GHRELIN'S POTENTIAL ROLE IN REPRODUCTION FOR THE BROILER BREEDER HEN

by

MARTHA ELIZABETH FREEMAN

(Under the Direction of Adam Davis)

ABSTRACT

In mammalian species ghrelin production increases with fasting and effects mammalian reproduction by decreasing gonadotropin secretion from the pituitary, however, there has been little research into ghrelin's effects on avian reproduction. While it is known in avian species that fasting promotes ovarian regression, identification of the hormonal signaling pathways that translate the lack of sufficient energy intake into a reduction in reproductive capability has not occured. Therefore the goal of this research was to explore if ghrelin is a direct link between nutritional status and reproduction in avian species by (1) determining if ghrelin or GHSR mRNA expression is present within the avian ovary, (2) investigating if fasting changes GHSR mRNA expression in the ovary or plasma levels of ghrelin, (3) exploring the effects of gonadotropins and sex steroids upon GHSR mRNA expression in granulosa cells, and (4) investigating if ghrelin influences progesterone secretion or GHSR mRNA expression in granulosa cells.

INDEX WORDS: broiler breeder hen, ghrelin, reproduction, feed deprivation

GHRELIN'S POTENTIAL ROLE IN REPRODUCTION FOR THE BROILER BREEDER HEN

by

MARTHA ELIZABETH FREEMAN

B.S., University of Georgia 2000

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

Masters of Science

Athens, GA

2008

©2008

Martha Elizabeth Freeman

All Rights Reserved

GHRELIN'S POTENTIAL ROLE IN REPRODUCTION FOR THE BROILER BREEDER HEN

by

MARTHA ELIZABETH FREEMAN

Major Professor: Adam Davis

Committee: Mark Compton
Jeanna Wilson

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2008

DEDICATION

To Brad, without whom I would accomplish nothing, and to Katie, who inspired me to go back to school in the first place.

ACKNOWLEDGEMENTS

First and foremost, I need to admit that I would not be where I am today without the assistance and support of Dr. Davis. I am lucky that he has provided me with a job that I love as well as the opportunity to advance my education. I am simply unable to convey the magnitude of just how much I owe to him and how I greatly appreciate it.

I would also like to thank my committee members, Dr. Compton and Dr. Wilson, as well as Dr. Lacy for their help and encouragement over the years. Last of all, a special thanks to all the graduate students I've worked with over the years whom helped me to realize that one of my greatest aspirations was to continue learning.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Chapter 1

Mammalian Ghrelin

1.1 General Overview

Ghrelin is a 28 amino acid hormone that was purified from rat stomach in 1999 (Kojima et al. 1999). It was originally identified as the endogenous ligand for an orphan G-protein coupled receptor, the growth hormone secretogogue receptor (GHSR). Since ghrelin's discovery, it has been found not only to be a stimulator of growth hormone secretion, but also a potent orexigenic compound that also has central and peripheral effects upon metabolism. In addition to modulating a plethora of metabolic processes, ghrelin has been found to influence reproduction both centrally and peripherally in mammalian species (van der Lely et al. 2004).

1.2 Synthesis

In humans, the mature ghrelin peptide is formed by several post-translational modifications of its 117 amino acid precursor, preproghrelin. A signal peptide of 23 amino acids is cleaved from preproghrelin to yield a 94 amino acid long polypeptide called proghrelin (Figure 1.1), which can be further processed into unacylated ghrelin (UAG) or obestatin (Hosoda et al. 2003, Gualillo et al. 2006). UAG, which consists of the first 28 amino acids after the signal

peptide, undergoes an acylation of its third amino acid residue using N-octanoic acid to become acylated ghrelin, or ghrelin (Figure 1.1). This acylation is essential for ghrelin's ability to bind to its cell surface receptor, growth hormone secretatgogue receptor type 1a (GHSR1a) (Kojima et al. 1999). The enzyme responsible for the acylation has not been identified, however, the acylation occurs prior to ghrelin's secretion from the stomach (Hosoda et al. 2000). The processing of ghrelin in other mammalian species is the same as human ghrelin, however, there is some variation in the number of amino acids in preproghrelin based on insertions and deletions of the amino acids in the proghrelin sequence.

It was initially believed that UAG had no biological activity since it was unable to bind or displace ghrelin from GHSR1a (Hosoda et al. 2000). Subsequent research has indicated that UAG may decrease food intake (Asakawa et al. 2005), promote adipogenesis (Thompson et al. 2004), and decrease glucose output by hepatocytes (Gauna et al. 2005), however, its functional receptor has yet to be identified.

Obestatin was discovered in 2005, and has been reported to reduce food intake and weight gain in mice by binding to the orphan receptor, GPC39 (Zhang et al. 2005, Lagaud et al. 2007). However, other research groups have been unable to substantiate obestatin's effects upon feed intake or its ability to bind to GPR39 (Gourcerol et al. 2006, Lauwers et al. 2006, Yamamoto et al. 2007).

The post-translational processing of the preproghrelin peptide into UAG, ghrelin and obestatin make any mRNA expression data for preproghrelin very difficult to interpret. Since UAG and obestatin are proposed to have opposite effects on food intake compared to ghrelin, it seems unlikely that an increase in preproghrelin mRNA expression would lead to an increase in the production of all three hormones. A complete understanding of the mechanisms regulating

the processing of proghrelin to UAG and obestatin as well as the potential function of UAG and obestatin will be necessary before a complete understanding of ghrelin's biology can be achieved.

1.3 Secretion and Tissue Distribution of Ghrelin

Approximately two thirds of plasma total ghrelin (UAG + ghrelin) is produced by the stomach by cells within the gastric fundus mucosa. The remaining source of plasma total ghrelin is the small intestine (Date et al. 2000, Ariyasu et al. 2001, Gualillo et al. 2003). In humans, removal of the stomach will initially reduce plasma total ghrelin levels by approximately 50%, however, the levels of total plasma ghrelin subsequently normalize which indicates other tissues can compensate for loss ghrelin production by the stomach (Hosoda et al. 2003). Preproghrelin mRNA expression in humans has been detected by RT-PCR in nongastrointestinal tissues such as heart, adipose tissue, pancreas, adrenals, thyroid, pituitary, hypothalamus, placenta, ovary and testes, but the mRNA expression levels in these tissues are much lower than what is expressed by the digestive tract (Gnanapavan et al. 2002). Of the total plasma ghrelin content, the concentration of UAG is approximately 50 times higher than that of ghrelin (Hosoda et al. 2000, Murakami et al. 2002).

Initially, many researchers measured plasma total ghrelin levels based on the assumption that UAG was inactive and that increases in total ghrelin reflected increases in ghrelin. While the ratio between total ghrelin and ghrelin secretion have been reported to remain constant under a variety of conditions (Ariyasu et al. 2002, Murikami et al. 2002), the scientifically preferred method for determining plasma ghrelin concentration is to measure it directly. Furthermore,

since the N-octanoic acid modification of ghrelin is relatively unstable, collected plasma samples should be acidified immediately to prevent hydrolysis of ghrelin to UAG (Hosoda et al. 2004). Thus, research reports dealing with plasma ghrelin concentrations should be reviewed carefully to determine if total ghrelin or acylated ghrelin was being measured and if the samples were processed properly to prevent degradation of acylated ghrelin.

1.4 Physiological Effects of Ghrelin

1.4.1 Stimulation of Growth Hormone Secretion

Ghrelin was originally discovered as the natural ligand for a growth hormone secretagogue receptor within the pituitary. Therefore, it is not surprising that ghrelin causes the potent release of growth hormone in somatotrophs both in vitro and in vivo in a dose dependant manner (Kojima et al. 1999, Peino et al. 2000, Hataya et al. 2001).

1.4.2 Stimulation of Feeding Behavior

The most striking physiological effects of ghrelin, independent of GH releasing activity, is the stimulation of appetite and feeding behavior. Infusion of ghrelin, either intracerebroventricularly or peripherally, into mice or rats stimulates feeding behavior and if the injections are given long enough the animals will gain weight (Tschop et al. 2000, Wren et al. 2000, Kamegai et al. 2001). Antagonism of GHSR1a will reduce ghrelin's effects on food intake and body weight gain (Asakawa et al. 2003). Ghrelin's stimulation of appetite is mediated by the

neurotransmitters neuropeptide Y (NPY) and agouti related protein (AGRP) in the hypothalamic arcurate nucleus region of the brain (Chen et al. 2004). In mice and rats, ghrelin increases the hypothalamic expression of both NPY and AGRP mRNA in vivo and in vitro (Kamegai et al. 2000, Kamegai et al. 2001, Goto et al. 2006). Furthermore, intracerebroventricular injections of NPY or AGRP antagonists in rats suppress ghrelin induced feeding behavior (Nakazato et al. 2001). However, feed intake in mice is not completely dependent upon ghrelin production because in ghrelin knockout mice the absence of ghrelin does not impair either growth or appetite (Sun et al. 2003).

1.4.3 Effects on Insulin, Glucose and Glucagon Secretion

Research on ghrelin's positive effect on glucose secretion is consistent but the effect on insulin secretion is not. Injection of ghrelin elevates plasma glucose levels in humans and mice (Broglio et al. 2003, Salehi et al. 2004). In addition, Gauna et al. (2005) found that porcine hepatocytes cultured with ghrelin released more glucose in both a time and dose dependant manner than untreated control cells. Furthermore, intraperitoneal injection of a GHSR antagonist will decrease fasting glucose levels in mice (Dezaki et al. 2004). In both humans and rats, ghrelin has been reported to decrease (Broglio et al. 2001, Egido et al. 2002, Broglio et al. 2003) or increase (Adehgate and Ponery 2002, Date et al. 2002, Lee et al. 2002) plasma insulin concentrations. Since ghrelin increases plasma glucose levels the rise in plasma glucose concentrations may be enough to stimulate insulin production. This indirect influence on insulin production by ghrelin may account for some of the conflicting reports on how ghrelin impacts insulin production.

Studies concerning glucagon are limited, but Salehi et al. (2004) reported that ghrelin strongly stimulates glucagon release in murine islet cells in vitro. However, the same results have yet to be duplicated in vivo (Egido et al. 2002, Salehi et al. 2004).

1.4.4 Influence on Adipocytes

In 2000, Tschop et al. reported that ghrelin not only indirectly effected adiposity by acting as an orexigenic peptide, but also reduced lipolysis in mice and rats. Chronic intracerebroventricular administration of ghrelin also regulates adipocyte metabolism by increasing glucose and triglyceride uptake as well as lipogenesis in white adipose cells in rats (Theander-Carrillo et al. 2006). Choi et al. (2003) also reported that rat adipocytes cultured with ghrelin had increased fat synthesizing capabilities, but Zhang et al. (2004) reported that adipocytes from a cell line overexpressing ghrelin had decreased rates of adipogensis compared to cells with typical ghrelin expression. However, both research groups agreed that ghrelin increased the rate of preadipocytes differentiating into adipocytes.

Ghrelin also influences the secretion of leptin from fat cells. Leptin is produced by adipocytes in response to a positive energy balance and there is a negative correlation between plasma levels of total ghrelin and leptin (Otto et al. 2001, Tolle et al. 2003, Tschops et al. 2001). Interestingly, in cultured rat adipocytes ghrelin increases leptin secretion and the addition of a ghrelin antagonist will decrease leptin secretion (Giovambattista et al. 2006). Regardless of the effects of ghrelin upon leptin secretion in cultured adipocytes, it has been well documented that in vivo ghrelin and leptin antagonize each other within the arcuate nucleus by modulating NPY

mRNA expression levels, and thereby feeding behavior (Shintani et al. 2001, Traebert et al. 2002, Kohno et al. 2007).

1.5 Ghrelin Receptor

1.5.1 GHSR Characterization

A receptor for ghrelin was identified in the hypothalamus several years before the discovery of ghrelin (Howard et al. 1996). This orphaned, G-coupled protein receptor attracted the attention of researchers due to its potent stimulation of growth hormone (GH) release when it bound synthetic ligands. There are two forms of GHSR in mammalian species, GHSR1a and GHSR1b, which are both derived from a single gene and are formed by alternate splicing of the GHSR mRNA transcript (Howard et al. 1996).

The GHSR gene is composed of two exons; the first exon encodes transmembrane domains one to five and the second exon encodes transmembrane domains six and seven. GHSR1a is encoded by both exons and is composed of 366 amino acids. The protein structure of GHSR1a is predicted to consist of an extracellular N-terminal domain, 7 transmembrane domains, and an intracellular C-terminal domain (Howard et al. 1996). GHSR1b is composed of 289 amino acids and lacks the first 77 amino acids encoded by the beginning of the second exon. It is predicted that this truncated form of GHSR has only the first 5 of the 7 transmembrane domains (Figure 1.2). GHSR1b is unable to bind ghrelin, or any other human GH secretagogue, and its functional activity, if any, has yet to be determined (Howard et al. 1996).

1.5.2 Ghrelin Binding and Cellular Activation

GHSR1a binds ghrelin and synthetic forms of ghrelin that contain at least the first 5 amino acids and the acylated serine in position 3, with high affinity (Howard et al. 1996, Kojima et al. 1999, Matsumoto et al. 2001). Ligand binding to GHSR1a activates G-protein subunits that in turn activate intracellular signaling cascades. As reviewed by Camina (2006), the GHSR1a G-protein subunits activated and their activation of effector molecules varies widely depending on the cell type to which ghrelin binds. The best characterized GHSR1a/G-protein activation is the one leading to cellular depolarization when a G-protein activates inositol 1,4,5-triphosphate (IP3) and dicecylglycerol (DAG) production. Other less characterized pathways include G-protein activation of the mitogen-activated protein kinase (MAPK) pathway through various intermediatory kinase systems (Camina 2006).

1.5.3 Tissue Distribution of GHSR Expression

The highest expression levels of GHSR1a mRNA are found in somatotrophs, cells responsible for GH secretion within the pituitary, and in the hypothalamus in the arcuate nucleus, an area crucial for neuroendocrine regulation of appetite stimulation (Gaun et al. 1997**,** Kojima and Kangawa 2005). However, GHSR1a mRNA is expressed at lower levels in a variety of other tissues such as heart, lung, liver, pancreas, intestine, adipocytes, thyroid, spleen, adrenal, ovarian and testicular tissue (Guan et al. 1997, Kojima et al. 2001, Gnanapavan et al. 2002, Barreiro et al. 2003, Gaytan et al. 2003). GHSR1b mRNA has been detected within the same tissues in which GSHR1a has been identified (Gnanapavan et al. 2002) but the mRNA expression of GHSR1b

tends to be less than the expression of GHSR1a (Korbonits et al. 2001, Gauna et al. 2005). Although GHSR1b cannot bind ghrelin, its synthesis could downregulate the availability of GHSR1a binding sites.

There is increasing evidence that GHSR1a is not the exclusive receptor for ghrelin. Ghrelin binding has been demonstrated in cardiomyocytes, chondrocytes, and in some regions of the pituitary, but GHSR1a mRNA expression is not detected in these tissues by RT-PCR (Muccioli et al. 2002, Petersenn 2002, Camina 2006). In addition, it has been reported that ghrelin and UAG may compete for binding to a common unidentified receptor in cardiomyocytes, adipocytes, and breast cancer cell culture lines (Cassoni et al. 2001, Baldanzi et al. 2002, Zhang et al. 2004).

