to six large yellow yolk-filled follicles, referred to as hierarchical follicles, that range in diameter from approximately 12 to 40 mm in diameter. There are several additional follicles, called small yellow follicles (SYF), measuring 5 to 12 mm in diameter and yellow yolk deposition has begun in these follicles. In addition to the SYF, there are a large number of white follicles that are less than 5 mm in diameter. The SYF and white follicles are referred to as pre-hierarchical follicles.

The large yellow hierarchical follicles are named according to size and expected time of ovulation. The largest follicle, designated F1, will ovulate within 24 hours. The next largest follicle is called the F2 follicle and will ovulate approximately 24-26 hours after the ovulation of the F1 follicle. The remaining large yolk filled hierarchical follicles are named accordingly (F3-Fn). With the ovulation of each F1 follicle, the next follicle in position advances one position forward in the naming hierarchy while a new follicle is recruited into the hierarchy from the pool of SYF. Meanwhile, some of the small white follicles begin the uptake of yellow yolk and advance to the pool of small yellow follicles. The vast majority of small yellow and large white

follicles will never advance into the hierarchy (Gilbert, et al., 1983) and instead will undergo atresia by apoptosis (Johnson, et al., 1996).

3.2. Avian follicular tissues and follicular maturation

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

In general terms, follicular maturation can be described by the accumulation of yolk and the development of endocrine capabilities within the follicular tissues (Huang and Nalbandov, 1979). The theca cells of the small yellow and white follicles are steroidogenically competent and are the primary source of plasma estrogen (Lee and Bahr, 1989; Senior and Furr, 1975). However, the granulosa cells of these small follicles are steroidogenically incompetent because they lack P450 side chain cleavage (P450 SCC) enzyme activity which catalyses the initial step in the metabolic pathway that converts cholesterol to steroid hormones (Li and Johnson, 1993).

Follicular maturation is primarily regulated 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. Before granulosa cells mature and become LH dependent, they are sensitive to FSH. FSH promotes granulosa cell proliferation and maturation (Davis, et al., 2000a; Davis, et al., 2001) and helps maintain the follicular hierarchy through the prevention of atresia (Palmer and Bahr 1992; Johnson, et al., 1996; Johnson, et al.,

1999). In addition, FSH induces LH receptor, steriodogenic acute regulatory protein (StAR) and P450 SCC enzyme expression in granulosa cells for subsequent steroid production (Li and Johnson, 1993; Johnson and Bridgham, 2001), and stimulates progesterone (P4) production (Calvo and Bahr, 1983; Robinson, et al., 1988; Davis, et al., 1999; Davis, et al., 2001; Johnson, et al., 2004). Taken together, these data suggest that the pre-hierarchical follicles with granulosa cells (which are the most sensitive to FSH) avoid atresia and becomes increasingly responsive to LH, thus acquiring the capability of producing steroids. Interestingly, only one follicle from the cohort of small yellow follicles has an up-regulated level of FSH receptors, and it is likely it is the one that advances into the hierarchy (Woods and Johnson, 2005).

3.3. Avian ovulation

Follicle recruitment into the hierarchy is coupled with an increase in LH receptor expression and acquisition of functional P450 SCC enzyme activity within the granulosa layer (Li and Johnson, 1993; Kato, et al., 1995). Once the selection of a follicle occurs, the granulosa cells become dependent predominantly on LH signaling rather than FSH signaling (Calvo and Bahr, 1983). LH's primary function in granulosa cells of the hierarchical follicles is to stimulate progesterone production (Robinson, et al., 1988). However, significant secretion of progesterone in response to LH occurs only from F1 follicle of the hen prior to ovulation (Huang and Nalbandov, 1979). As reviewed by Etches (1996), plasma progesterone produced by F1 follicle triggers secretion of luteinizing hormone releasing hormone (LHLH-1) from the hypothalamus. LHRH-1 travels to the anterior pituitary through the hypothalamus-pituitary portal vascular system and initiates the secretion of LH from the anterior pituitary. LH and progesterone then engage in a positive feedback loop which generates the preovulatory surge of both LH and

progesterone 4 to 6 hours prior to the ovulation of the F1 follicle. LH and progesterone bind to their respective receptors in the cells along the stigma of the F1 follicle. This binding activates the receptors to stimulate production of enzymes such as collagenase that degrade the tissue along the stigma and promote the rupture of the F1 follicle and thus induce ovulation (Isola, et al., 1987; Yoshimura and Bahr, 1991).

3.4. Feed restriction in broiler breeder hens

Through genetic selection and better nutrition and bird management, today's broilers reach a market weight of 2 to 2.5 kilograms in 5 to 6 weeks. To support this rapid growth rate, broilers have been bred to possess nearly insatiable appetites. These voracious appetites and rapid growth rate are problematic for optimal reproductive performance in the genetically similar parent stocks of broilers. Optimum reproductive efficiency in broilers is dependent in large part on attaining and maintaining an ideal body weight to support reproduction, consuming a nutritionally adequate diet, and being photostimulated. Although the ideal body weight for reproduction is similar to market size, the optimum sensitivity to photostimulation for reproduction in broilers 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 restricted. Feed restriction of broiler breeder hens is a neccessary management tool that increases 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) 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 that have been feed-restricted produce more eggs (Yu, et al., 1992a; Heck, et al., 2004; Bruggeman, et al., 2005; 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., 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 are still plagued by an unacceptable incidence of atresia of large yellow follicles and internal ovulations. It is currently unknown why broiler breeders fed *ad libitum* produce excessive numbers of follicles at the same 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. Typically, feed allocations are 60-80 percent less during the rearing period and 25-50 percent less during the laying period than what breeder hens would consume *ad libitum*. There are also conflicting research reports on the optimal 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. McDaniel et al. (1981) and 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 restricted during both periods. Robinson et al. (1992a) 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 1-7 weeks of age followed by feed restriction from 7-15 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 and then once a day when egg production reaches 5% for the flock. This feed is quickly consumed by the birds resulting in a 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 depresses 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.,1992b). 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 23-59 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 lay in the twice a day feeding treatment. 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).

3.5. Summary

The broiler breeder hen ovary provides a unique opportunity to explore the role of visfatin and nesfatin in ovarian development. The histological structure of avian preovulatory follicles as well as the arrangement of the preovulatory follicles in a size hierarchy relative to ovulation makes the hens ovary an ideal model to determine the roles visfatin and nesfatin may have on ovarian development. Nutrition and energy balance are critical for the hen which can

only afford to continuously produce lipid and protein-packed, yolk-filled ova when there is the excess energy and nutrient resources to do so. In broiler breeders, the role of nutrition in reproduction is further complicated by the need to feed restrict broiler breeder hens to prevent excessive weight gain that is detrimental to reproductive efficiency. However, management practices in implementing this caloric restriction may negatively impact reproduction. The negative impact of fasting on reproduction could be mediated in part by nesfatin and visfatin.

CHAPTER 4

STATEMENT OF PURPOSE

NUCB2 and visfatin are energy balance regulating hormones that are expressed in brain nuclei and peripheral tissues and both are implicated in regulating food intake, metabolism and reproduction in mammals. While visfatin has been characterized in some chicken tissues, nesfatin has not yet been explored in any avian species. Neither visfatin nor nesfatin expression has been characterized during follicular development in the hen ovary. However, as hormones that potentially regulate the effects of nutrient status on reproductive function, characterization of these hormones in the ovary of the broiler breeder hen could be important for determining why follicular development is abnormal in these hens relative to laying hens. Visfatin and nesfatin, which appear to influence reproduction in mammalian species, may be of critical importance in broiler breeders which have to be severely feed restricted not only during rearing but during production. Therefore, the main objective of this research is to characterize the expression of nesfatin and visfatin in broilers breeders in order to determine whether these two hormones are correlated with nutritional status and reproduction in avian species. Specifically, the current research will be conducted in order (1) to determine if nesfatin mRNA is expressed in the broiler chicken, (2), to explore whether nesfatin and visfatin are expressed in the theca and granulosa tissues of hierarchical and prehierarchical follicles and (3) to investigate whether fasting changes nesfatin and visfatin mRNA expression in the developing follicles of the ovary.

CHAPTER 5

MATERIAL AND METHODS

5.1. Experiment 1

The purpose of this experiment was to determine the tissue distribution of NUCB2 and visfatin mRNA in key metabolic and digestive tissues in growing broilers.