1.5.4 Regulation of GHSR1a mRNA expression

Since its discovery in 1996, there has been little research on the regulation of GHSR1a expression. Furthermore, despite its widespread tissue distribution most studies concerning its regulation have pertained to the brain, in particular the arcuate nucleus and the pituitary. GHSR1a mRNA expression increases in the hypothalamus and pituitary when rats are fasted or when ghrelin is injected into the hypothalamus (Kim et al. 2003, Nogueiras et al. 2004). However, the addition of ghrelin to cultured porcine pituitary cells decreased GHSR1a mRNA expression (Luque et al. 2004). Leptin injected into rat hypothalmic arcuate nuclei decreased GHSR1a mRNA expression within the arcuate nucleus (Nogueiras et al. 2004). Due to the inverse relationship that exists between leptin and ghrelin, it was not unexpected that leptin would decrease the sensitivity of the arcuate nucleus to ghrelin.

Age and body weight may also play a role in GHSR1a regulation. GHSR1a mRNA expression in the brain is significantly higher in young mice (1-2 months of age) than in older mice (over 6 months of age, Sun et al. 2007). In addition, lean ewes have significantly higher GHSR1a mRNA expression in the hypothalamus when compared to obese ewes (Kurose et al. 2005).

1.6 Regulation of Ghrelin Secretion

1.6.1 Energy Balance

Plasma ghrelin concentrations and mRNA expression within the stomach directly reflect energy balance within mammals. Rats that have been fasted for 24 or 48 hours, and consequently have a negative energy balance, have higher levels of ghrelin mRNA expression in their stomachs, as well as increased plasma levels of total ghrelin, when compared to rats that have been fed (Toshinai et al. 2001). Six hours after refeeding the fasted rats, there was a decrease in stomach ghrelin mRNA expression and plasma levels of total ghrelin (Toshinai et al. 2001). Mechanical distention of the stomach with non-nutritive substances, such as water, will not suppress plasma ghrelin levels in mice or rats (Tschop et al. 2000, Williams et al. 2003), but providing total parenteral nutrition will reduce plasma ghrelin levels (Qader et al. 2005). Furthermore, the nutrient composition of the diet consumed has an impact on ghrelin secretion. Ingesting a high-carbohydrate meal will suppress plasma total ghrelin levels more than a high-fat meal in both humans and rats (Monteleone et al. 2003, Gomez at al. 2004, Sanchez et al. 2004).

1.6.2 Body Composition and Leptin

In addition to nutrient intake, body composition and leptin have also been found to influence ghrelin plasma levels. Plamsa ghrelin levels correlate negatively with body mass index (English et al. 2002, Hansen et al. 2002). For example, obese humans have lower plasma levels of ghrelin than normal weight controls (Shiiya et al. 2002) and weight loss will increase the plasma levels of total ghrelin in obese humans (Hansen et al. 2002). Not surprisingly, humans suffering from anorexia nervosa or bulimia have higher plasma levels of ghrelin than normal weight individuals (Tanaka et al. 2002, Tolle et al. 2003) and weight gain will lower plasma total ghrelin levels in patients with anorexia (Otto et al. 2001).

Given the association between plasma ghrelin concentrations and body mass, it is not surprising that researchers have examined the potential role of leptin in regulating ghrelin secretion. Leptin signals energy abundance and peripheral injections can cause weight loss by decreasing feed intake and increasing metabolism in rodents. Since ghrelin production indicates a lack of energy intake and leptin production indicates sufficient or excess energy, it is not surprising that a negative correlation between leptin and ghrelin secretion has been observed both in vivo and in vitro in humans and rats (Kamegai et al. 2004, Erdmann et al. 2005). Barazzoni et al. (2003) reported that calorie restricted rats given peripheral injections of leptin had decreased plasma levels of total ghrelin compared to saline injected control rats. This study suggested that leptin directly influences ghrelin secretion rather than indirectly increasing ghrelin secretion by causing weight loss. However, peripheral leptin injections had no effect on plasma ghrelin levels in normal weight humans (Chan et al. 2004).

1.6.3 Insulin and Glucagon

Since insulin and glucagon play an integral role in energy balance, their effects on ghrelin secretion have been investigated. While some research groups have reported that insulin does not affect (Caixas et al. 2002, Schaller et al. 2003, Arafat et al. 2005) or increases (Toshninai et al. 2001) ghrelin production, other researchers reported that insulin inhibits ghrelin production (Flanagan et al. 2003, Kamegai et al. 2004, Lippl et al. 2004). The lack of consistency in insulin's effect on ghrelin production has been further complicated by research involving diabetics. Obese type 2 diabetics, whom have high levels of circulating insulin as well as high blood glucose levels, have lower plasma ghrelin concentrations than obese nondiabetic patients (Katsuki et al. 2004, Erdmann et al. 2005) but glucose given intravenously or orally to type 2 diabetics decreased plasma ghrelin levels without increasing plasma insulin concentrations (Shiiya et al. 2002, Briatore et al. 2003) which suggested that plasma glucose rather than insulin influenced ghrelin production. However humans with uncontrolled type 1 diabetes, as well as rats with streptozotocin induced diabetes, have very little or no insulin production and very high blood glucose levels yet these subjects do not exhibit the postprandial decrease in plasma ghrelin concentrations as seen in normal controls (Murdolo et al. 2003, Gelling et al. 2004) which suggests that insulin, not high levels of blood glucose, may directly inhibit ghrelin secretion.

Similar to insulin, there have been conflicting research reports on the role of glucagon in regulating ghrelin secretion. In humans, intravenous administration of glucagon had no effect (Broglio et al. 2004) or decreased (Arafat et al. 2005, Hirsh et al. 2005, Banasch et al. 2006) plasma total ghrelin concentrations. However, Kamegai et al. (2004) reported that ghrelin secretion increased in a dose dependant manner when isolated rat stomachs were perfused with

glucagon. Furthermore, intravenous injection glucagon increased ghrelin mRNA expression and ghrelin and UAG protein expression, in rat stomachs (Katayama et al. 2007).

1.6.4 Thyroid Hormone

The relationship between thyroid hormone, a key hormone in maintaining energy balance, and ghrelin production is starting to be clarified. Hyperthyroidism in humans lowers ghrelin mRNA expression within the stomach and plasma ghrelin concentrations (Caminos et al. 2002, Riis et al. 2003, Altinova et al 2006). Because thyrotoxicosis results in weight loss and hyperphagia, the suppressive effects of hyperthyroidism on ghrelin production was initially unexpected. However, further research has suggested the hyperinsulinemia associated with hyperthyroidism may cause a depression in ghrelin secretion (Gimenez-Palop et al. 2005). Hypothyroidism in humans has been reported to increase (Caminos et al. 2002) or not affect total plasma levels of ghrelin (Riis et al. 2003, Gimenez-Palop et al. 2005).

1.7 Ghrelin and Reproduction

1.7.1 Age and Gender Influences upon Ghrelin Secretion

Ghrelin mRNA expression within the stomach and plasma total ghrelin levels decrease with age when comparing prepubertal to post pubertal mice and humans (Lui et al. 2002, Whatmore et al. 2003). However, while there is no sex related differences in ghrelin mRNA or protein expression in young rats and prepubertal children, once puberty is attained ghrelin

mRNA expression within the stomach and total plasma ghrelin concentration declines significantly faster in males than females throughout puberty (Gualillo 2001b, Whatmore et al. 2003). Initially there was speculation that the decrease in plasma ghrelin levels during puberty was linked to increased plasma concentrations of sex hormones. In human males this may be true since testosterone administration to peripubertal boys will decrease plasma total ghrelin levels (Lebenthal et al. 2006). However, estrogen administration to peripubertal girls does not decrease plasma total ghrelin concentrations (Lebenthal et al. 2006). The effect of the primary sex hormones on plasma ghrelin in boys, but not girls, may explain the faster rates of decline of plasma ghrelin levels in males than females during puberty.

Hyperghrelinemia delays puberty in male rats but has no effect on puberty onset in females (Fernandez-Fernandez et al. 2005a, Martini et al. 2006). Prepubertal males receiving subcutaneous injections of ghrelin have lower levels of plasma LH than normal controls, as well as decreased plasma testosterone levels. However, females undergoing the same treatment had no changes in plasma LH, FSH, or estradiol concentrations (Fernandez-Fernandez et al. 2005a). These results also suggest that male reproduction may be more sensitive to the influence of ghrelin than female reproduction.

After puberty, there has been some controversy on whether ghrelin secretion in adult humans is sexually dimorphic. Greenman et al. (2004) reported that women have higher plasma levels of ghrelin than men. Barkan et al. (2003) reported that women have higher levels of circulating ghrelin than men only when the women are in the late follicular phase of their ovulatory cycle. The variation in plasma ghrelin levels in women related to their ovulatory cycle

may explain why Purnell et al. (2003) reported no difference in plasma total ghrelin levels between men and women since the female subjects were not selected or categorized based on their ovulatory cycle status.

Regardless of differences in ghrelin secretion due to gender and age, sex steroid hormones in adult humans appear to modulate ghrelin secretion based on research with nonreproductive individuals. Postmenopausal women and men suffering from hypogonadism have lower levels of plasma total ghrelin than individuals with normal plasma concentrations of sex steroid hormones and plasma total ghrelin levels increase after estrogen or testosterone therapy (Pagotto et al. 2003, Kellokoski et al. 2005). Furthermore, women who are anorexic and consequently have markedly decreased estradiol levels and elevated ghrelin levels will still have an increase in ghrelin levels when given oral estrogen, even if the anorexia is not resolved (Grinspoon et al. 2004).

1.7.2 Ghrelin's Effects upon Gonadotropin Secretion

In female and male rats, female monkeys, ewes, and human males intracerebroventricular or peripheral injected ghrelin will decrease the pulse frequency of luteinizing hormone (LH) release from the pituitary and plasma LH levels regardless of whether or not the animal is gonadally intact (Furata et al. 2001, Tena-Sempere et al. 2002, Fernandez-Fernandez et al. 2004, Vulliemoz et al. 2004, Iqbal et al. 2006, Kluge et al. 2007). The effect on LH secretion appears to be mediated by gonadotropin releasing hormone (GnRH), since GnRH secretion by hypothalamic fragments from ovariectomized female rats is inhibited by ghrelin as is pituitary cell responsiveness to GnRH as measured by LH production (Fernandez-Fernandez et al. 2005b).

Interestingly, ghrelin will stimulate LH and FSH secretion in a dose dependant manner when it is added to isolated pituitary cells collected from ovariectomized female rats (Fernandez-Fernandez et al. 2004, Fernandez-Fernandez et al. 2005b, Lebrethon et al. 2006). The biological basis for this unique effect in vitro of ghrelin on gonadotropin secretion is not known, but it has not affected the overall conclusion that ghrelin inhibits LH production in vivo and provides a mechanism that when there is caloric insufficiency, further energy demands of reproduction (Barreiro and Tena-Sempere 2004, Tena-Sempere 2005).

1.7.3 Ghrelin and GHSR in Male Reproduction

Both ghrelin and GHSR1a expression have been characterized within rat and human testes. Ghrelin mRNA and protein is expressed in mature leydig cells while GHSR1a mRNA and protein is expressed in leydig and sertoli cells as well as germ cells associated with sperm production (Barreiro et al. 2002, Tena-Sempere et al. 2002, Barreiro et al. 2003, Gaytan et al. 2004). FSH and ghrelin, but not LH, increase GHSR1a mRNA expression in cultured rat testicular tissue (Barreiro et al. 2003).

Peripheral injection of ghrelin into human males lowers plasma testosterone levels (Kluge et al. 2007). This is likely due to ghrelin inhibiting LH production, since LH is the primary stimulus for leydig cells to produce testosterone (Moyle and Ramachandran 1973). However, ghrelin produced by leydig cells, possibly in response to LH stimulation (Barreiro et al. 2002), may have a role in fine tuning testosterone production by the leydig cells (Barreiro and Tena-Sempere 2004). Neither ghrelin or GHSR1a mRNA or protein is expressed in leydig cells before they are steriodogenically competent (Barreiro et al. 2002, Barreiro et al. 2003). Once the

testes are capable of producing steroids, rat testicular tissue incubated with ghrelin produces less testosterone than untreated control tissue (Tena-Sempere et al. 2002). This direct effect of ghrelin on testosterone production is associated with a decrease in mRNA expression of steriodogenic enzymes, such as steriodogenic acute regulatory protein (StAR) and cholesterol side chain cleavage enzyme (P_{450}SSC), required for testosterone synthesis (Tena-Sempere et al. 2002), and would diminish the stimulatory effect of LH on testosterone production.

Ghrelin can also effect cell growth within the testes. Intratesticular injection of ghrelin in rats will decrease the mRNA expression of stem cell factor (SCF) mRNA expression (Barreiro et al. 2004) which is an important growth factor for leydig cell precursors as well as a major paracrine stimulator for germ cell development into functional spermatids (Hakovirta et al. 1999, Yan et al. 2000a, Yan et al. 2000b). Ghrelin also decreases proliferative activity of immature rat leydig cells differentiating into adult leydig cells (Barreiro et al. 2004).

1.7.4 Ghrelin and GHSR in Female Reproduction

Although there is evidence of ovarian production of ghrelin and for the presence of GHSR1a in most ovarian tissue, ghrelin's role in follicular development has not been widely researched and remains unclear. Ghrelin and GHSR1a mRNA and protein are expressed in mature human and rat ovaries (Caminos et al. 2003, Gaytan et al. 2003). Ghrelin mRNA expression within the rat ovary is cyclic in nature, with the lowest expression found during proestrus and highest expression level occurring during the luteal phase (Caminos et al. 2003). In humans, ghrelin protein is present in interstitial cells and granulosa cells, but not detectable in

oocytes or theca cells (Gaytan et al. 2003). In addition, ghrelin protein is not detectable in newly formed or regressing corpus luteum, but is detected in the young and mature corpus luteum (Gaytan et al. 2003).

GHSR1a mRNA expression is more widely distributed than ghrelin within the human ovary since in addition to being expressed in granulosa and interstitial cells it is also found in theca cells, oocytes and steriodogenic corpus luteum cells at all stages of development (Gaytan et al. 2003). In early antral follicles, the level of GHSR1a mRNA expression in the granulosa cells is less than in theca cells, but the GHSR1a mRNA expression levels in both the theca and granulosa cells increase as the follicle matures (Gaytan et al. 2003).

Ghrelin seems to play a significant role in pregnancy. Ghrelin mRNA and protein are expressed by rodent endometrial and placental cells (Gualillo et al. 2001a) and are secreted by endometrial cells into uterine fluid (Kawamura et al. 2003). The injection of exogenous ghrelin during early pregnancy significantly decreased litter size in rats, most likely due to an inhibition in the development of preimplantation embryos (Fernandez-Fernandez et al. 2005a). However, placental ghrelin production may be crucial to some aspects of fetal development. Ghrelin mRNA and protein expression in human and rat placenta sharply peaks during the last half of gestation (Gualillo et al. 2001a). Furthermore, exogenous ghrelin given to pregnant rats late in gestation increases fetal body weight and immunization against ghrelin will decrease fetal body weight (Hayashida et al. 2002, Nakahara et al. 2006). In addition, ghrelin will increase the proliferation of rat fetal skin cells in vitro (Nakahara et al. 2006). Thus, during implantation and early pregnancy ghrelin may serve to link nutrient status with the demands of pregnancy by

preventing pregnancy or limiting litter size in females with energy insufficiency. Interestingly, during the later stages of pregnancy ghrelin from placental production may actually promote fetal growth due to the stimulatory effect of growth hormone secretion.

1.8 Summary

In summary, mammalian ghrelin mediates a wide array of metabolic and reproductive functions, however, there are conflicting research regarding the some of the potential functions of ghrelin. In part the discrepancies from early research appear to result from inappropriate experimental design due assumptions about ghrelin's posttranslational processing and the discovery that UAG possesses biological activities. In spite of the conflicting research reports, it is clear that ghrelin plays a key role in signaling a negative energy balance and stimulating appetite in mammalian species. Furthermore, ghrelin plays a role in mammalian reproduction. At the central level, ghrelin has a negative impact on reproduction by decreasing LH secretion in both males and females. In the testes, locally produced ghrelin seems to play a role in fine tuning testosterone production, but ghrelin's influence upon the ovary has yet to be elucidated. High levels of ghrelin in females suffering from caloric insufficiency may prevent pregnancy. However, placental derived ghrelin may play a key role in stimulating fetal development by increasing growth hormone release in the fetus.

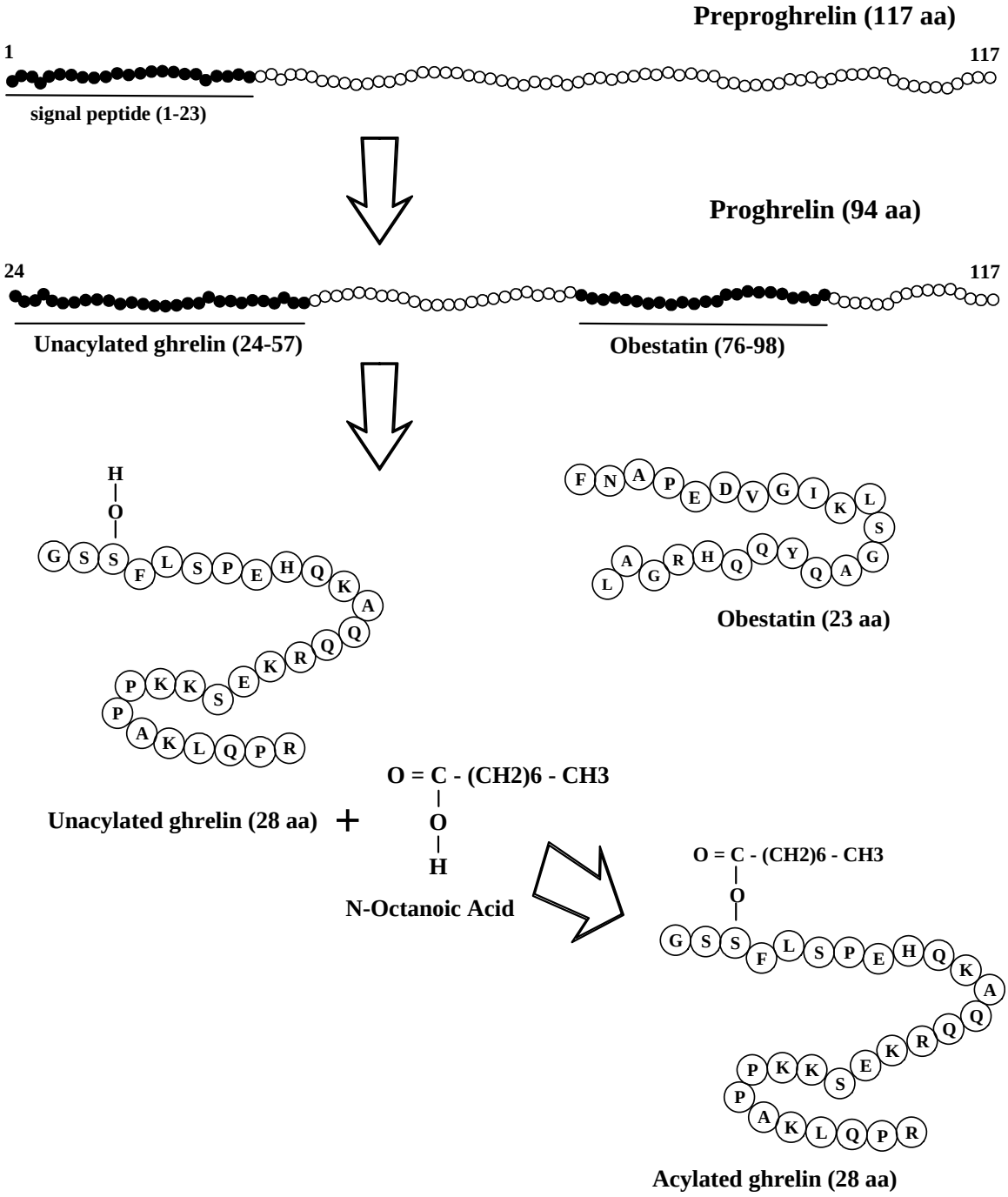

Figure 1.1. Post-translational processing of human preproghrelin in to unacylated ghrelin (UAG), acylated ghrelin, and obestatin.

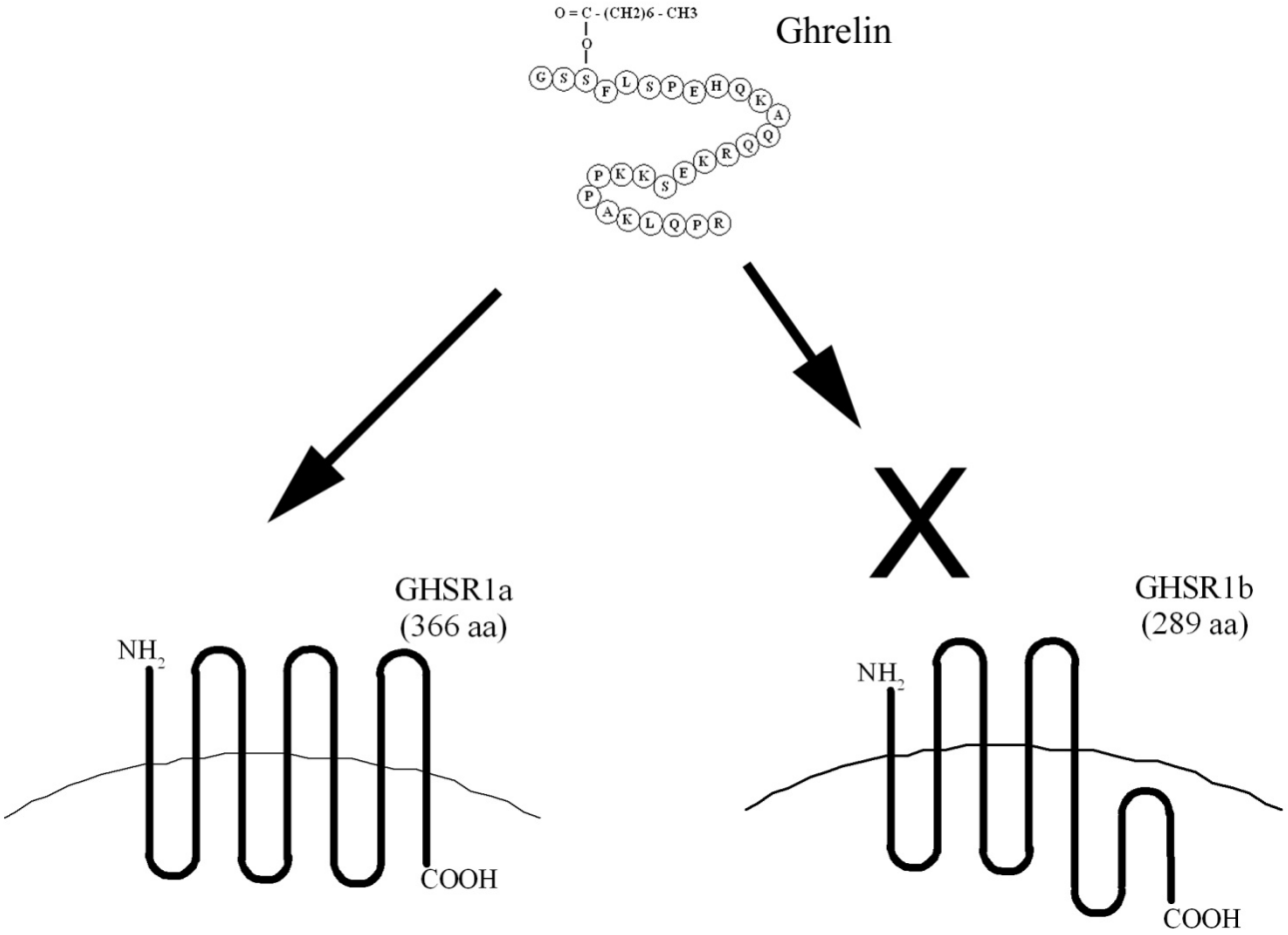

Figure 1.2. Predicted structures of mammalian GHSR1a and GHSR1b and the ability of acylated ghrelin to bind to these receptors.

Chapter 2

Avian Ghrelin and Ghrelin receptor

2.1 Avian Ghrelin Synthesis and Distribution

Avian ghrelin was cloned in 2002 by Kaiya et al. Avian preproghrelin is composed of 116 amino acids and it shares very little amino acid sequence homology with mammalian preproghrelin except in the core UAG sequence (Yuan et al. 2007). Similar to the processing of human ghrelin (Figure 1.1) the first 23 amino acids of chicken preproghrelin are cleaved to yield proghrelin. Although the chicken proghrelin sequence has no amino acid deletions in the 28 amino acid UAG core sequence that follows the signal sequence, it is processed differently than human ghrelin. N-terminal sequencing of isolated chicken ghrelin reveals that it consists of only 26 amino acids (Kaiya et al. 2002). When UAG is cleaved from proghrelin the amino acid residues in position 27 and 28 are left with the proghrelin portion of the protein (Kaiya et al. 2002, Yuan et al. 2007). The first seven amino acids of avian ghrelin are identical to those found in mammalian ghrelin (Figure 2.1), and as in mammalian species it is the third amino acid residue, a serine, that is acylated (Kaiya et al. 2002).

The tissue distribution pattern of avian ghrelin mRNA and protein expression is similar to that of mammalian species. The highest expression levels are found in the proventriculus, the glandular portion of the avian stomach, followed by the small intestines (Kaiya et al. 2002, Wada et al. 2003, Richards et al. 2005). Avian ghrelin mRNA is also expressed in the pancreas, adipose

tissue, lung, spleen and brain, but at levels lower than what is detected in the digestive tract (Kaiya et al. 2002, Wada et al. 2003, Richards et al. 2005, Kaiya et al. 2007). It is assumed that the proventriculus is the main source of circulating ghrelin in avian species (Richards et al. 2005, Kaiya et al. 2007).

2.2 Avian GHSR

Avian GHSR was characterized in 2003 by two separate research groups (Tanaka et al. 2003, Geelissen et al. 2003). Both groups reported that chickens have a GHSR gene structure analogous to that seen in humans with the avian GHSR gene being composed of two exons. The GHSR1a mRNA sequence in chickens codes for a protein of 347 amino acids. Alternate splicing of the GHSR transcript yields two other forms of the chicken ghrelin receptor, GHSR1aV and GHSRtv (Tanaka et al. 2003, Sirotkin et al. 2006). GHSR1aV lacks the first 16 amino acids coded by exon 2 and thus is predicted to lack transmembrane region 6 (Tanaka et al. 2003) and to have its C-terminal region located on the extracellular side of the cell membrane (Figure 2.2). The GHSR1tv transcript forms from a premature splicing from exon 1, retention of a 126 bp fragment from intron 1, and premature initiation of exon 2, all of which results in a shift in the open reading frame of the message that results in a new stop codon at amino acid 221. It is unclear if both of these truncated forms of the chicken GHSR receptor are even translated from their altered mRNA sequences (Sirotkin et al. 2006). GHSR1a mRNA expression in avian species is very high in the hypothalamus and pituitary, and lower levels of expression are found in the proventriculus, duodenum, adrenals, ovary, testes, liver, muscles, heart and skin (Geelisson et al. 2003, Richards et al. 2005). Although GHSR1aV and GHSR1tv are assumed to

have no biological activities (Tanaka et al. 2003, Sirotkin et al. 2006) the mRNA transcript for both of these splice variants is detected by RT-PCR in the tissues in which GHSR1a is expressed, although at lower levels (Geelisson et al. 2003, Richards et al. 2005, Sirotkin et al. 2006).

Limited research on the regulation of avian GHSR expression has been reported. Geelissen et al. (2003) reported that GHSR1a mRNA was down regulated in the pituitary after intracerebroventricular injections of ghrelin, growth hormone or corticosterone. Richards et al. (2005) found that 48 hours of fasting decreased GHSR1a mRNA expression in the pancreas, but did not alter GHSR1a expression in the proventriculus or pituitary.

2.3 Ghrelin Secretion and Impact upon Avian Metabolism

2.3.1 Regulation of Secretion

In 2005, Richards et al. reported that broilers fasted for 48 hours had increased ghrelin mRNA levels in the proventriculus, but that plasma concentrations of total ghrelin remained unchanged. In addition, ghrelin mRNA expression remained high in the proventriculus 12 hours after the birds had been refed. Rodents, on the other hand, have an increase in both ghrelin mRNA levels in the stomach and plasma levels of total ghrelin when they are fasted and both decrease within 6 hours after refeeding (Toshinai et al. 2001). Research reports in quail and male leghorn chicks in which acylated ghrelin was specifically measured indicate that plasma ghrelin levels increased and subsequently decreased upon refeeding (Shousha et al. 2005, Kaiya et al. 2007). However, it was again noted by Kaiya et al. (2007) that the level of ghrelin mRNA in the

proventriculus remained high 24 hours after the Leghorn chicks had been refed (Kaiya et al. 2007). This discrepancy after refeeding between plasma ghrelin concentrations and ghrelin mRNA expression levels within the proventriculus needs further investigation, but may indicate that after refeeding, UAG is being made from the increased mRNA levels but that subsequent acylation is not occurring.

Immunohistochemistical analyses determined adult male Leghorn chickens have higher levels of acylated ghrelin protein expression in the proventriculus than newly hatched chicks (Wada et al. 2003). There was no difference in the number of cells expressing ghrelin mRNA and acylated ghrelin protein in the proventriculus of adult Leghorns (Wada et al. 2003). In contrast, male Leghorn chicks had a greater number of ghrelin mRNA expressing cells than cells producing acylated ghrelin protein (Wada et al. 2003). The lower level of acylated ghrelin protein in newly hatched chicks may be due to the lack of N-octanoic acid. Yamoto et al. (2005) reported that oral doses or intraperitoneal injection of N-octanoic acid increased the number of cells expressing acylated ghrelin protein in the proventriculus of Leghorn chicks while chicks given saline exhibited no increase in acylated ghrelin expressing cells.