Animals

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

Tissue collection

At 5 weeks of age, the broilers were killed by cervical dislocation for tissue collection. Approximately 300 mg of proventriculus, duodenum, jejunum, ileum, gizzard, liver, spleen, kidney, liver, adductor longus (leg muscle), pectoralis major (breast muscle), abdominal fat and

heart tissue was collected from 12 individual birds. Immediately after collection each tissue was placed in 3 mL (7 mL for fat tissue) of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA). Individual homogenized tissue solutions were frozen and stored at -80ºC for future RNA extraction.

5.2. Experiment 2

The aim of the second experiment was to characterize the mRNA expression pattern of NUCB2 and visfatin in the preovulatory follicles of the broiler breeder hen.

Animals

Seven hundred day of hatch female Cobb 500 fast-feathering broiler breeder chicks were split into 3 groups and each group was reared in a room measuring 7.32 × 9.14 m with pine shaving litter. From 1 to 3 days of age, the chicks were given 24 hours of light per day, and then from 4 through 14 days of age, the amount of light was decreased 2 hours per day until birds were provided 8 hours of light per day. The 8 hour per day lighting schedule was then maintained until the birds reached 21.6 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 were given ad libitum access to water from nipple drinkers. Feed was distributed everyday by an automatic chain feeder. From 2 to 23 weeks of age the birds were fed a standard developer diet and were feed restricted following the primary breeder guidelines. However, although the degree of feed restriction followed guidelines, the birds were provided a restricted feed allotment on a daily basis instead of doubling the feed allotment and providing it every other day which is the industry norm. A random selection of 10% of the birds from each room was weighed every week to determine and adjust feed allocations so that body weight gain of the pullets matched the

recommended guidelines of the primary breeder. All animal procedures were approved by the Animal Care and Use Committee at the University of Georgia.

At 21.6 weeks of age, pullets were assigned to 30 laying pens to ensure that the weight profile in each pen was similar. Each laying pen contained on average 46 pullets and 4 roosters. Each pen measured 3.65 × 2.75 m, and the floor space of each pen consisted of two-thirds pine shavings litter and one-third elevated slats. Each pen had one 6-hole nest box located on the slat area and was equipped with 10 nipple drinkers. In the laying pens, the hens and roosters were hand-fed by using plastic feeder pans. Each pen contained 3 hen feeder pans, which were fitted with rooster exclusion grills. The feeding system provided 9.14 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.

Photostimulation for reproduction occurred at 21 weeks of age by providing 14 hours of light (lights on at 0600 hours), and this photoperiod was maintained until the experiment ended. At 23 weeks of age, all the hens and roosters were switched to a broiler breeder layer diet while being maintained on an everyday feeding schedule. The amount of feed provided was still restricted following the primary breeder guidelines and weekly body weight gains.

Tissue collection

At 28 weeks of age, 12 birds were killed by cervical dislocation 2-4 hours prior to ovulation. The ovary was collected from each hen. The 4 largest hierarchical follicles F1, F2, F3 and F4, small yellow follicles (SYF, >5 to 12mm in diameter), and large white follicles (LWF, >2-5mm in diameter) were separated from each collected ovary. The granulosa cell layer was manually separated from the theca cell layers of each hierarchical follicle (Huang and Nolbandov 1979). For the prehierarchical follicles (SYF and LWF), the theca and granulosa

cells were not separated. The theca layers collected from each individual F1 through F4 follicle and the pooled theca and granulosa layers of the 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 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.

5.3. Experiment 3

This experiment was completed to determine if the mRNA expression of NUCB2 or visfatin in the hierarchical or prehierarchical follicles of broiler breeder hens was influenced by fasting.

Animals

The Cobb 500 broiler breeder hens utilized for this experiment were between 45 and 55 weeks of age. The birds were reared as previously described (Spradley, et al., 2008) using a skip a day feed restriction program. At 21 weeks of age the pullets were placed in individual cages and were photostimulated to initiate reproduction with a lighting program that provided 14L:8D (lights on at 06:30 hours) per day. The hens were given free access to water and were fed a standard broiler breeder layer diet each morning at 08:00 hours. The daily amount of feed provided to the hens was determined using the guidelines of the primary breeder (Cobb-Vantress 2005a, Cobb-Vantress 2005b) based on the weekly body weight measurements and egg production rates of the hens. Eggs were collected twice daily and individual hen egg production

was recorded. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Tissue collection

Four hens in midlaying sequence were divided into 2 treatment groups. The hens in one treatment group continued to receive their daily allotment of feed while the hens in the other treatment group did not receive food. After 72 hours of fasting, all 4 hens were killed and the ovary was collected from each hen. The theca and granulosa layers from each of the F1, F2, F3 and F4 follicles were collected as described in experiment 2. The theca and granulosa layers from the SYF (5 to 10 mm in diameter) and LWF follicles (2 to 5 mm in diameter) were separated enzymatically (Davis, et al., 2000a). The individual theca and granulosa samples for each follicle size from one hen of each treatment were combined with the corresponding samples from the other hen of that treatment for RNA extraction. This collection procedure was repeated 2 more times to give 3 total replications for each treatment (n = 3).

5.4. RNA Extraction

Total RNA was extracted from tissue samples using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA samples were stored at -80ºC. 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. Based on the quality of the RNA, the best 6 replicate samples were chosen out of the 12 replicate samples for experiments 1 and 2 for the determination of the mRNA expression of visfatin and NUCB2.

5.5. Real time RT-PCR

Extracted RNA samples were DNase treated using TURBO-DNA-free kit (Ambion, Austin, TX) to remove any potential genomic DNA contamination. Taqman minor groove-binding probes and primers (Table 5.1) for detecting NUCB2 (GenBank accession # NM_001006468.1), visfatin (GenBank accession #NM_001030728) and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession #M11213) were designed using Primer Express software (Version 4.0, Applied Biosystems). 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 concentration as described by the manufacturer (Applied Biosystems, Foster City, CA).

Reverse-transcription cDNA synthesis reactions were performed using the TaqMan Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. For two-step real-time PCR, 100 ng of cDNA was used for each sample for the NUCB2, visfatin and GAPDH amplification. The reactions were performed in a 25ul volume of reaction buffer containing 1x Taqman Universal PCR Master Mix (Applied Biosystems) and 900nM of either, NUCB2, visfatin or GAPDH primer pairs and 25nM of the appropriate probe. The reactions were completed in an ABI 7500 thermocycler (Applied Biosystems). The thermocycler conditions were 10 minutes at 95ºC and 40 cycles each 15 seconds at 95ºC and 1 minute at 60ºC. The reactions for each sample were performed in duplicate for NUCB2, visfatin and GAPDH assays. The Ct (the cycle number at which the fluorescence exceeds the threshold level) was

determined for each reaction using the Sequence Detection Software (version 1.2.2, Applied Biosystems) and quantification was completed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the NUCB2 and visfatin Ct's were determined for each sample and then normalized to the GAPDH Ct from the same sample (GAPDH Ct subtracted from the NUCB2 Ct or visfatin Ct yielded the ΔCt). After all the ΔCt values were obtained for an experimental replicate, the ΔCt values for each individual NUCB2 or visfatin were compared to the sample within the replicate that had the highest mRNA expression for NUCB2 or visfatin using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Thus, all data for NUCB2 or visfatin is expressed as the fold-difference relative to sample with the highest expression.

5.6. Statistics

Data from each experiment were subjected to ANOVA according to the General Linear Model (GLM) with replicate and tissue as factors in experiment 1 and 2 and replicate, feeding state and tissue as factors in experiment 3. Tukey's multiple-comparison procedure (Neter et al. 1990) was used to detect significant differences among individual tissues and follicle sizes. Differences were considered significant when $P < 0.05$. All statistical procedures were completed with the Minitab statistical software package (Release13, State College, PA).

Table 5.1 Real-time RT-PCR primer and probe designs for NUCB2, visfatin and GAPDH

Gene	Primer	Oligonucleotide Primer and Probe Sequence	Product Size (Base Pairs)
NUCB2	Forward	5'-TGACATCAACAAGGACAGACTAGTAACTC-3'	60
	Reverse	5'-TTGATCTAGGGTCTCCCAGCTATC-3'	
	Probe	5'-AGTTCCTGCGAGCTACA-3'	
Visfatin	Forward	5'-AAAGATCCACTTCCGGGATATTC-3'	62
	Reverse	5'-CATCATCCTTCCCCCAAGCT-3'	
	Probe	5'-CTGCTGAACACAGTACCA-3'	
GAPDH	Forward	5'-TTGGCATTGTGGAGGGTCTT-3'	87
	Reverse	5'-GGGCCATCCACCGTCTTC-3'	
	Probe	5'-TGACCACTGTCCATGCCAT-3'	

CHAPTER 6

RESULTS

6.1. Experiment 1

In broiler chickens, the mRNA for NUCB2 and visfatin were detected by RT-PCR in all tissues examined. The mRNA expression level of NUCB2 for spleen was greater than all other tissues examined (Figure 6.1). The mRNA expression of NUCB2 in leg and breast muscle (pectoralis major) was very low relative to most tissues, and thus the NUCB2 expression data for the muscle tissues was presented in a separate figure (Figure 6.2) with a different y-axis scale than in Figure 6.1. The mRNA expression level of NUCB2 in liver is presented in both Figures 6.1 and 6.2 to provide reference.