2.3.2 Physiological Effects of Ghrelin upon Metabolism in Avian Species

Similar to mammalian species, ghrelin has been found to be a potent in vivo and in vitro stimulator of growth hormone release in Leghorn chickens (Ahmed and Harvey 2002, Baudet and Harvey 2003). However, ghrelin's effects upon feeding behavior are not as clearly defined in avian species as they are in rodents and humans. Since the discovery of ghrelin in avian species, there has been only one report of peripherally injected ghrelin stimulating feeding behavior in

birds and that occurred in adult quail (Shousha et al. 2005). Intracerebroventricular injection of ghrelin into broiler chicks or peripheral injection of ghrelin into Leghorn chicks inhibited (Furuse et al. 2001, Saito et al. 2002, Saito et al. 2005) or had no effect (Kaiya et al. 2007) on feed intake.

If ghrelin has an effect on feed intake in poultry, the mechanism by which ghrelin influences feed intake in chickens may be different than in mammals. In mammalian species, ghrelin's orexigenic effects are due to its stimulation of NPY and AGRP production production in the hypothalamus (Chen et al. 2004). While intracerebroventricular injection of NPY in chickens does stimulate feeding behavior (Ando et al. 2001), intracerebroventricular injection of ghrelin has no effect on NPY mRNA expression (Saito et al. 2005). Furthermore, intracerebroventricular co-injection of ghrelin and NPY into male Leghorn chicks inhibits the normal NPY induction of food intake (Saito et al. 2005).

Previous research has determined that corticotropin releasing factor (CRF), which stimulates corticosterone release from the adrenal glands, reduces feed intake in chickens (Furuse et al. 1997, Denbow et al. 1999). Kaiya et al. (2002) observed that peripheral ghrelin injection increased plasma corticosterone concentrations in broiler chicks (Kaiya et al. 2002). Furthermore, when ghrelin was injected intracerebroventricularly in young Leghorn chicks, plasma corticosterone levels increased in a dose dependant manner (Saito et al. 2005, Kaiya et al. 2007). Saito et al. (2005) also reported that when ghrelin is administered intracerebroventricularly along with a CRF receptor antagonist ghrelin's ability to increase plasma corticosterone levels and cause anorexia were attenuated. Collectively, these results suggest that ghrelin in the avian brain does not influence the NPY system and may inhibit food intake by increasing CRF production.

Given the variability of previous research reports regarding ghrelin effects on feed intake it should not be surprising that reports on ghrelin's influence on plasma metabolites are also inconsistent. Kaiya et al. (2007) reported that peripheral ghrelin administration caused a decrease in plasma glucose levels in 8-day old male laying chicks. On the other hand, peripherally injected ghrelin did not have any effect on plasma glucose, triglyceride, free fatty acids, protein, or T3 levels in week old male Ross broiler chicks (Geelissen et al. 2006).

2.4 Summary

Ghrelin and its functional receptor have been identified in avian species. Similar to mammalian species, ghrelin in avian species is predominantly produced by the digestive tract and its production appears to increase with fasting. However, even though avian ghrelin biology is just starting to be elucidated, it appears that, unlike mammalian species, ghrelin may actually inhibit food intake in avian species. More research needs to be performed to clarify ghrelin's action upon avian metabolism and food intake.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Species
G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Human
G	S	S	F	L	S	P	E	H	Q	K	A	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Rat/Mouse
G	S	S	F	L	S	P	E	H	Q	K	L	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Dog
G	S	S	F	L	S	P	E	H	Q	K	V	Q	Q	R	K	E	S	K	K	P	A	A	K	L	K	P	R	Pig
G	S	S	F	L	S	P	T	K	K	N	I	Q	Q	Q	K	D	T	R	K	P	T	A	R	L	H			Chicken

Figure 2.1. Comparison of the ghrelin amino acid sequences between species. Note that the first 7 amino acid residues of the ghrelin protein sequence are conserved across all species.

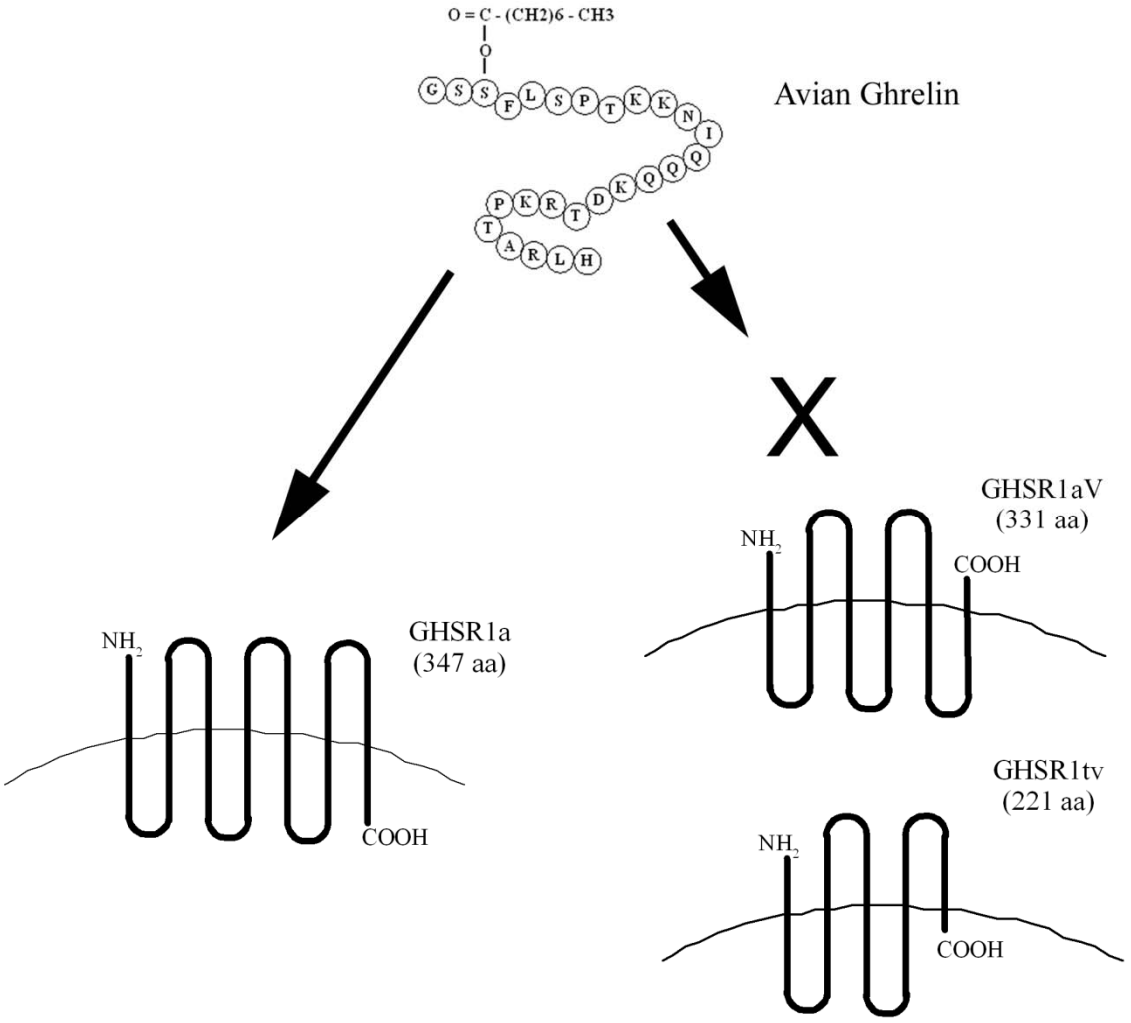

Figure 2.2. Predicted structures of avian ghrelin receptors GHSR1a, GHSR1aV and GHSRtv and the ability of acylated ghrelin to bind to these receptors.

Chapter 3

Ghrelin and Avian Reproduction

3.1 The Avian Ovary

The ovary of the mature hen consists of a visually evident hierarchy of follicles relative to size and time to ovulation. In the laying hen, there are commonly four to six large yellow yolk – filled follicles, termed hierarchical follicles, that are approximately 12 - 40 mm in diameter. These follicles are accompanied by several 5 to 12 mm diameter follicles in which yellow yolk deposition has begun and a large number of small white follicles that are less than 5 mm in diameter. The large yolk filled follicles are named according to size and time to ovulation. The largest follicle is termed the F_1 follicle and will ovulate within the next 24 hours. The next largest follicle is named the F_2 follicle and it will ovulate 24-26 hours after the ovulation of the F_1 follicle. The remaining large yolk filled follicles are named accordingly (F_3-F_n). With each ovulation all the hierarchical follicles advance one position forward in the naming hierarchy and a new follicle enters into the hierarchy from the pool of small yellow follicles. In addition, some of the larger white follicles will start the uptake of yellow yolk and become small yellow follicles. A vast majority of the small yellow and large white follicles will never advance into the hierarchy, but will instead undergo atresia (Gilbert et al. 1983).

3.2 Avian Follicular Tissues and Follicular Maturation

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

In general terms, follicular maturation can be characterized by the accumulation of yolk and the development of endocrine capabilities within the follicular tissues (Huang and Nalbandov, 1979). The theca cells of the small yellow and white follicles are steroidogenically competent and are the primary source of plasma estrogen **(**Senior and Furr 1975, Lee and Bahr 1989). However, the granulosa cells of these small follicles are steroidogenically incompetent since they lack P_{450} side chain cleavage (P_{450}SCC) enzyme activity, which catalyzes the initial step in the metabolic pathway that converts cholesterol to steroids (Li and Johnson, 1993).

Follicular maturation is regulated by two pituitary 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 dependant, they are sensitive to FSH. FSH promotes granulosa cell proliferation and maturation (Davis et al. 2000, Davis et al. 2001), helps maintain the follicular hierarchy through the prevention of atresia (Palmer and Bahr 1992, Johnson et al 1996, Johnson et al. 1999), induces LH receptor, steriodogenic acute regulatory protein (StAR), and P_{450}SCC enzyme expression in granulosa cells for subsequent steroid production (Li and Johnson 1993, Johnson and Bridgham 2001,

Johnson et al. 2004) 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 results suggest that the prehierachial follicle which has granulosa cells that are the most responsive to FSH avoids 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 the one that advances into the hierarchy (Woods and Johnson, 2005).

Follicle recruitment into the hierarchy is coupled with an increase in LH receptor expression and acquisition of functional P_{450}SCC enzyme activity within the granulosa layer (Li and Johnson 1993, Kato et al. 1995). Once the selection of a follicle occurs, granulosa cells rely 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 the F_1 follicle of the hen prior to ovulation (Huang and Nalbandov, 1979). As reviewed by Etches (1996), plasma progesterone produced by the F_1 follicle triggers secretion of luteinizing hormone releasing hormone (LHRH-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 before ovulation of the F_1 follicle.

3.3 Effects of Feed Restriction in Hens

Food restriction of hens is common within the poultry industry. In older laying hens, food is removed for an extended period of time to induce molt to cause the regression of the ovary and oviduct and the cessation of egg production (Brake and Thaxton, 1979). Broiler breeder hens are feed restricted on a daily basis during rearing and production to control weight gains that are detrimental to egg production and egg quality. Broiler breeder hens who are fed ad libitum generally have higher body weights (Robbins et al. 1986, Bruggeman et al.1999, Richards et al. 2003; Bruggeman et al. 2005, Onagbesan et al. 2006), an earlier age of sexual maturation and age of first egg (Heck et al. 2004, Bruggeman et al. 2005), but produce fewer total eggs over the entire laying period than broiler breeder hens that are feed restricted (Yu et al. 1992, Bruggeman et al. 1999).

Despite the improvement of egg production of broiler breeders hens resulting from feed restriction that limits body weight gain, these hens still have inferior total egg production when compared to commercial laying hens even though they have the follicles to produce just as many eggs. Follicular development of broiler breeder hens is often abnormal as characterized by the double hierarchies, erratic ovulations and atresia of large yellow follicles (Renema et al. 1999). The abnormal follicular development and lack of egg production may actually be related to the industry's feed restriction practices for broiler breeder hens. Broiler breeder hens are typically fed every other day during rearing and then provided a restricted level of feed once a day after the flock reaches 5% egg production. The feed provided is quickly consumed, and the hens are left to fast until the next feeding period. Morris and Nalbandov (1961) suggested that a lack of gonadotropin secretion may be responsible for the decreased egg production in fasted hens.

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 fed control cockerals. In addition, fasting laying hens have lower plasma concentrations of LH after 48 hours of fasting and lower estradiol and progesterone concentrations after 24 hours of fasting when compared to ad libitum fed control hens (Tanabe et al. 1981). More recently, egg production was reported to be improved by reducing the fasting duration between meals in broiler breeder hens by feeding them twice a day rather than once a day (Spradley 2007). In addition, reducing the fasting period by feeding once a day instead of every other day during the period after photostimulation for reproduction until the flock reached 8% egg production significantly enhanced total egg production per hen (Gibson 2006).

The research of Gibson (2006) and Spradley (2007) reinforces the hypothesis that nutritional status and caloric intake are both intricately connected to reproductive function in birds. The complex hormonal interactions defining how nutrition affects reproduction have yet to be clarified but a key component of this interaction may be ghrelin. Initial research in mammalian species indicates that ghrelin can influence the reproductive axis, however very little research has been performed on ghrelin and reproduction in avian species. In 2005, Yoshimura et al. reported that ghrelin mRNA and protein was expressed in the mucosal epithelium layers in the infundibulum and magnum of mature quail, yet there was no ghrelin expression found in the oviducts of immature quail. In addition, the density of ghrelin immunoreactive protein in the magnum decreased after the passage of the ovum. The authors speculated that maternal ghrelin deposited in the albumin may play a role in chick embryo development. Finally, Sirotkin et al. (2006) found ghrelin and GHSR mRNA to be present in follicular wall fragments of developing hierarchal follicles.

Determining the potential roles of ghrelin in follicular development may be easier in avian species than in mammalian species. The histological structure of the hen's ovarian follicle allows for the manual separation of granulosa and theca tissues in the hierarchal follicles and for the enzymatic separation of these tissues in the non-hierarchal follicles. Such tissue specific studies are not as feasible in mammals where the theca and granulosa cells of the follicle are more intermixed and not easily separated. Furthermore, the well defined follicular hierarchy and the predictable timing of ovulation in avian species allows for identification of regulatory mechanisms to be correlated with time to ovulation and degree of follicular maturation.

3.4 Summary

The broiler breeder hen ovary provides a unique opportunity to explore the role of ghrelin in ovarian development. Restricting calorie intake is essential in broiler breeder hens to prevent excessive weight gain that is detrimental to reproductive efficiency. At the same time, management practices in implementing this caloric restriction may negatively impact reproduction. The negative impact of fasting on reproduction could be mediated in part by ghrelin. Finally, the histological structure of avian preovulatory follicles as well as the arrangement of the preovulatory follicles in a size hierarchy relative to ovulation make it an ideal model to determine the role ghrelin may have on ovarian development.