Visfatin mRNA expression in the spleen was not significantly different from its expression level found in the duodenum and jejunum (Figure 6.3). Visfatin mRNA expression in leg and breast muscle were not different (Figure 6.4). As with NUCB2, the expression of the visfatin mRNA was very low in leg and breast muscle relative to most tissues and thus the expression data for visfatin was presented in Figures 6.3 and 6.4 with hepatic mRNA expression of visfatin serving as a reference point between the two figures.

6.2. Experiment 2

In broiler breeder hens, NUCB2 and visfatin mRNA were detected in both hierarchal and non-hierarchal follicles. The overall expression of NUCB2 mRNA was greater in granulosa

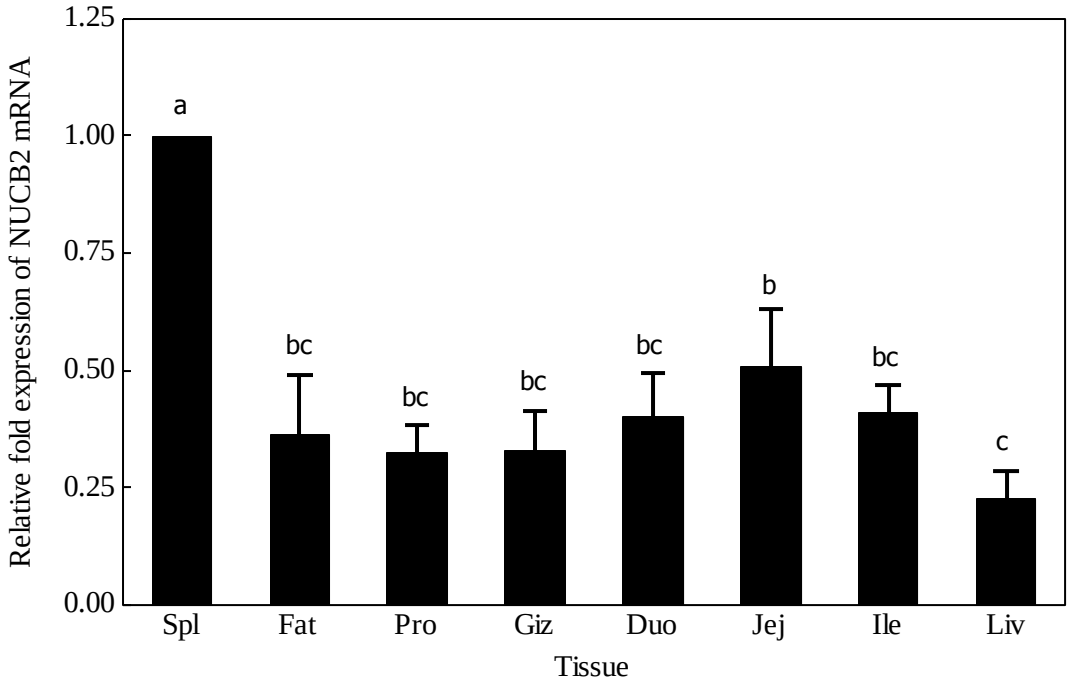

Figure 6.1: The relative fold expression of NUCB2 mRNA in tissues isolated from 5 week old broilers (experiment 1). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. $^{a-c}$Means with different letters differ, $P < 0.05$. Abbreviations: Spl = spleen, Pro = proventriculus, Giz = gizzard, Duo = duodenum, Jej = jejunum, Ile = ileum, Liv = liver.

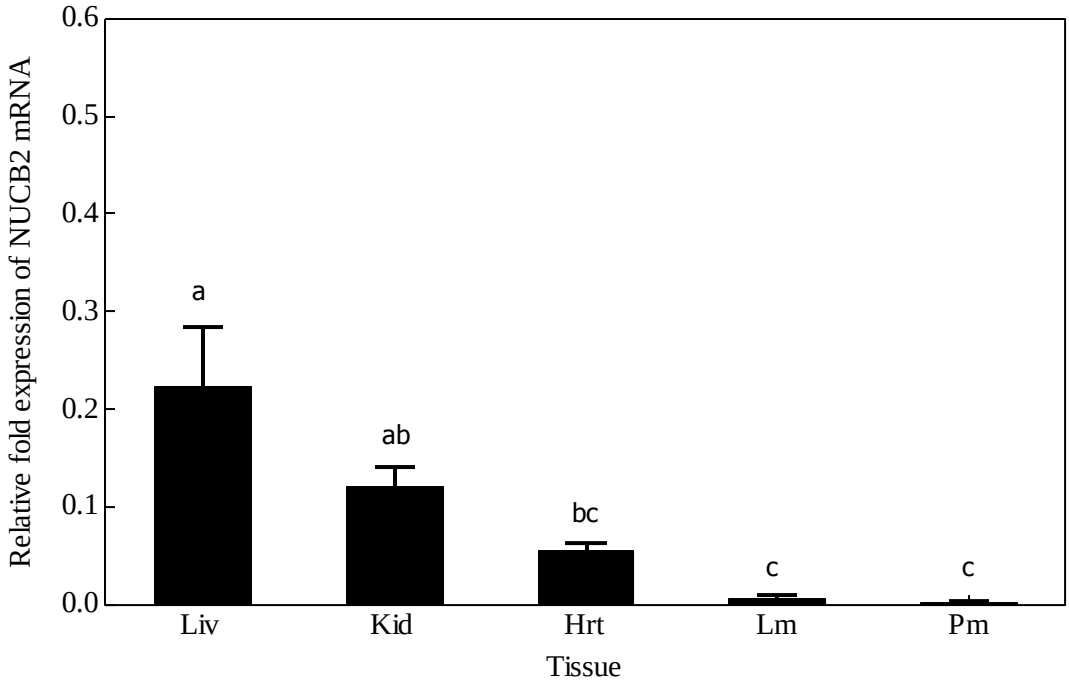

Figure 6.2: The relative fold expression of NUCB2 mRNA in tissues isolated from 5 week old broilers (experiment 1). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. $^{a-c}$Means with different letters differ, $P < 0.05$. Abbreviations: Liv = liver, Kid = kidney, Hrt = heart, Lm = leg muscle (adductor longus), Pm = pectoralis major.

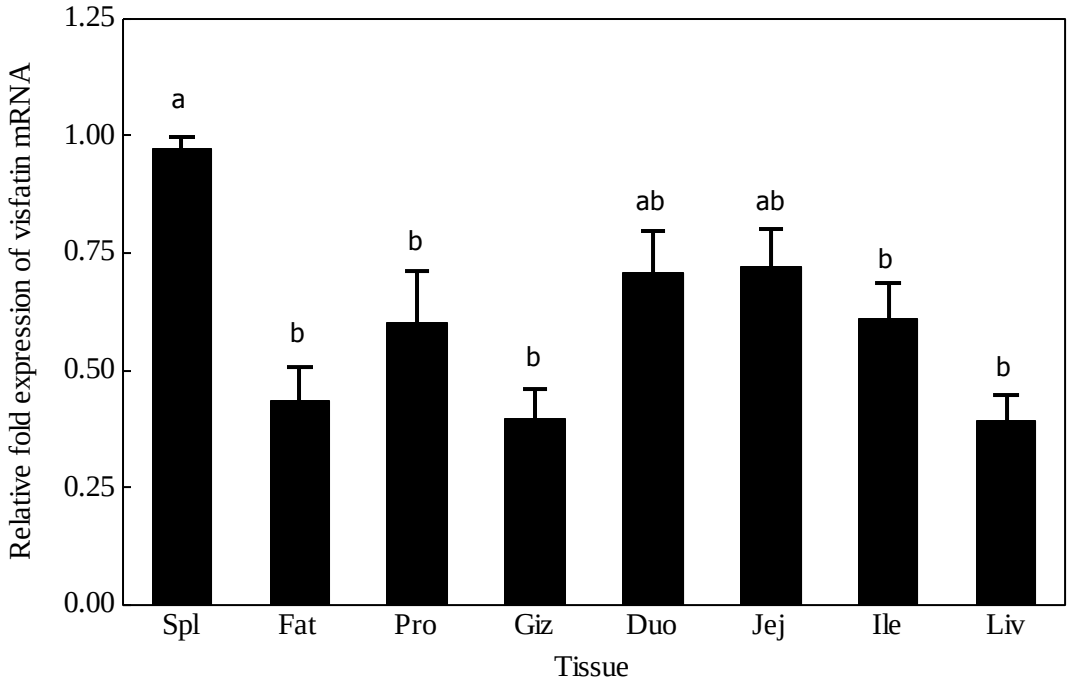

Figure 6.3: The relative fold expression of visfatin mRNA in tissues isolated from 5 week old broilers (experiment 1). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. $^{a-b}$Means with different letters differ, $P < 0.05$. Abbreviations: Spl = spleen, Pro = proventriculus, Giz = gizzard, Duo = duodenum, Jej = jejunum, Ile = ileum, Liv = liver.