Chapter 4

Statement of Purpose

In mammalian species, ghrelin production increases with fasting and ghrelin has been shown to affect mammalian reproduction by decreasing gonadotropin secretion from the pituitary, however, there has been very little research into ghrelin's effects on avian reproduction. While it is known in avian species that fasting promotes ovarian regression, identification of the hormonal signaling pathways that translate the lack of sufficient energy intake into a reduction in reproductive capability has not occured. Therefore the goal of this research was to explore the possibility that ghrelin may be a direct link between nutritional status and reproduction in avian species by (1) determining if ghrelin or GHSR mRNA expression is present within the avian ovary, (2) investigating if fasting changes GHSR mRNA expression in the ovary or plasma levels of ghrelin, (3) exploring the effects of gonadotropins and sex steroids upon GHSR mRNA expression in granulosa cells, and (4) investigating if ghrelin influences progesterone secretion or GHSR mRNA expression in granulosa cells.

Chapter 5

Materials and Methods

5.1 Animals

Cobb 500 slow - feathering pullets were reared from 1 day of age in floor pens using standard broiler breeder pullet diets and a skip a day feed restriction program. The pullets received 8 hours of light each day. Ten percent of the pullets were randomly selected and weighed each week to determine feed allocation so that the body weight gain of the pullets matched the recommended guidelines of the primary breeder (Cobb-Vantress 2005a). 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 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.

5.2 Experiment 1

To determine if ghrelin or GHSR mRNA are expressed in preovulatory follicles of the broiler breeder hen, three, 42-week old broiler breeder hens were killed by cervical dislocation 2 - 4 hours prior to ovulation and the ovary and a 200 mg portion of glandular proventriculus tissue were collected from each hen. The theca and granulosa layers from each of the F_1, F_2, F_3, and F_4 follicles were manually separated from one another (Huang and Nalbandov 1979) while the theca and granulosa layers from the small yellow (SY) follicles (5 - 10 mm in diameter) and large white (LW) follicles (2 - 5 mm in diameter) were separated enzymatically (Davis et al. 2000). The proventriculus and the individual theca and granulosa tissue for each follicle size obtained from 1 hen were combined with corresponding samples from the other 2 hens for RNA extraction and subsequent RT-PCR detection of ghrelin and GHSR mRNA.

5.3 Experiment 2

To determine if GHSR mRNA expression increased in the preovulatory follicles, the pituitary, and the proventriculus of fasted broiler breeder hens, 4 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, all 4 hens were killed and the pituitary, a 200 mg portion of glandular proventriculus tissue, and the ovary were collected from each hen. The theca and granulosa layers from each of the F_1, F_2, F_3, and F_4 follicles as well as the SY and LW follicles were collected from each hen as described in experiment 1. The individual theca and granulosa samples for each follicle size, as

well as the pituitary and proventriculus tissue from one hen of each treatment was combined with the corresponding samples from the other hen of that treatment for RNA extraction and subsequent real-time RT-PCR analysis of GHSR mRNA expression. This collection procedure was repeated 2 more times to give 3 total replications for each treatment (n = 3). The hens utilized for the 3 replicate tissue collections were between 45-55 weeks of age.

5.3 Experiment 3

To determine if plasma total and acylated ghrelin levels in broiler breeder hens increased under fasting conditions, 18 hens that were 51 weeks of age and were consistently producing eggs were selected. The hens were weighed, fed their daily allotment of feed (time 0 hours) and then fasted for the following 96 hours. Water was provided ad libitum to the hens throughout the experiment. Blood was collected from the brachial vein of all the hens at 6 and 96 hours after feeding and placed in an ETDA vacutainer (Becton Dickinson, Franklin Lakes, NJ). Blood samples were collected from 10 of the 18 hens at 24, 48, and 72 hours after feeding. Except for one hen, the hens bled at 48 hours were different than those bled at 24 hours and similarly those bled at 72 hours were different than those bled at 48 hours. Blood samples were placed on ice immediately after collection and later centrifuged at 1000 x *g* 4 ^{o}C for 10 minutes. Plasma was collected and 50 µL of 1N HCl and 10 µL of a 10 mg/mL solution of phenylmethanesulfonyl fluoride (PMSF) were added per 1 mL of plasma to prevent degradation of the acylated ghrelin protein (Hosoda et al. 2004). Plasma samples were stored at -80 ^{o}C for future analysis of total and acylated ghrelin concentrations. Egg production was recorded throughout the experiment,

and at the end of the experiment the hens were weighed and then killed by cervical dislocation for a visual inspection of ovarian morphology.

5.5 Experiment 4

To investigate whether GHSR mRNA expression in granulosa cells is influenced by gonadotropins the F_1, F_3 and SY follicles were removed from three, 45-week old hens and pooled by size. The hens were killed by cervical dislocation 2 - 4 hours prior to ovulation. Granulosa cells were isolated, dispersed and washed as previously described (Davis et al. 2000). Cell number and viability were estimated using a hemocytometer with trypan blue exclusion, with cell viability was greater than 95%. Dispersed cells from each follicle size were cultured in 6-well tissue culture plates at a density of 2.5 x 10^6 cells/well with 4 mL of M199 culture media as previously described (Davis et al. 2000), except the lipoprotein supplement was not added to the M199 culture media. Cells were cultured for 24 hours with 0 or 50 ng/mL cell culture media of ovine LH (Lot AFP8468A) or human recombinant FSH (Lot AFP5551B). Both the LH and FSH were generously provided by Dr. A. F. Parlow of the National Hormone and Peptide Program, Torrance, CA. For each follicle size there were 3 wells per treatment and at the end of the experiment, the cells from these three wells were collected in solution D, combined (Chomczynski and Sacchi, 1987) and then frozen at -80 ^{o}C for future RNA extraction and real-time RT-PCR analysis of GHSR mRNA expression. The culture media from each treatment and follicle size was saved and stored at -80 ^{o}C for subsequent analysis of progesterone content. This experiment was repeated 4 more times over the subsequent 3 weeks to generate 5 replicates (n = 5).

5.6 Experiment 5

To determine if GHSR mRNA expression in granulosa cells is regulated by sex steroid hormones, the F_1, F_3 and SY follicles were removed from three, 50-week old broiler breeder hens which were killed by cervical dislocation 2 - 4 hours prior to ovulation. The same procedures utilized in experiment 4 were used in this experiment, except that dispersed cells were cultured in the absence or presence of 1×10^{-6} testosterone (Steraloids, Newport, RI) or 17-β-estradiol (Sigma, St. Louis, MO).

5.7 Experiment 6

To determine if ghrelin influences the mRNA expression of its receptor in granulosa cells, F_1, F_3 and SY follicles were removed from three, 50 week old hens which were killed by cervical dislocation 2 - 4 hours prior to ovulation. The same procedures utilized in experiment 4 were used in this experiment except that cells were cultured with 0, 50 ng/mL of chicken ghrelin (Phoenix Pharmaceuticals, Belmont, CA), 50 ng/mL of ovine LH, or 50 ng/mL of both ghrelin and LH. This procedure was repeated 3 more times over the subsequent week to generate 4 replicates (n = 4). In addition, the cell culture media from this experiment was saved not only for determining progesterone content, but also IGF-1 content.

5.8 RNA Extraction

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

5.9 RT-PCR

Primers were specifically designed for chicken ghrelin (GenBank accession # NM_001001131), GHSR (GenBank accession # AB095995), and the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession # K01458). Primers were manufactured by the University of Georgia Molecular Genetics and Instrumentation Facility (MGIF). The forward primer for ghrelin was 5'-ATACAAGAAAACCCAACAGCAAGAT-3' while the reverse primer was 5'-ACTAAGGAAGGAAATAAAATAAGC-3'. For GHSR, the forward primer was 5'-GGTGGGCGTCGACGACGACAATG-3' and the reverse primer was 5'-GGCTGATCACTGCTATCTCCAAGG-3'. The forward primer for GAPDH was 5'-GACGTGCAGCAGGGAACACTA-3' and the reverse primer was 5'-CCTCTGTCATCTCTCCACAGC-3'. Reverse transcription cDNA synthesis reactions were performed using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA), following the manufacturer's protocol. The polymerase chain reaction (PCR) was done as previously described (Davis and Johnson, 1998) using 350 ng of cDNA per reaction for the theca

and granulosa samples and 115 ng of cDNA for the proventriculus samples. The amount of cDNA utilized for the proventriculus samples was decreased because it was known to have the highest levels of ghrelin mRNA expression in avian species (Kaiya et al. 2002, Richards et al. 2005). The annealing temperature was 50 ^{o}C for all 3 primer sets. A 10 uL aliquot of each PCR reaction was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for PCR product visualization.

5.10 Real time RT-PCR

Extracted RNA samples were DNase treated using the TURBO-DNA-free kit (Ambion, Austin, TX) to remove any potential genomic DNA contamination. Primers and TaqMan probes specific for chicken GHSR (GenBank accession # AB095995) and GAPDH (GenBank accession # M11213) were generated using Primer Express software version 2.0 (Applied Biosystems, Table 5.1) and were synthesized by Applied Biosystems. The GHSR assay was designed to amplify both GHSR1a and GHSR1aV. The probes for GHSR and GAPDH were labeled at the 5' end with FAM (6-carboxyfluorescein) as the reporter dye and at the 3' end with TAMRA (6-carboxy-*N*,*N*,*N*',*N*'-tetramethylrhodamine) as the quencher dye. Primer and probe sets were validated for real-time PCR by determining the optimal amplification efficiency and primer/probe concentrations as described by the manufacturer (Applied Biosystems, Foster City, CA).

cDNA for two step real time PCR was generated using the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol. Each 25 uL sample reaction contained 100 ng cDNA, 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM

of the appropriate primer pair and 25 nM of the appropriate probe. The reactions were completed in an ABI 7500 Thermocycler (Applied Biosystems). The thermocycler conditions were 10 minutes at 95 ^{o}C and 40 cycles each of 15 seconds at 95 ^{o}C and 1 minute at 60 ^{o}C. The reactions for each sample were performed in duplicate for both the GHSR and GAPDH assays. The C_T (the cycle number at which the fluorescence exceeds the threshold level) was determined for each reaction using the Sequence Detection software (version 1.2.2, Applied Biosystems) and quantification was completed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the GHSR C_T was determined for each sample and then normalized to the GAPDH C_T from the same sample (GHSR C_T - GAPDH C_T = ΔC_T). After all these ΔC_T values were obtained for a replicate experiment the ΔC_T values were all compared relative to the sample with the highest GHSR mRNA expression for a given tissue or follicle size using the $2^{-\Delta\Delta Ct}$ method and data was then expressed as the fold-difference relative to the sample with the highest expression.

5.11 Total and Acylated Ghrelin RIA

Plasma total and acylated ghrelin concentrations for broiler breeder hen plasma samples were determined by RIA using Millipore's (Billerica, MA) ghrelin total RIA kit (catalogue # GHRT-89HK) and ghrelin active RIA kit (catalogue # GHRA-88HK) following the manufacture's protocol. The manufacturer of these kits indicate they have a 100% specificity for human, rat and canine total and acylated ghrelin. The kits were originally designed and sold by Linco (St. Charles, MO) until Linco was acquired by Millipore. RIA samples were counted with a Wallac Wizard 1470 gamma counter (Perkin Elmer, Waltham, MA).

5.12 Progesterone RIA

Cell culture media progesterone concentrations were determined by RIA using the Coat-A-Count Progesterone kit (Diagnostic Products Corporation, Los Angeles, CA, catalogue # TKTPG) following the manufacturer's protocol.

5.13 IGF-I Two-site Immunoradiometric Assay (IRMA)

Cell culture media concentrations of total insulin-like growth factor-I (IGF-I) were determined by immunoradiometric assay (IRMA) following the manufacturer's protocol. The ACTIVE® Insulin-Like Growth Factor-I Coated-Tube IRMA Kit (catalogue # DSL-5600) was obtained from Diagnostic Systems Laboratory's (Webster, TX).

5.14 Statistics

Data from each experiment were subjected to ANOVA according to the General Linear Model (GLM). Tukey's multiple-comparison procedure (Neter et al. 1990) was used to detect significant differences among follicle sizes and cell culture treatments. Differences were considered significant when $P < 0.05$. All statistical procedures were completed with the Minitab statistical software package (Release 13, State College, PA).

Table 5.1. Real-time RT-PCR primer and probe designs for GHSR and GAPDH.

	GHSR	GAPDH
Forward Primer	CAG TAC CGG CCC TGG AAC TT	TTG GCA TTG TGG AGG GTC TT
Reverse Primer	TGA GCG CGG TGA TGT TGA	GGG CCA TCC ACC GTC TTC
Probe [1]	CTG CAC CTA CTC CAC CAT	TGA CCA CTG TCC ATG CCA T

[1] The probes for GHSR and GAPDH were labeled at the 5' end with FAM as the reporter dye and at the 3' end with TAMRA as the quencher dye.

Chapter 6

Results

6.1 Experiment 1

Ghrelin mRNA was not detected by RT-PCR in either the theca or the granulosa cells of any of the preovulatory follicles (Figure 6.1). However, GHSR mRNA was detected in theca and granulosa cells from hierarchal follicles as well as nonheirarchal follicles (Figure 6.1). All three variations of the spliced mRNA transcript of the chicken GHSR message (GHSR1a, GHSR1aV, and GHSR1tv) were amplified in follicular tissue (Figure 6.1).

6.2. Experiment 2

In fed hens, GHSR mRNA was detected in the theca and granulosa cells of hierarchal and nonheirarchal follicles and there was a significant difference in GHSR mRNA expression between the theca and granulosa cells of the F_2 follicle (Figure 6.2). The overall expression of GHSR mRNA across all follicle sizes was significantly higher in the theca than in the granulosa tissue (Figure 6.3). In hens that had been fasted for 72 hours the mRNA expression of GHSR was significantly higher in the theca cells than granulosa cells for all follicle sizes (Figure 6.2). Furthermore, the overall expression of GHSR mRNA in the theca tissue from the F_1 through F_4, SY and LW follicles of fasted hens was significantly higher than in the theca tissue of fed hens

(Figure 6.3). The overall expression of GHSR mRNA in granulosa cells from the $F_1 – F_4$, SY, and LW follicles did not differ between fasted and fed hens (Figure 6.3).

Fasting did not influence GHSR mRNA expression levels in the pituitary or proventriculus of broiler breeder hens (Figure 6.4). The mRNA expression for GHSR in the F_1 theca of fasted hens did not differ from the expression level found in the pituitary of fed or fasted hens.

6.3. Experiment 3

Since the RIA kits for total and acylated ghrelin had not been verified for use in avian species, both kits were tested using serial dilutions of synthesized acylated chicken ghrelin (Phoenix Pharmaceuticals) and broiler breeder hen plasma samples. The total ghrelin kit generated plasma ghrelin concentrations which varied from hen to hen. However, when a plasma sample was diluted, the diluted plasma's value had the same concentration as the undiluted sample even though the dilution factor had not yet been taken into account. Furthermore, dilutions of purified chicken acylated ghrelin all generated the same concentration value with the assay.

The acylated ghrelin kit detected varying concentrations of the purified acylated ghrelin and had an acceptable linearity based on the dilution of broiler breeder plasma samples. The assay tended to underestimate expected ghrelin concentrations by 10 - 25% as plasma samples were diluted. This was in contrast to the manufacturer's results with human plasma in which the expected ghrelin concentrations were all overestimated by 10 – 40% as the plasma samples were

diluted. Interassay variation for chicken plasma samples was approximately 25% for the acylated ghrelin kits.