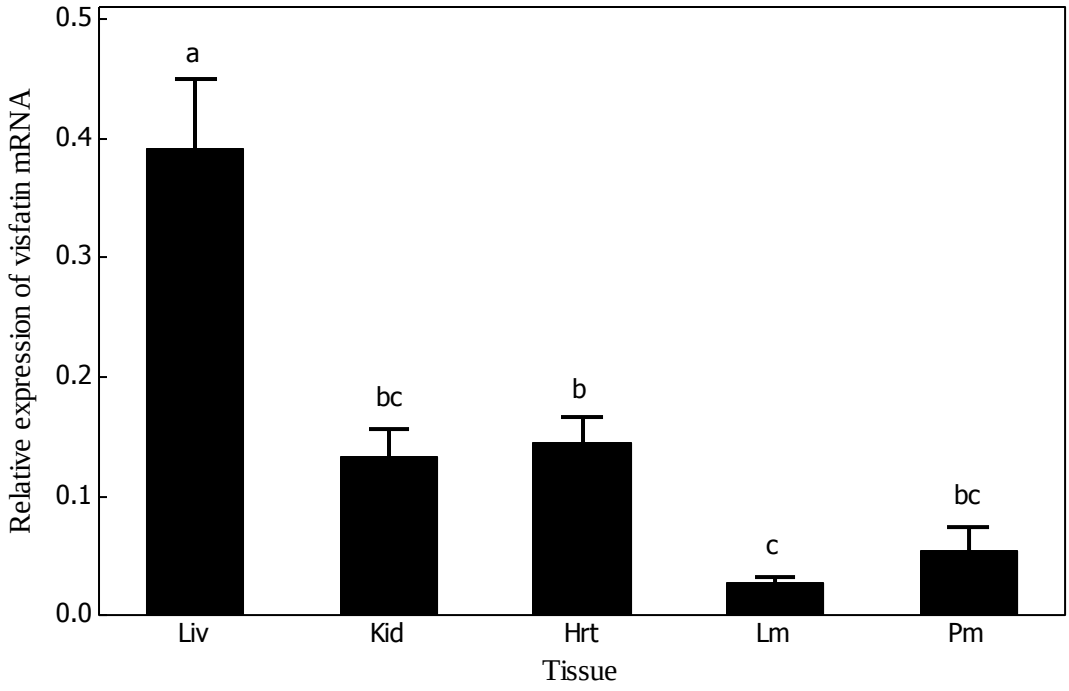

Figure 6.4: The relative fold expression of visfatin mRNA in tissues isolated from 5 week old broilers (experiment 1). The mRNA expression data were normalized with glyceraldehydes-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. $^{a-c}$Means with different letters differ, $P < 0.05$. Abbreviations: Liv = liver, Kid = kidney, Hrt = heart, Lm = leg muscle (adductor longus), Pm = pectoralis major.

tissue than in theca tissue from the four largest (F1 through F4) hierarchical follicles (Figure 6.5). Granulosa tissue isolated from the F4 follicle had greater mRNA expression of NUCB2 than granulosa tissue isolated from the F1 or F2 follicles (Figure 6.5). The mRNA expression of NUCB2 was greater in the granulosa tissue than corresponding theca tissue for each of the four largest hierarchical preovulatory follicles (Figure 6.5). The mRNA expression level of NUCB2 in the combined theca and granulosa tissue isolated from either small yellow or large white follicles did not differ (Figure 6.6).

Similar to NUCB2, the overall expression of visfatin mRNA was greater in granulosa tissue than in theca tissue from the F1 through F4 preovulatory follicles (Figure 6.7). However, within individual follicles the mRNA expression of visfatin was only greater in granulosa tissue relative to the theca tissue in the F4 follicle (Figure 6.8). Visfatin mRNA expression was more abundant in F4 granulosa tissue than in granulosa tissue from the F2 and F3 follicles (Figure 6.8). The mRNA expression level of visfatin in the combined theca and granulosa tissue isolated from either small yellow or large white follicles did not differ (Figure 6.8).

6.3. Experiment 3

The overall mRNA expression of NUCB2 from theca and granulosa tissue isolated from broiler breeder hens that were fed or fasted for 72 hours did not differ significantly (Figures 6.9 and 6.10). Similar to the finding in experiment 2 conducted with breeder hens in the early egg production (28 weeks of age), the expression of NUCB2 mRNA is higher in granulosa tissue than theca tissue (Figure 6.10). Because there were no significant differences in NUCB2 mRNA expression between feeding state the individual granulosa and theca mRNA expression of NUCB2 for fed and fasted

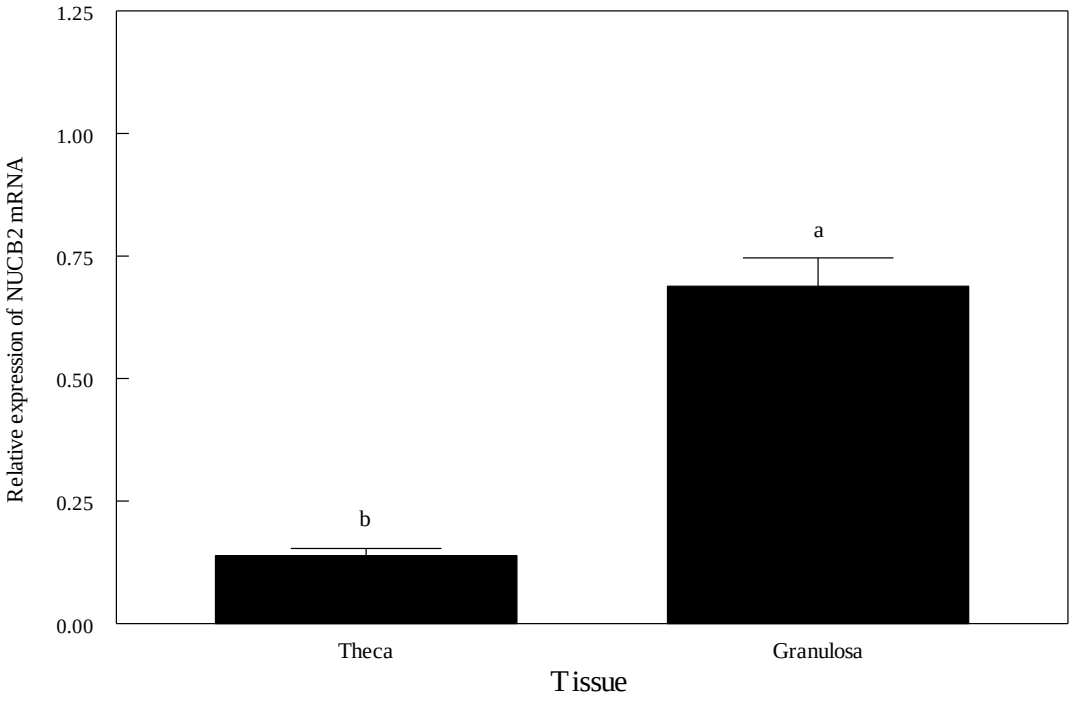

Figure 6.5: The overall relative fold expression of NUCB2 mRNA in theca and granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens (experiment 2). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 24 (6 samples from each of the 4 follicle sizes). $^{a-b}$Means with different letters differ, $P < 0.05$.