Plasma acylated ghrelin levels in fasted broiler breeder hens were significantly elevated at 24, 72, and 96 hours after feeding when compared to a 6 hour after feeding baseline level (Table 6.1). The mean ± SEM body weight of the hens (n = 18) just before they were fed at time 0 hours was 4,233 ± 74 g. Ninety-six hours after being fed the mean ± SEM body weight of the hens had significantly decreased ($P < 0.01$) to 3,701 ± 75 g. Despite the weight loss, 50% of the hens were still producing eggs (egg laid in the previous 12 hours or hard shelled egg present in the oviduct when killed) and had no visual signs of hierarchal follicular regression. Of the remaining 9 hens, 6 had hierarchal follicles showing visual signs of regression and 3 hens had complete regression of the follicular hierarchy.

6.4. Experiment 4

The mRNA expression of GHSR in cultured granulosa cells from all 3 follicle sizes following 24 hours of culture in M199 was significantly increased compared to the GHSR mRNA expression in freshly dispersed granulosa cells from these follicles (Figure 6.5). Addition of LH or FSH to granulosa cells cultured from the F_1 and F_3 follicles significantly reduced the mRNA expression of GHSR in comparison to untreated controls (Figure 6.5). Progesterone accumulation in the media of the granulosa cells cultured with LH was higher than the progesterone accumulation in the untreated control cells for all follicle sizes except for the SY follicles (Table 6.2). The addition of FSH to the granulosa cell culture media significantly

increased the accumulation of progesterone for the granulosa cells from all follicle sizes (Table 6.2).

6.5. Experiment 5

Similar to what was observed in experiment 4, the mRNA expression of GHSR in untreated granulosa cells from all three follicle sizes was significantly increased compared to the GHSR mRNA expression in freshly dispersed granulosa cells (Figure 6.6). Compared to untreated control cultures, the addition of testosterone to the cell culture media decreased GHSR mRNA expression in the granulosa cells from F_1 and F_3 follicles, but had no effect on GHSR mRNA expression in SY follicles (Figure 6.6). The addition of 17-β-estradiol had no effect on granulosa GHSR mRNA expression for any follicle size (Figure 6.6). Progesterone accumulation in the media of granulosa cells from the F_1, F_3, and SY follicles cultured with testosterone was significantly higher than in the media of control granulosa cell cultures from these follicles (Table 6.3). The addition of 17-β-estradiol to the cultured granulosa cells had no effect on progesterone accumulation (Table 6.4).

6.6. Experiment 6

The addition of ghrelin to cultured granulosa cells from the F_3 follicle increased GHSR mRNA expression (Figure 6.7). In cultured granulosa cells obtained from the F_1 and SY follicles, the addition of ghrelin had no effect on GHSR mRNA expression (Figure 6.7). In experiments 4

and 5 the expression of GHSR mRNA in freshly dispersed granulosa cells from the F_1, F_3 and SY follicles was very low and in the current experiment GHSR mRNA was undetectable in freshly dispersed cells from these follicles.

Progesterone accumulation in the media of granulosa cells cultured with ghrelin was the same as in the media of the untreated controls (Table 6.4). Ghrelin also did not alter the ability of LH to stimulate progesterone production in cultured granulosa cells (Table 6.4). In addition, based on mRNA yields and visual observations granulosa cell proliferation and livability were not influenced by the addition of ghrelin to the cell culture media (data not shown).

IGF-I concentrations in the cell culture media did not vary with follicle size or treatment (data not shown).

Table 6.1. Plasma ghrelin concentrations in broiler breeder hens 6, 24, 48, 72, or 96 hours after feeding.

Time Duration	Sample Size	Ghrelin Concentration[1]
(hr)	(n)	(pg/mL)
6	18	45.0 ± 3.7
24	10	82.7 ± 8.9*
48	10	64.6 ± 15.7
72	10	85.9 ± 11.6*
96	18	58.2 ± 5.5*

[1] Values are means ± SEM.
* Significantly different ($P < 0.05$) from 6 hour value.

Table 6.2. Progesterone concentration in granulosa cell culture media from F_1, F_3 or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 50 ng/mL culture media of LH or FSH.

Follicle Size	Treatment	Progesterone Concentration[1]
		(ng/mL)
F_1	Control	356 ± 35 [a]
F_1	LH	3,600 ± 390 [b]
F_1	FSH	1,800 ± 346 [c]
F_3	Control	134 ± 9 [a]
F_3	LH	1,900 ± 179 [b]
F_3	FSH	1,460 ± 154 [c]
SY	Control	0.17 ± 0.08 [a]
SY	LH	0.96 ± 0.46 [ab]
SY	FSH	1.28 ± 0.54 [b]

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

Table 6.3. Progesterone concentration in granulosa cell culture media from F_1, F_3 or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 1 x 10^{-6} M of estrogen or testosterone.

Follicle Size	Treatment	Progesterone Concentration[1]
		(ng/mL)
F_1	Control	335 ± 39.0 [a]
F_1	Estrogen	297 ± 40.0 [a]
F_1	Testosterone	619 ± 68.0 [b]
F_3	Control	150 ± 16.0 [a]
F_3	Estrogen	137 ± 12.0 [a]
F_3	Testosterone	679 ± 36.0 [b]
SY	Control	0.40 ± 0.10 [a]
SY	Estrogen	0.29 ± 0.10 [a]
SY	Testosterone	3.24 ± 0.49 [b]

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

Table 6.4. Progesterone concentration in granulosa cell culture media from F_1, F_3 or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 50 ng/mL culture media of ghrelin (Ghr), luteinizing hormone (LH) or 50 ng/mL of both ghrelin and LH.

Follicle Size	Treatment	Progesterone Concentration[1]
		(ng/mL)
F_1	Control	320 ± 94 [a]
F_1	Ghr	342 ± 96 [a]
F_1	LH	2,857 ± 337 [b]
F_1	Ghr + LH	2,882 ± 399 [b]
F_3	Control	165 ± 22 [a]
F_3	Ghr	159 ± 18 [a]
F_3	LH	1,790 ± 127 [b]
F_3	Ghr + LH	1,710 ± 95 [b]
SY	Control	undetectable
SY	Ghr	undetectable
SY	LH	undetectable
SY	Ghr + LH	undetectable

[a-b] Values with different superscripts for a given follicle size differ, ($P < 0.05$).
[1] Values are means ± SEM, n = 3 replicate experiments.

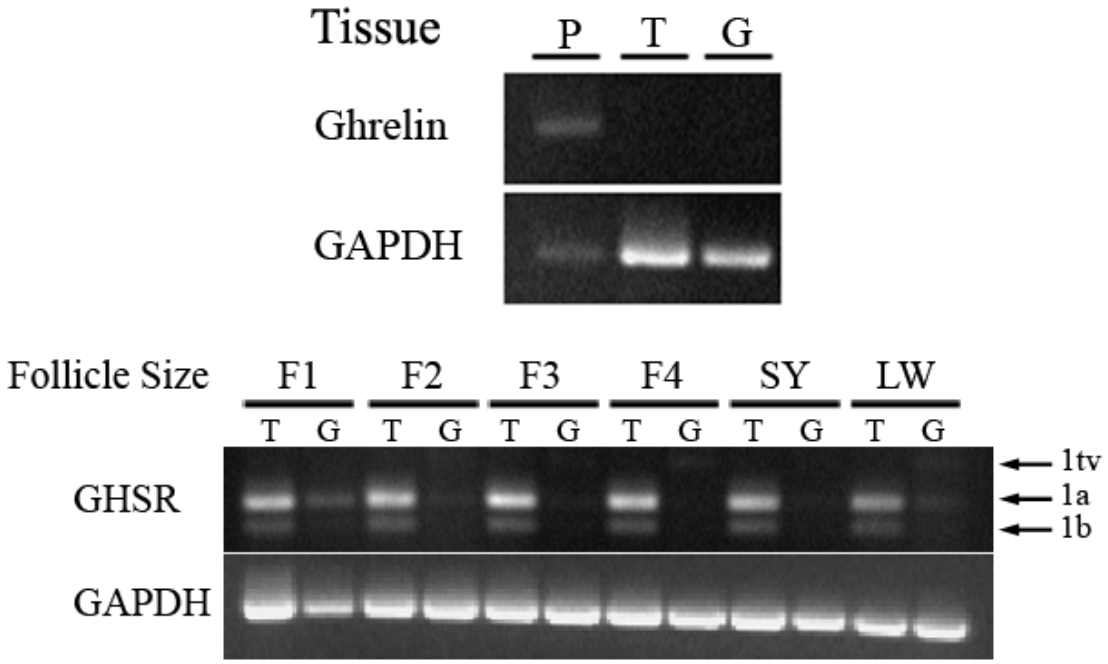

Figure 6.1. Ethidium bromide stained ghrelin, ghrelin receptor (GHSR), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RT-PCR products. For each 50 uL RT-PCT reaction 350 ng of cDNA was used except for the proventriculus (P) sample in which only 115 ng was used. Ten uL of each RT-PCR reaction was electrophoresed on a 1.5% agarose gel and ran at 90V for 60 minutes. Although a RT-PCR reaction for ghrelin, GHSR, and GAPDH (control) was run with each sample, only the theca (T) and granulosa (G) sample from the F_1 follicle is shown for ghrelin since no products were detected with ethidium bromide staining.

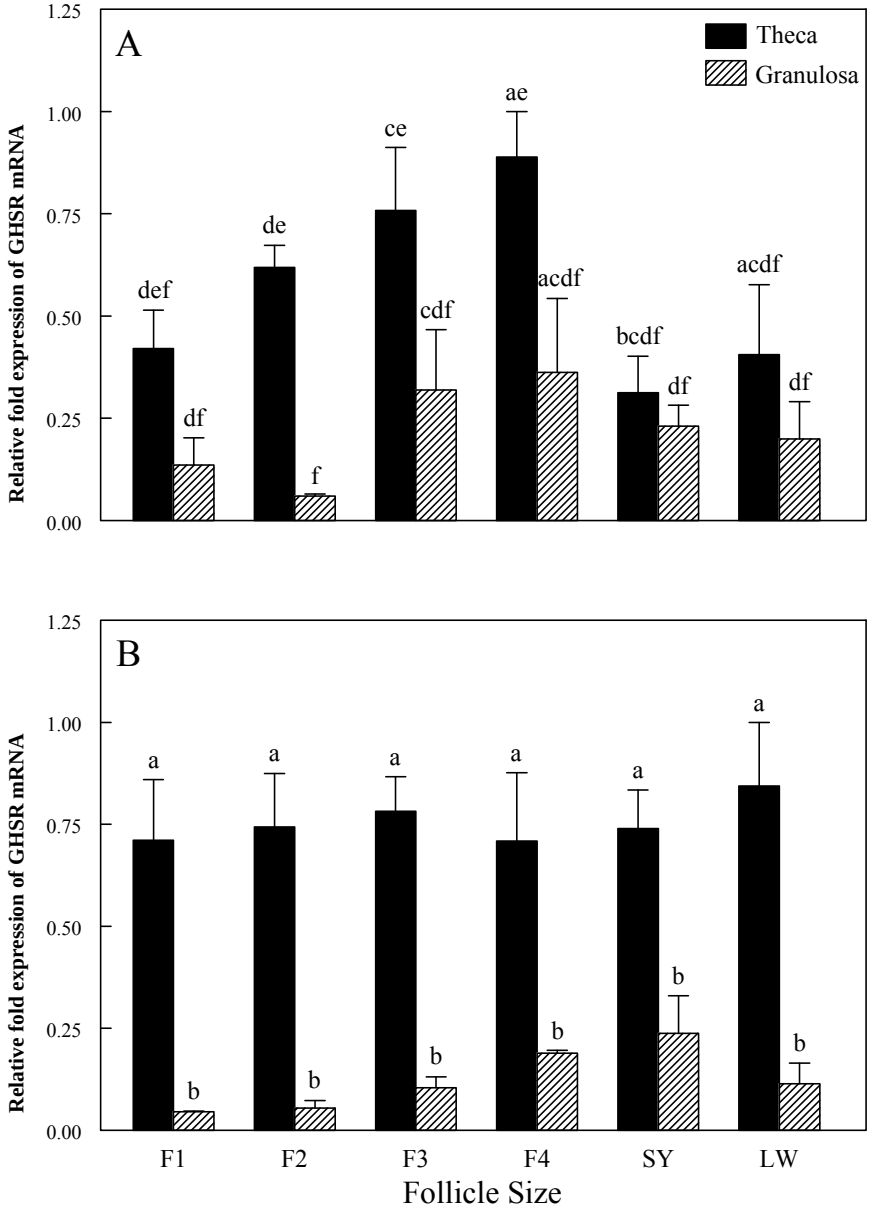

Figure 6.2. Ghrelin receptor (GHSR) mRNA expression in theca and granulosa tissue from preovulatory follicles of fed (A) and fasted (B) hens. GHSR mRNA expression values were normalized with GAPDH and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 3. Means without a common letter differ, ($P < 0.05$).

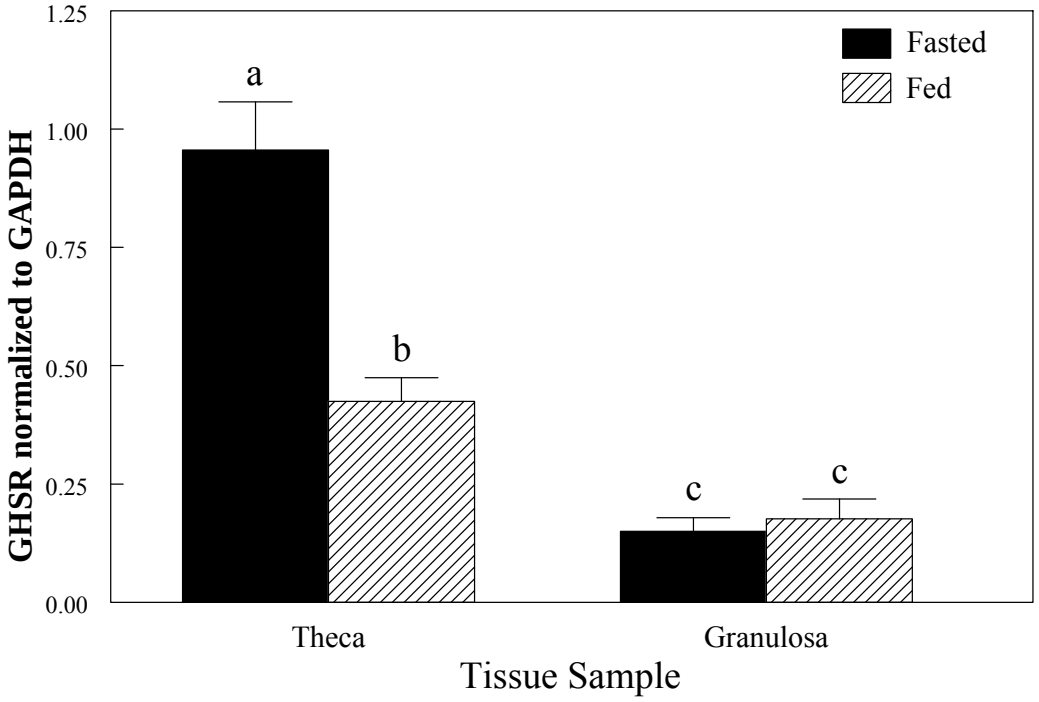

Figure 6.3. The overall ghrelin receptor (GHSR) mRNA expression in theca and granulosa tissue from F_1-F_4, SY, and LW preovulatory follicles of fed and fasted hens. GHSR mRNA expression values were normalized with GAPDH and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 18. Means without a common letter differ, ($P < 0.05$).