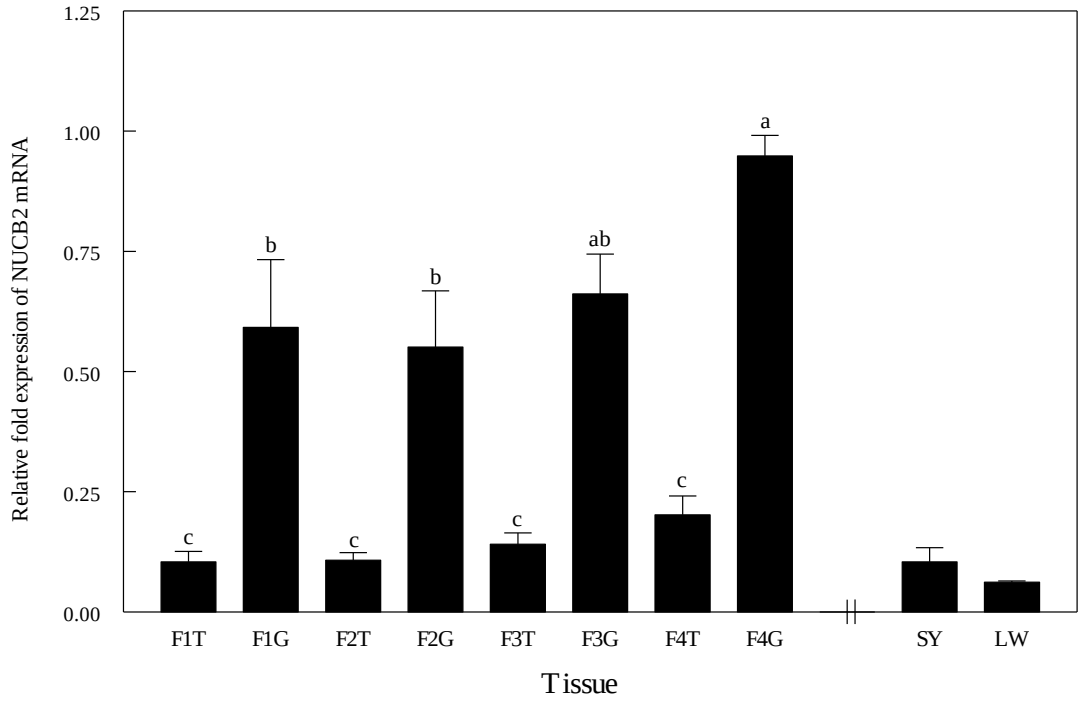

Figure 6.6: The relative fold expression of NUCB2 mRNA in theca (T) and granulosa (G) tissue collected from the four largest hierarchical (F1 through F4) and from the combined theca and granulosa tissue of small yellow (SY) and large white (LW) follicles of 28 week old broiler breeder hens (experiment 2). 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. $^{a-c}$Means with different letters differ, $P < 0.05$. Because the SY and LW follicle samples are a combination of theca and granulosa tissue, the NUCB2 mRNA expression level from these follicles were not statistically compared to the individually isolated theca and granulosa samples obtained from the hierarchical follicles. However, the mRNA expression level of NUCB2 did not differ between SY and LW follicles.

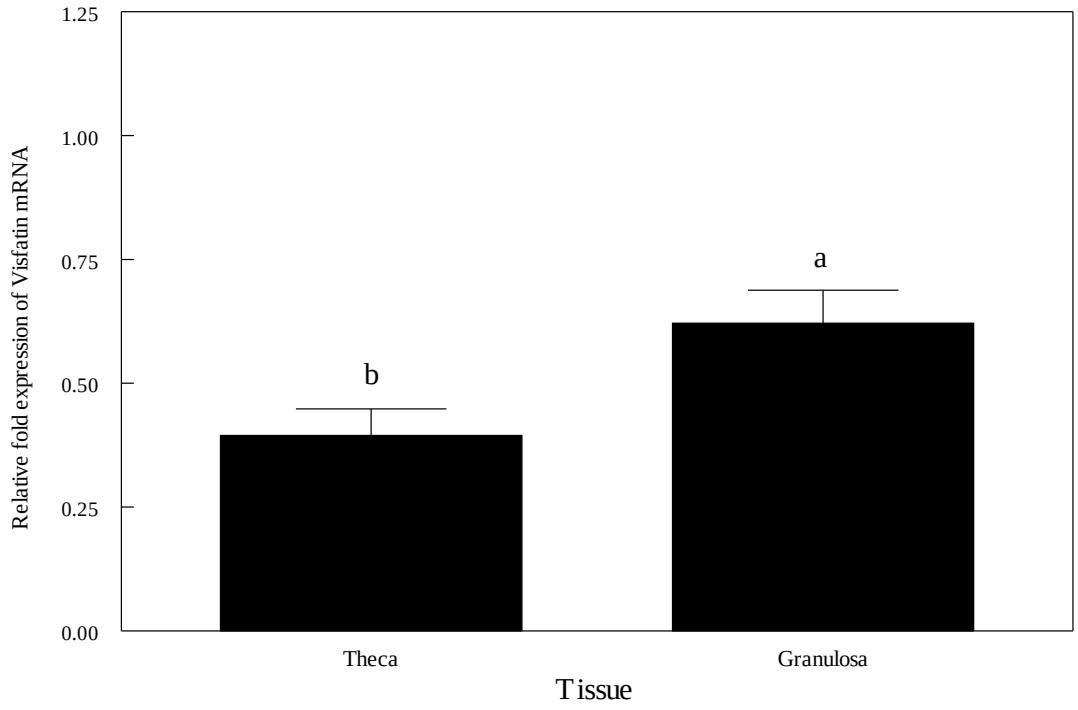

Figure 6.7: The overall relative fold expression of visfatin mRNA in theca and granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles of 28 week old broiler breeder hens (experiment 2). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 24 (6 samples from each of the 4 follicle sizes). $^{a-b}$Means with different letters differ, $P < 0.05$.

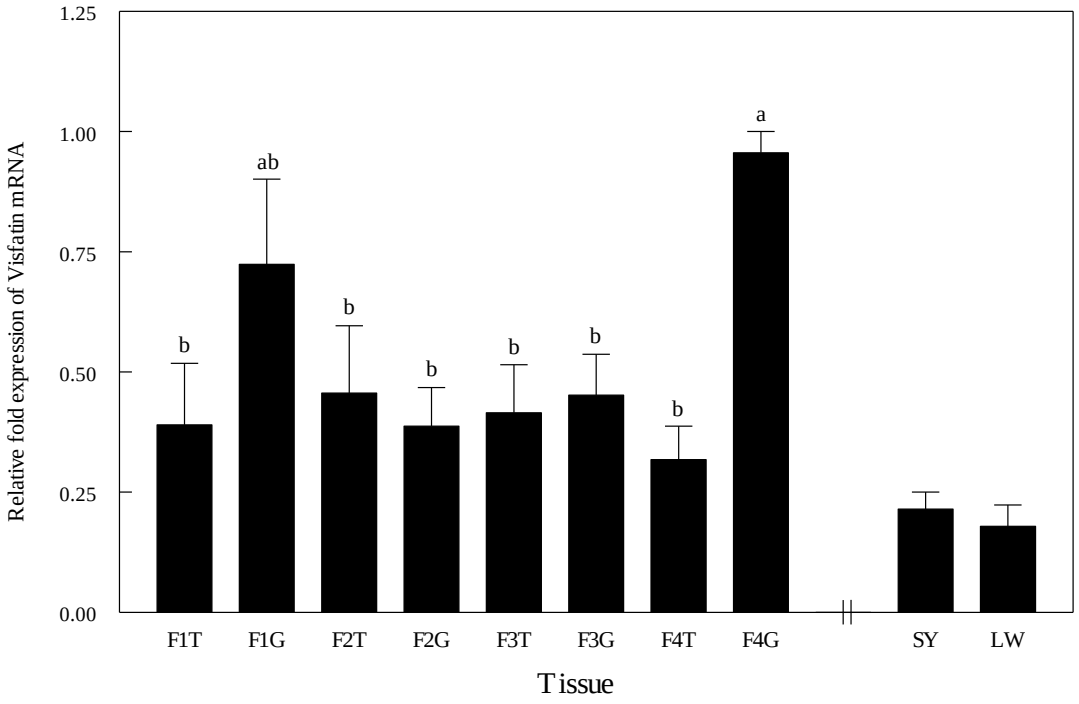

Figure 6.8: The relative fold expression of visfatin mRNA in theca (T) and granulosa (G) tissue collected from the four largest hierarchical (F1 through F4) and from the combined theca and granulosa tissue of small yellow (SY) and large white (LW) follicles of 28 week old broiler breeder hens (experiment 2). 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. $^{a-b}$Means with different letters differ, $P < 0.05$. Because the SY and LW follicle samples are a combination of theca and granulosa tissue, the visfatin mRNA expression level from these follicles were not statistically compared to the individually isolated theca and granulosa samples obtained from the hierarchical follicles. However, the mRNA expression level of visfatin did not differ between SY and LW follicles.