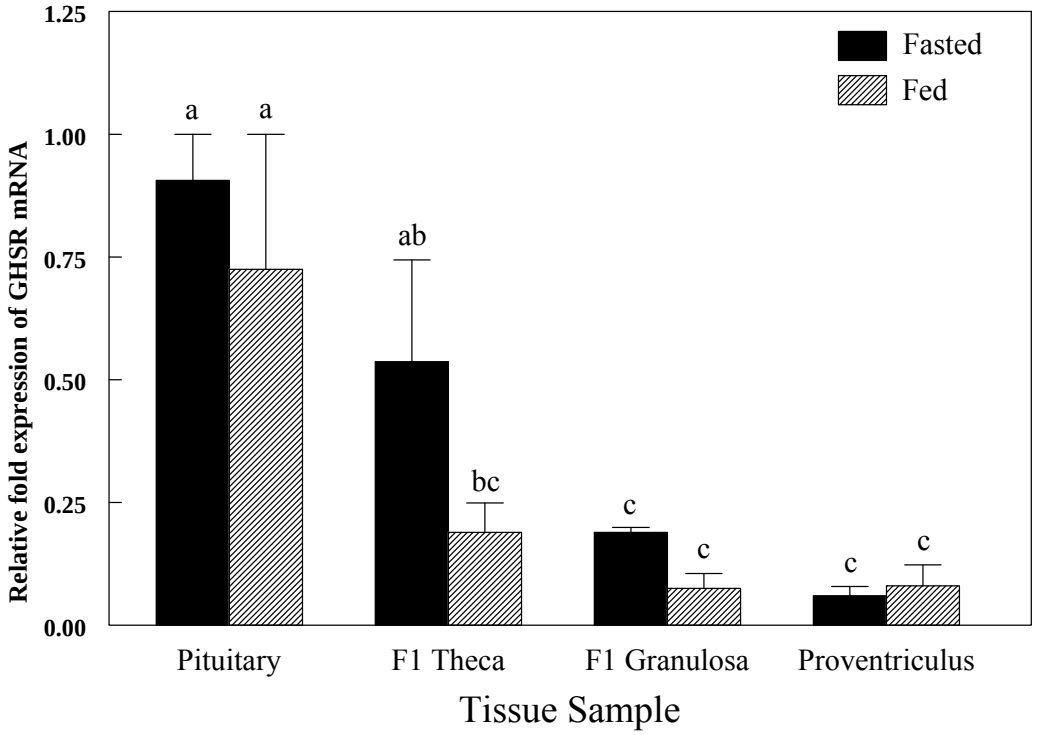

Figure 6.4. GHSR mRNA expression in pituitary, F_1 theca, F_1 granulosa, and glandular proventriculus tissue of fasted and fed hens. GHSR mRNA expression values were normalized with GAPDH and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 3. Means without a common letter differ, ($P < 0.05$).

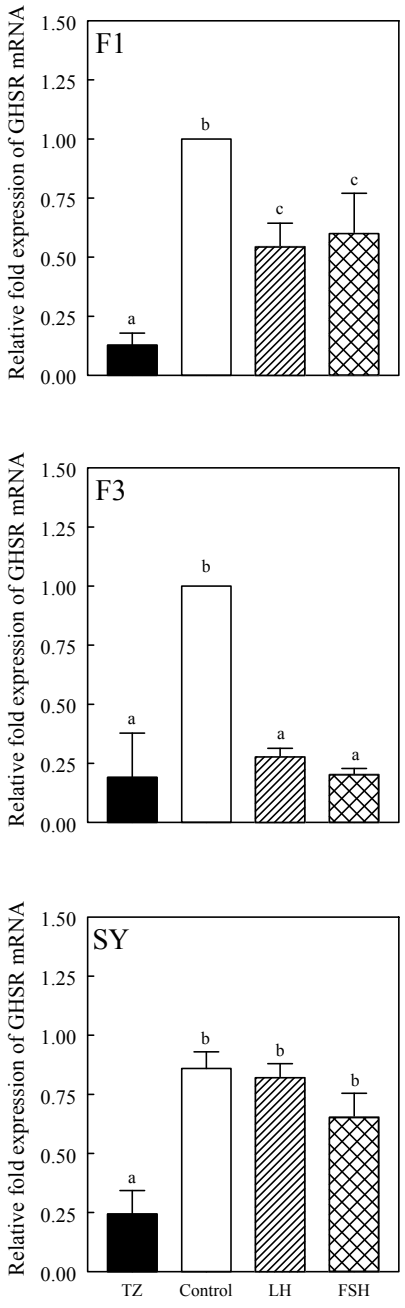

Figure 6.5. The relative fold expression of ghrelin receptor (GHSR) mRNA in granulosa cells from the F1, F3, and small yellow (SY) follicles cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH). GHSR mRNA expression values were normalized by GAPDH and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 5. Means for follicle size with different letters differ, ($P < 0.05$).

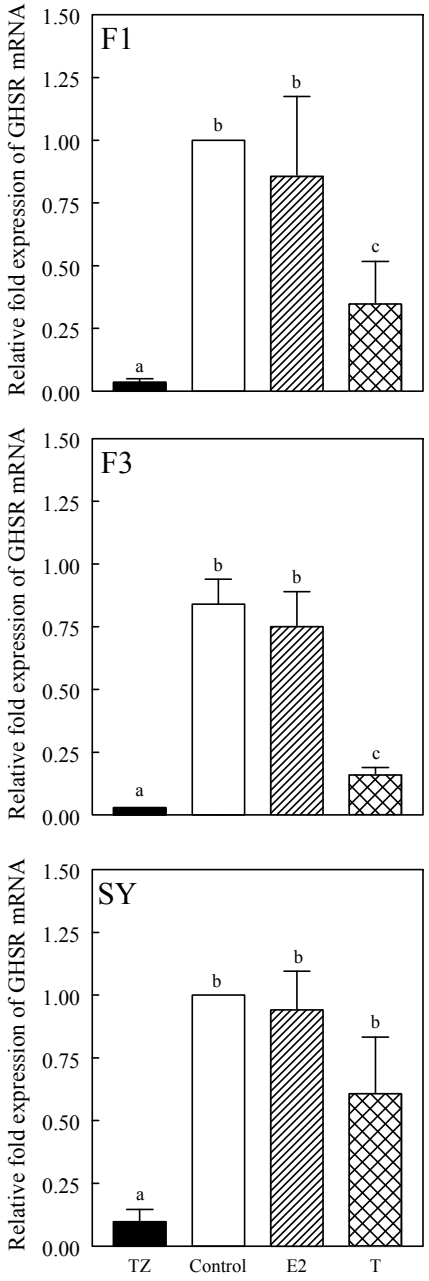

Figure 6.6. The relative fold expression of ghrelin receptor (GHSR) mRNA in granulosa cells from the F1, F3, and small yellow (SY) follicles cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6}M culture media of 17-β-estradiol (E2) or testosterone (T). GHSR mRNA expression values were normalized by GAPDH and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 5. Means for follicle size with different letters differ, ($P < 0.05$).

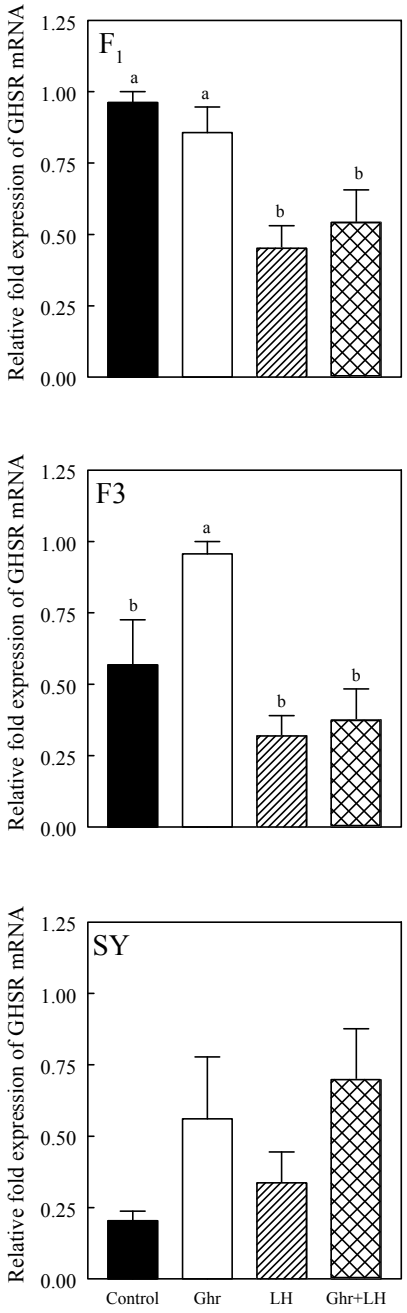

Figure 6.7. The relative fold expression of ghrelin receptor (GHSR) mRNA in granulosa cells from the F1, F3, and small yellow (SY) follicles cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of ghrelin (Ghr), luteinizing hormone (LH) or 50 ng/mL of both ghrelin and LH. GHSR mRNA expression values were normalized by GAPDH and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4. Means for follicle size with different letters differ, ($P < 0.05$).

Chapter 7

Discussion

In the current research, GHSR mRNA expression was detected in both the theca and granulosa cells of hierarchal and nonhierarchical follicles. This finding expands on the report of Sirotkin et al. (2006) in which GHSR was detected in follicular wall fragment samples containing both granulosa and theca cells. Based on mRNA expression of GHSR, the theca layer may be more sensitive to ghrelin than the granulosa layer. Furthermore, this sensitivity to ghrelin maybe enhanced in theca cells during periods of caloric insufficiency, since fasting increased the mRNA expression of GHSR in theca cells. The level of GHSR mRNA expression in the F_1 theca from fasted broiler breeder hens was not different from the expression level found in the pituitary which was previously reported to have the highest level of GHSR mRNA expression among chicken tissues (Geelissen et al. 2003). The increase in GHSR mRNA expression in theca cells from hierarchical and prehierarchical follicle combined with the increase in plasma acylated ghrelin levels in broiler breeder hens during fasting indicates that ghrelin may play a role in linking caloric insufficiency with delaying or terminating preovulatory follicular development in the broiler breeder hen.

Follicular atresia in the hen is initiated by the granulosa cells (Tilly et al. 1991, Johnson et al. 1996) and this is why the current research utilized granulosa cell cultures even though these cells have lower expression levels of GHSR mRNA than theca cells. Although the addition of synthetic chicken acylated ghrelin did not cause cell death or decrease progesterone secretion in

cultured granulosa cells, the results from the granulosa cell culture experiments indicate that GHSR expression is normally inhibited in granulosa cells. GHSR mRNA expression was consistently induced in the untreated granulosa cells isolated from the F_1, F_3 and SY follicles that were cultured for 24 hours compared to freshly dispersed granulosa cells from these follicles. The induction of GHSR mRNA expression during granulosa cell culture may reflect the removal of inhibitory factors which normally prevent the expression of GHSR in vivo. In granulosa cells cultured from the hierarchal F_1 and F_3 follicles, LH, FSH and testosterone all reduced GHSR mRNA expression compared to untreated controls and all 3 of these hormones would be available to play a similar role in vivo in the actively laying broiler breeder hen. The ability of LH to suppress GHSR mRNA expression in granulosa cells from the F_1 and F_3 follicles is particularly interesting because in mammalian species ghrelin is known to inhibit LH production and release (Furata et al. 2001, Fernandez-Fernandez et al. 2004, Vulliemoz et al. 2004, Kluge et al. 2007). Further research is needed to determine if the increase in plasma acylated ghrelin concentrations associated with fasting in broiler breeder hens might lower plasma LH concentrations and thus be partially responsible for the observed increase in GHSR mRNA levels in the theca cells of preovulatory follicles in fasted broiler breeder hens. In granulosa cells from the F_3 follicle the current research indicates that ghrelin can increase the expression of its own receptor and thus ghrelin's role in potentially increasing GHSR mRNA expression in theca cells also needs further investigation.

The current research supports the reports by Shousha et al. (2005) and Kaiya et al. (2007) that fasting increases acylated ghrelin concentrations in avian species. However, the current research is the first to report on acylated ghrelin levels in adult chickens and to measure plasma ghrelin concentrations over an extended period of fasting in the same birds. Richards et al.

(2005) reported that total (UAG + ghrelin) ghrelin levels in broiler chicks did not increase upon fasting but they used the total ghrelin RIA kit that we could not validate in the current research. In their report, (Richards et al. 2005) it is not stated whether they specifically validated the kit for use with broiler plasma and this may explain why their results do not agree with the current and previous (Shousha et al. 2005, Kaiya et al. 2007) research in avian species.

Although the interassay variation and the variability in the obtained and expected values of diluted broiler breeder plasma samples were somewhat high, we feel confident that the Millipore acylated ghrelin kit effectively measured relative differences in plasma acylated ghrelin levels in broiler breeder hens. The acylated ghrelin kit was designed to specifically measure and differentiate between acylated ghrelin, which has the acylated serine residue in position 3 of the ghrelin amino acid sequence, and unacylated ghrelin. The acylated serine in amino acid position 3 as well as the first 7 amino acids of the ghrelin sequence do not vary between mammalian and avian species (Kaiya et al. 2002, Yaun et al. 2007) and this probably explains the success of this kit in measuring acylated ghrelin levels across species. Millipore notes in their protocol for the acylated ghrelin kit that the addition of HCl and PMSF to plasma samples to prevent the degradation of acylated ghrelin may cause precipitate formation in some samples. They recommend centrifugation of the samples to pellet the precipitate before using the plasma. Addition of HCl and PMSF did create a significant amount of precipitate in broiler breeder hen plasma samples and we would note, from our experience, extreme care must be taken to avoid even the slightest contamination of the precipitate if repeatable results with the acylated RIA kit are going to be obtained.

Previous reports on the effect of human recombinant ghrelin 1-18 analogue (contains the first 18 amino acids of the 28 amino acid long human ghrelin peptide) on progesterone secretion

from cultured fragments of follicular wall isolated from chicken hierarchical preovulatory follicles has been varied (Sirotkin et al. 2006, Sirotkin and Grossmann 2007a, Sirotkin and Grossman 2007b). Sirotkin et al. (2006) reported that a dose of 1 ng/mL culture media significantly stimulated progesterone secretion while doses of 10 or 100 ng/mL had no effect on progesterone secretion. Subsequently, Sirotkin and Grossmann (2007b) reported that the addition of 1, 10, and 100 ng/mL human recombinant ghrelin 1-18 analogue had no effect on progesterone secretion. Finally, Sirotkin and Grossman (2007a) reported that follicular wall fragments cultured in 1, 10 and 100 nM human recombinant ghrelin 1-18 analogue secreted significantly more progesterone than untreated control cells but that culturing follicular wall fragments in 100 nM human recombinant ghrelin (1-28 amino acids) and 1-5 analogue decreased progesterone secretion. The current research is the first to examine ghrelin's effect on progesterone secretion using purified granulosa cells from hierarchical and prehierarchical follicles utilizing synthetic chicken ghrelin and the amount of progesterone secreted by granulosa cells into the cell culture media was not affected by the addition of 50 ng/mL ghrelin to the culture media.