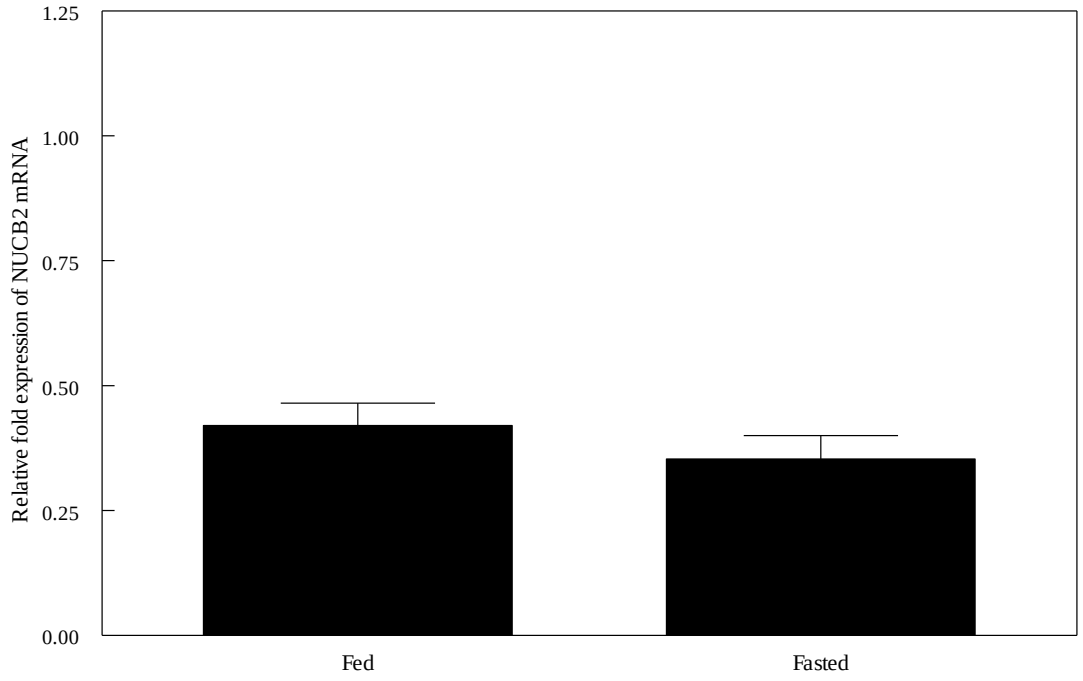

Figure 6.9: The overall relative fold expression of NUCB2 mRNA in theca and granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles and the small yellow and large white follicles from 45-55 week old broiler breeder hens fed daily or fasted for 72 hours (experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 36 [6 samples (3 theca and 3 granulosa) from each of the 6 follicle sizes].

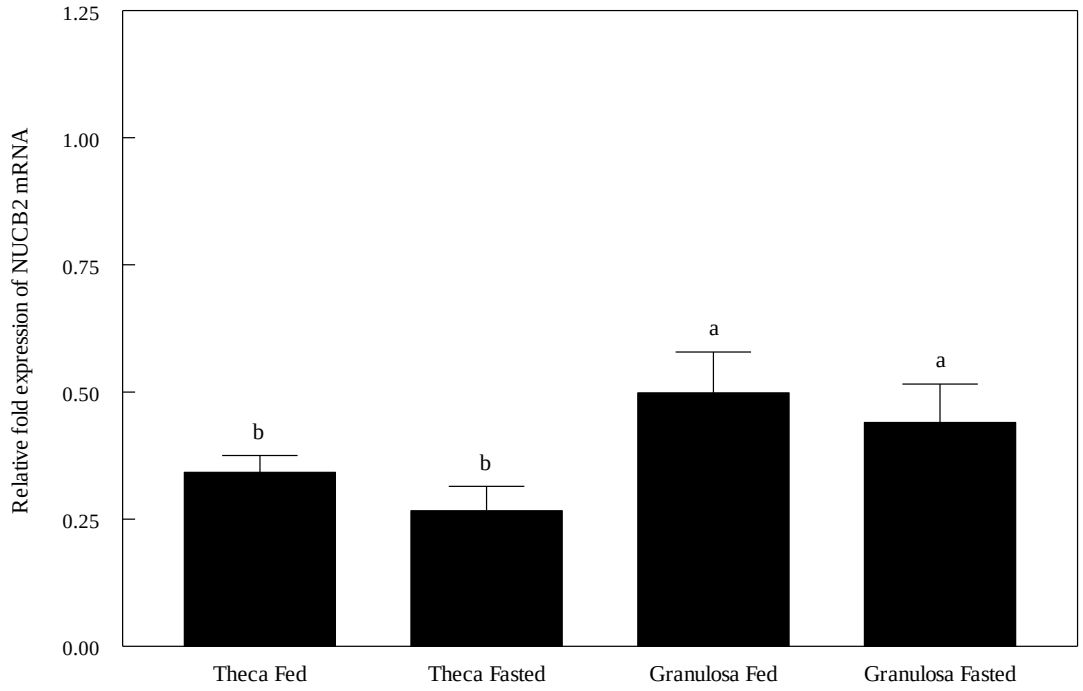

Figure 6.10: The overall relative fold expression of NUCB2 mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles, the small yellow and large white follicles from 45-55 week old broiler breeder hens fed daily or fasted for 72 hours (experiment 3). The 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). $^{a-b}$Means with different letters differ, $P < 0.05$.

birds was combined to give a total of 6 replicate samples (3 fasted plus 3 fed) for each tissue type at each follicle size. This allowed the mRNA expression of NUCB2 to be investigated in the separate granulosa and theca tissue of the small yellow and large white follicles, which was not possible with the combined theca and granulosa samples from these follicle sizes in experiment 2. The mRNA expression of NUCB2 in granulosa cells from large white follicles is less than the expression found in granulosa cells from small yellow follicles or from theca cells of the small yellow or large white follicles (Figure 6.11).

The overall mRNA expression of visfatin did not differ ($P = 0.058$) between the granulosa and theca samples of fed breeder hens versus broiler breeder hens fasted for 72 hours (Figure 6.12). However, a further examination of the data indicated that while granulosa mRNA expression of visfatin in the hierarchical and pre hierarchical follicles did not differ with feeding state, theca mRNA expression was decreased with fasting (Figure 6.13). In fed broiler breeder hens, the mRNA expression of visfatin in granulosa cells from the small yellow follicles is greater than from granulosa cells from the F2 follicle (Figure 6.14).

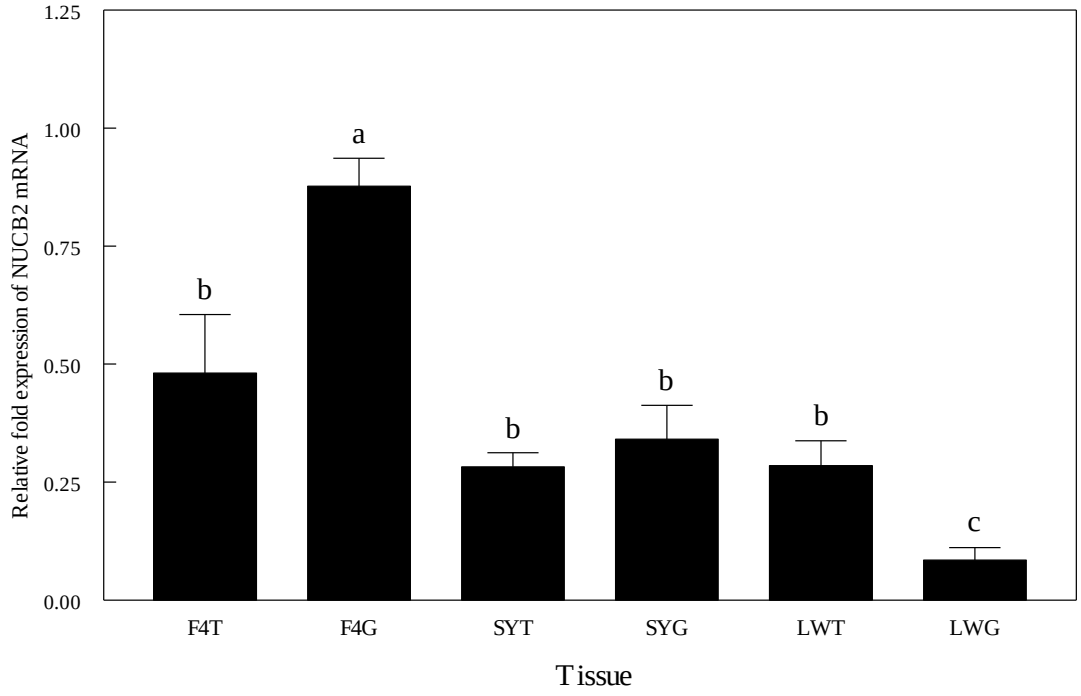

Figure 6.11: The relative fold expression of NUCB2 mRNA in theca (T)or granulosa (G) tissue collected from the fourth largest hierarchical follicle (F4), small yellow (SY) and large white (LW) follicles from 45-55 week old broiler breeder hens (experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. $^{a-c}$Means with different letters differ, $P < 0.05$.

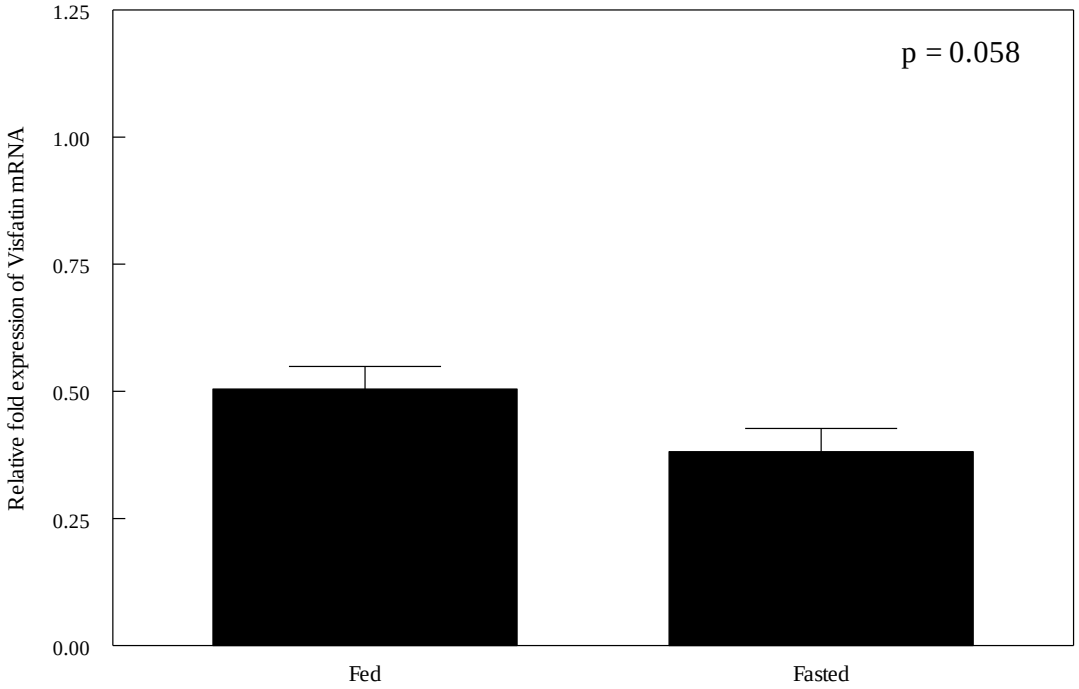

Figure 6.12: The overall relative fold expression of visfatin mRNA in theca and granulosa tissue collected from the four largest hierarchical (F1 through F4) and the small yellow and large white follicles from 45-55 week old broiler breeder hens fed daily or fasted for 72 hours (experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 36 [6 samples (3 theca and 3 granulosa) from each of the 6 follicle sizes].

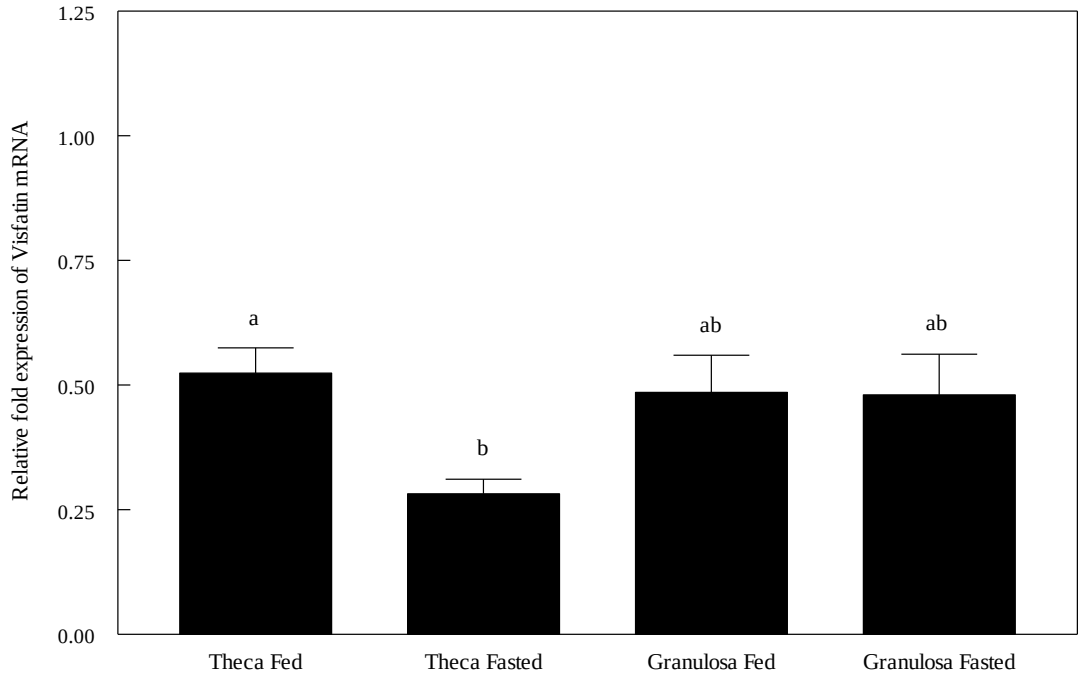

Figure 6.13: The overall relative fold expression of visfatin mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles, the small yellow and large white follicles from 45-55 week old broiler breeder hens fed daily or fasted for 72 hours (experiment 3). 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). $^{a-b}$Means with different letters differ, $P < 0.05$.

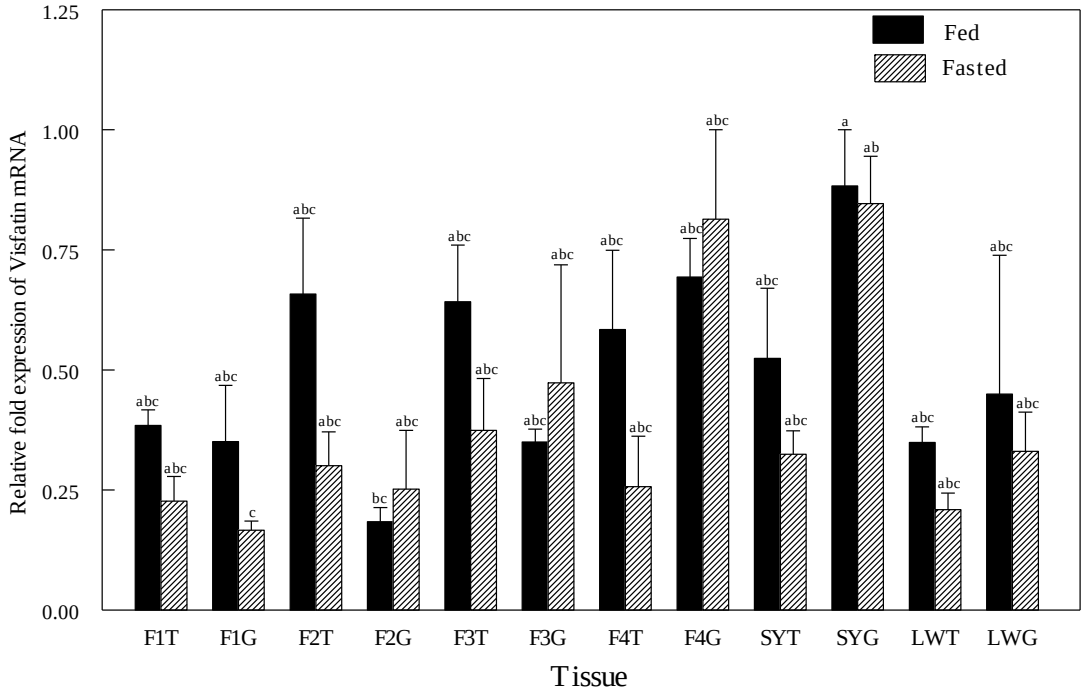

Figure 6.14: The relative fold expression of visfatin mRNA in theca (T)or granulosa (G) tissue collected from the four largest hierarchical preovulatory follicles (F1 through F4), small yellow (SY) and large white (LW) follicles from 45-55 week old broiler breeder hens fed daily or fasted for 72 hours (experiment 3). 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.

[a-c]Means with different letters differ, $P < 0.05$.

CHAPTER 7

DISCUSSION

The present research is the first to characterize the existence and expression of NUCB2 in any avian species, and it is the first to characterize the mRNA expression profile of visfatin and NUCB2 in the preovulatory follicles of the hen ovary. Both NUCB2 and visfatin mRNA were found to be expressed at all stages of follicular development and in both theca and granulosa cells with overall expression being greater in granulosa cells for both proteins. Expression of NUCB2 mRNA is highest in the follicles as they enter into the follicular hierarchy and transition from FSH dependency to LH dependency. Food deprivation of broiler breeders for 72 hours significantly decreases theca mRNA expression of visfatin, but fasting does not affect NUCB2 expression in the ovary.

7.1. Visfatin

In the current research visfatin mRNA expression was found in all tissues examined, with the highest expression found in spleen and the lowest expression found in kidney and muscle tissue. The widespread distribution of visfatin is not surprising given that visfatin is also known as NAMPT (nicotinamide phosphoribosyltransferase), an enzyme that functions to catalyze the rate limiting conversion of nicotinamide to nicotinamide mononucleotide, a substrate for NAD synthesis. Additionally, the high level of expression in spleen tissue from 5 week old broilers is not surprising, given that the spleen in chickens is an active lymphocytic organ secreting B

lymphocytes (Payne and Powell PC, 1984; Jeurissen et al., 1988). Visfatin was also discovered based on its ability to serve as a pre-B cell colony –enhancing factor that enhanced maturation of B-lymphocyte precursors (Samal et al., 1994). What was unexpected was the low level of visfatin expression in muscle tissue because in previous work with broilers, muscle tissues had the highest expression of visfatin relative to other tissues (Krzysik-Walker et al., 2008; Ons et al., 2010). However, neither of the previous research groups included spleen in their relative expression profiles. Nonetheless, when Krzysik-Walker et al., (2008) compared visfatin mRNA levels in pectoralis muscle, liver and abdominal fat, expression was the highest in muscle, lowest in abdominal fat and intermediate in liver in 8 week old broilers which does not agree with our data. Similarly Ons et al., (2010) reported that breast muscle had the highest level of expression of visfatin mRNA followed by abdominal fat in females and kidney in males with gastrocnemius (leg) muscle having less expression than breast muscle.

The reasons for the differences in tissue expression between the current research and previous research are not known. However, in the current and previous research the results are relative to the tissue with the most expression and neither of the previous reports included spleen. Additionally it is very clear from the research of Ons et al., (2010) that gender plays a significant role in visfatin mRNA expression in some tissues such as abdominal fat where female birds have more than double the mRNA levels found in males. Age also plays a significant role in visfatin mRNA expression levels in some tissues as the levels of visfatin mRNA in breast and liver tissue increase significantly between 4 and 8 week old broilers (Krzysik-Walker et al., 2008). In the current research, the female broilers used had just turned 5 weeks of age, in the other research in which breast muscle visfatin mRNA expression was so abundant the birds were either 6 or 8 weeks of age. Broilers gain 40 to 50 % of their body weight from week 5 to week 8, and thus it

may not be surprising for visfatin expression to increase significantly if it serves as a myokine as suggested by Krzysik-Walker et al., (2008). In addition, the current research was conducted using more specific MGB probe and primers for visfatin detection while the previous research used less specific Sybr Green detection methods.

In the current research, fasting broiler breeder hens for 72 hours resulted in a significant decrease in visfatin mRNA in theca tissue from preovulatory follicles. Previously, Ons et al. (2010) reported that food deprivation for 24 hours in 9 week old broilers resulted in a significant increase in hepatic visfatin mRNA concentrations while the levels in the hypothalamus and gastrocnemius muscle remained unchanged. Plasma visfatin levels were unchanged after a 48 hour fast in young male broilers (Krzysik-Walker et al., 2008). Overall the data suggest independent tissue regulation of visfatin production during fasting.

The decrease in theca visfatin mRNA expression could be critical in signaling follicular atresia. It is well known that follicular atresia occurs in hens that are deprived of food with subsequent regression of the ovary (Hocking et al., 1989) as might occur with feed restriction molting programs. The metabolic signal or signals involved in mediating the atretic response is unknown. Visfatin's role as a potential regulator is intriguing as decreases in visfatin are associated with cell death (Hasmann and Schemainda, 2003; van der Veer, et al., 2007) in other tissues. Furthermore, although atresia is thought to be initiated in the granulosa cell layer of birds as is thought to be the case in mammalian species (Tilly et al. 1991, Johnson et al. 1996), recent evidence has suggested that the theca cells actually produce a signal that initiates the granulosa cells to die (Armstrong and Rodgers, 2010). In birds, the theca tissue is highly vascular unlike the avascular granulosa layer, and thus would receive blood borne chemical messages that caloric intake to support reproduction no longer existed. Such messages could

stimulate the theca cells to produce less visfatin which in turn could either indirectly or directly stimulate the initiation of atresia by cellular apoptosis. As visfatin mRNA expression was found in the theca cells of all preovulatory follicles, it could regulate follicular atresia in both hierarchical and prehierarchical follicles.

Establishing a role for visfatin in follicular atresia needs much more research. It is intriguing that intracerebroventricular injection of visfatin stimulates feed intake in broiler chicks (Cline et al., 2007). Thus, visfatin at the brain could be stimulating feeding behavior and caloric intake to support reproduction while at the ovary a decrease in local visfatin production could initiate follicular atresia if caloric intake to support reproduction failed to occur. Besides the current research the only other research involving visfatin and reproduction is one report that indicates that visfatin is produced in rooster testes and that its expression increases significantly with sexual maturation (Ocon-Grove et al., 2010).

7.2. NUCB2

Similar to visfatin, NUCB2 mRNA was found in all tissues examined, with spleen expressing the greatest amount and muscles the least. In human and rodents, visceral adipose and brain have the highest level of NUCB2 mRNA expression, but relatively few studies exist and tissue characterization is limited (Oh et al., 2006; Hausman, et al., 2012). In the present research, both theca and granulosa cells produced NUCB2 mRNA. The granulosa cells of the hierarchical follicles produced more NUCB2 mRNA than theca cells, but this pattern of expression was not seen in the prehierarchical follicles. The granulosa cells from the LWF actually produced less NUCB2 mRNA than their corresponding theca cells. Expression of NUCB2 mRNA was also greater in granulosa cells from the F4 follicle than from granulosa cells

from the F1, F2 and prehierarchical follicles. Collectively this suggests NUCB2 may play an important role in the transition of follicles after selection into the follicular hierarchy. During this time the granulosa cells transition from FSH dependency with abundant FSH receptor expression (You et al., 1996), to LH dependency with the acquisition of LH receptors (Johnson, eta l., 1996) in preparation for ovulation of the F1 follicle. Future research will have to determine if NUCB2 has a positive effect on LH receptor expression in granulosa cells from recently selected hierarchy follicles.

The role of NUCB2 in mammalian reproduction is unclear. One preliminary report indicates that a functional knockdown of endogenous nesfatin tone in the hypothalamus of female pubertal rats delays vaginal opening and reduces the weight of ovaries as well as the levels of LH (Garcia-Galiano, et al., 2010). In prepubertal rats but not adult female rats, the icv infusion of nesfatin stimulates an increase in LH and FSH secretion (Garcia-Galiano, et al., 2010). Finally, NUCB2 mRNA expression has been detected in human, rat and mouse testis as well as rat ovary as reported by Garcia-Galiano, et al., (2010).

In the current food experiment, deprivation did not alter the mRNA expression of NUCB2 in the theca or granulosa tissue of the hen ovary. In rats, fasting decreased NUCB2 mRNA levels in gastric mucosa (Stengel et al., 2009) and in the hypothalamus (Garcia-Galiano, et al., 2010). In goldfish however, fasting for 3 or 7 days decreased NUCB2 mRNA in the hypothalamus but increased hepatic mRNA levels (Gonzalez et al., 2010). Thus, as discussed with visfatin earlier, the effects of food deprivation on NUCB2 mRNA expression may vary and be tissue specific.

7.3. Summary

Both visfatin and NUCB2 have the potential to influence follicular development and maturation in the hen. As both have been tied to feed intake and energy balance in either mammalian or avian species, NUCB2 and visfatin could link nutritional status with the reproductive axis. Our results support such a potential role for visfatin. Finally, further research is needed with both molecules to investigate their potential roles as hormones and their involvement with reproduction. Identifying the cell surface receptors for both protein hormones would be one of the most pressing research needs.

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