In summary, the results indicate that the theca and granulosa cells of the preovulatory follicles of the hen ovary are sensitive to ghrelin and that sensitivity to plasma ghrelin in the theca cells increases with fasting. In addition, the plasma concentration of acylated ghrelin increases in fasted broiler breeder hens. Thus, ghrelin in the broiler breeder hen may serve a pivotal role in regulating the progression of follicular development with the hen's food intake and energy balance. In light of the cell culture results in the current research, which indicate that ghrelin does not appear to cause granulosa cell apoptosis, but that the receptor for ghrelin appears to be down-regulated in the granulosa cells of preovulatory follicles from fed, egg

producing broiler breeder hens, further research is needed to define the reproductive role of ghrelin in the broiler breeder hen.

Chapter 8

References

Adeghate E and Ponery A. 2002. Ghrelin stimulates insulin secretion from the pancreas of normal and diabetic rats. Journal of Neuroendocrinology 14:555-560.

Ahmed S, Harvey S. 2002. Ghrelin: a hypothalamic GH-releasing factor in domestic fowl (*Gallus domesticus*). Journal of Endocrinology 172:117-125.

Altinova A, Toruner F, Akturk M, Elbeg S, Yetkin I, Cakir N, Arslan M. 2006. Reduced serum acylated ghrelin levels in patients with hyperthyroidism. Hormone Research 65:295-299.

Ando R, Kawakami S, Bungo T, Ohgushi A, Takagi T, Denbow D, Furuse M. 2001. Feeding response to several neuropeptide Y receptor agonists in the neonatal chick. European Journal of Pharmacology 427:53-59.

Arafat M, Otto B, Rochlitz H, Tschop M, Bahr V, Mohlig M, Diederich S, Spranger J, Pfeiffer A. 2005. Glucagon inhibits ghrelin secretion in humans. European Journal of Endocrinology 153:397-402.

Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K. 2001. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. The Journal of Clinical Endocrinology and Metabolism 86:4753-4758.

Ariyasu H, Takaya H, Hosoda H, Iwakura H, Ebihara K, Mori K, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. 2002. Delayed short term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. Journal of Endocrinology 143:3341-3350.

Asakawa A, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino M, Kasuga M. 2003. Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. Gut 52:947–952.

Asakawa A, Inui A, Sakamaki R, Shunfuku N, Ueta Y, Meguid M, Kasuga M. 2005. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54:18-24.

Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Grantata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigagli F,

Prat M, Mucciolo G, Ghigo E, Graziani A. 2002.Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and IP#-kinase/AKT. Journal of Cell Biology 159:1029-1037.

Banasch M, Bulut K, Hagemann D, Schrader H, Holst J, Schmidt W, Meier J. 2006. Glucagon-like peptide 2 inhibits ghrelin secretion in humans. Regulatory Peptides 137:173-178.

Barazzoni R, Zanetti M, Stebel M, Biolo G, Cattin L, Guarnieri G. 2003. Hyperleptinemia prevents increased plasma ghrelin concentration during short term caloric restriction in rats. Gastroenterology 124:1188-1192.

Barreiro M, Gaytan F, Caminos J, Pinilla L, Casanueva F, Aguilar E, Dieguez C, Tena-Sempere M. 2002. Cellular localization and hormonal regulation of ghrelin expression in rat testis. Biology of Reproduction 67:1768-1776.

Barreiro M, Suominen J, Gaytan F, Pinilla L, Chopin L, Casanueava F, Dieguez C, Aguilar E, Toppari J, Tena-Sempere M. 2003. Developmental, stage specific, and hormonally regulated expression of growth hormone secretagogue receptor messenger mRNA in rat testis. Biology of Reproduction 68:1631-1640.

Barreiro M, Gaytan F, Castellano J, Suominen J, Roa J, Gaytan M, Aguilar E, Dieguez C, Toppari J, Tena-Sempere M. 2004. Ghrelin inhibits the proliferative activity of immature leydig cells in vivo and regulates stem cell factor messenger ribonucleic acid expression in rat testes. Endocrinology 145:4825-4834.

Barreiro M, Tena-Sempere M. 2004. Ghrelin and reproduction: a novel sign linking energy status and fertility. Molecular and Cellular Endocrinology 226:1-9.

Barkan A, Dimaraki E, Jessup S, Symons K, Ermolenko M, Jaffe C. 2003. Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affect by the ambient growth hormone levels. The Journal of Clinical Endocrinology and Metabolism 88:2180-2184.

Baudet M, Harvey S. 2003. Ghrelin-induced GH secretion in the domestic fowl in vivo and in vitro. Journal of Endocrinology 179:97-105.

Brake J, Thaxton P. 1979. Physiological changes in caged layers during a forced molt. 2. Gross changes in organs. Poultry Science 58:707-716.

Briatore L, Andraghetti G, Cordera R. 2003. Acute plasma glucose increase, but not early insulin response, regulates plasma ghrelin. European Journal of Endocrinology 149: 403-406.

Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely A, Deghenghi R, Ghigo E. 2001. Ghrelin, a natural GH secretagogue produced by the stomach, induces insulin secretion in humans. The Journal of Clinical Endocrinology and Metabolism 86:5083-5086.

Broglio F, Gottero C, Benso A, Prodam F, Destefanis S, Gauna C, Maccario M, Deghenghi R, van der Lely A, Ghigo E. 2003. Effect of ghrelin on the insulin and glycemic response to glucose, arginine, or free fatty acids load in humans. The Journal of Clinical Endocrinology and Metabolism 88:4268-4272.

Broglio F, Gottero C, Prodam F, Destefanis S, Gauna C, Me E, Riganti F, Vivenza D, Rapa A, Martina V, Arvat E, Bona G, van der Lely A, Ghigo E. 2004. Ghrelin secretion is inhibited by glucose load and insulin induced hypoglycaemia but unaffected by glucagon and arginine in humans. Clinical Endocrinology 61:503-509.

Bruggeman V, Onagbesan O, D'Hondt E, Buys N, Safi M, Vanmontfort D, Berghman L, Vandesande F, Decuypere E. 1999. Effects of timing and duration of feed restriction during rearing on reproductive characteristics in broiler breeder females. Poultry Science. 78: 1424-1434.

Bruggeman V, Onagbesan O, Ragot O, Metayer S, Cassy S, Favreau F, Jego Y, Trevidy J, Tona K, Williams J, Decuypere E, Picard M. 2005. Feed allowance- genotype interactions in broiler breeder hens. Poultry Science. 84: 298-306.

Camina J. 2006. Cell biology of the ghrelin receptor. Journal of Neuroendocrinology 18:65-76.

Calvo F and Bahr, J. 1983. Adenylyl cyclase system of the small preovulatory follicles of the domestic hen: responsiveness to follicle-stimulating hormone and luteinizing hormone. Biology of Reproduction 29:542-547.

Caminos J, Seoane L, Tovar S, Casanueva F, Dieguez. 2002. Influence of thyroid status and growth hormone deficiency on ghrelin. European Journal of Endocrinology 147:159-163.

Caminos J, Tena-Sempere M, Gaytan F, Sanchez-Criado J, Barreiro M, Nogueras R, Casanueva F, Aguilar E, Dieguez C. 2003. Expression of ghrelin in the cyclic and pregnant rat ovary. Endocrinology 144:1594-1602.

Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, Deghenghi R, Reissmann T, Ghigo E, Muccioli G. 2001. Identification, characterization and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. Journal of Clinical Endocrinology and Metabolism 86:1738-1745.

Caixas A, Bashore C, Nash W, Pi-Sunyer F, Lafferere B. 2002. Insulin, unlike food intake, does not suppress ghrelin in human subjects. Journal of Endocrinology and Metabolism 87:1902-1906.

Chan J, Bullen J, Lee J, Yiannakouris N, Mantzoros C. 2004. Ghrelin levels are not regulated by recombinant leptin administration and/or three days of fasting in healthy subjects. Journal of Clinical Endocrinology and Metabolism 89: 335-343.

Chen H, Trumnauer M, Chen A, Weingarth D, Adams J, Frazier E, Shen Z, Marsh D, Feighner S, Guan X, Ye Z, Nargund R, Smith R, van der Ploeg L, Howard A, Macneil D, Qian S. 2004. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. Endocrinology 145:2607-2612.

Choi K, Roh S, Hong Y, Shrestha Y, Hishikawa D, Chen C, Kojima M, Kangawa K, Sasaki S. 2003. The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. Endocrinology 144:754-759.

Chomczynski P, Sacchi N. 1987. Single step method of RNA isolation by acid guanidinium thiocynate-phenol-choloform extraction. Analytical Biochemistry 162:156-159.

Cobb 500 slow feather breeder management supplement. 2005a. Cobb-Vantress Inc. Siloam Springs, Arkansas.

Cobb breeder management guide. 2005b. Cobb-Vantress Inc. Siloam Springs, Arkansas.

Date Y, Kojima M, Sawaguchi A, Mondal M, Suganuma T, Matsukura K, Nakazato M. 2000. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141:4255-4261.

Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal M, Hosdoa H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S. 2002. Ghrelin is present in pancreatic □-cells of humans and rats and stimulates insulin secretion. Diabetes 51:124-129.

Davis A, Johnson P. 1998. Expression pattern of messenger ribonucleic acid for follistatin and the inhibin/activin subunits during follicular and testicular development in Gallus domesticus. Biology of Reproduction 59:271-277.

Davis A, Brooks C, Johnson P. 2000. Estradiol regulation of follistatin and inhibin α- and β_B-subunits in avian granulosa cells. General and Comparative Endocrinology 119:308-316.

Davis A, Brooks C, Johnson P. 2001. Activin A and gonadotropins regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNA in avian granulosa cells. Biology of Reproduction 65:1352-1358.

Denbow D, Snapir N, Furuse M. 1999. Inhibition of food intake by CRF in chickens. Physiology and Behavior 66:645-649.

Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K, Yada T. 2004. Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca^{2+} signaling in β-cells. Diabetes 53:3142-3155.

Etches R, Cheng K. 1981. Changes in the plasma-concentrations of luteinizing-hormone, progesterone, estradiol and testosterone and in the binding of follicle-stimulating-hormone to the theca of follicles during the ovulation cycle of the hen (*Gallus domestus*). Journal of Endocrinology 91:11-22.

Etches, R. J. (1996). "Reproduction in Poultry." CAB International, Oxon, UK.

Egido E, Rodriguez-Gallardo J, Silvestre R, Marco J. 2002. Inhibitory effect of ghrelin on insulin and pancreatic somatostatin secretion. European Journal of Endocrinology 146:241-244.

English P, Ghatei M, Malik I, Bloom S, Wilding J. 2002. Food fails to suppress ghrelin levels in obese humans. Journal of Clinical Endocrinology and Metabolism 87:2984:2987.

Erdmann J, Lippl F, Wagenpfeil S, Schusdziarra V. 2005 Differential association of basal and postprandial plasma ghrelin with leptin, insulin, and type 2 diabetes. Diabetes 54:1371-1378.

Fernandez-Fernandez R, Tena-Sempere M, Aguilar E, Pinella L. 2004. Ghrelin effects on gonadotropin secretion in male and female rats. Neuroscience Letters 362:103-107.

Fernandez-Fernandez R, Navarro V, Barreiro M, Vigo E, Tovar S, Sirotkin A, Casanueva F, Aguilar E, Dieguez C, Pinella L, Tena-Sempere M. 2005a. Effect of chronic hyperghrelinemia on puberty onset and pregnancy outcome in the rat. Endocrinology 146:3018-3025.

Fernandez-Fernandez R, Tena-Sempere M, Navarro V, Barreiro M, Castellano J, Aguilar E, Pinella L. 2005b. Effects of ghrelin upon gonadotropin-releasing hormone and gonadotropin secretion in adult female rats: In vivo and in vitro studies. Neuroendocrinology 82:254-255.

Flanagan D, Evans M, Monsod T, Rife F, Heptulla R, Tamborlane W, Sherwin R. 2003. The influence of insulin on circulating ghrelin. American Journal of Physiology Endocrinology and Metabolism 284:E313-E316.

Furata M, Funabashi T, Kimura F. 2001. Intracerebroventricular administration of ghrelin rapidly suppresses pulsatile luteinizing hormone secretion in ovariectomized rats. Biochemical and Biophysical Communications 288:780-785.

Furuse M, Matsumoto M, Saito N, Sugahara K, Hasegawa S. 1997. The central corticotropin-releasing factor and glucagon-like peptide1 in food intake in the neonatal chick. European Journal of Pharmacology 339:211-213.

Furuse M, Tachibana T, Ohgushi A, Ando R, Yashimatsu T, Denbow D. 2001. Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neuroscience Letters 301:123-126.

Gauna C, Delhanty P, Hofland L, Janssen J, Broglio F, Ross R, Ghigo E, van der Lely A. 2005. Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes. The Journal of Clinical Endocrinology and Metabolism 90:1055-1060.

Gaytan F, Barreiro M, Chopin L, Herington A, Morales C, Pinilla L, Casanueva F, Aguilar E, Dieguez C, Tena-Sempere M. 2003. Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. The Journal of Clinical Endocrinology and Metabolism 88:879-887.

Gaytan F, Barreiro M, Caminos J, Chopin L, Herington A, Moreales C, Pinella L, Paniagua R, Nistal M, Casanueva F, Aguilar E, Dieguez C, Tena-Sempere M. 2004. Expression of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in normal human testis and testicular tumors. The Journal of Clinical Endocrinology and Metabolism 89:400-409.

Geelissen S, Beck I, Darras V, Kuhn E, van der Geyten S. 2003. Distribution and regulation of chicken growth hormone segretagogue receptor isoforms. General and Comparative Endocrinology 134:167-174.

Geelissen S, Swennen Q, Van der Geytan S, Kuhn E, Kaiya H, Kangawa K, Decuypere E, Buyse J, Darras. 2006. Peripheral ghrelin reduces food intake and respiratory intake in chicken. Domestic Animal Endocrinology 30:108-116.

Gelling R, Overdun J, Morrison C, Morton G, Frayo S, Cummings D, Schwartz M. 2004. Effect of uncontrolled diabetes on plasma ghrelin concentrations and ghrelin-induced feeding. Endocrinology 145:4575-4582.

Gibson C. 2006. Influence of feeding methods during early lay in broiler breeder hens. Master's thesis 1-59. University of Georgia, Athens GA.

Gilbert A, Perry M, Waddington D, Hardie M. 1983. Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (*Gallus domesticus*). Journal of Reproduction and Fertility 69:221-227.

Gimenez-Palop O, Gimenez-Perez G, Maurico D, Berlanga E, Potau N, Vilardell C, Arroyo J, Gonzalez-Clemente J, Ciazas A. 2005. Circulating ghrelin in thyroid dysfunction is related to insulin resistance and not to hunger, food intake or anthropomorphic changes. European Journal of Endocrinology 153:73-79.

Giovamattista A, Pieraria J, Suescun M, Calacra R, Gaillard R, Spinedi E. 2006. Direct effect of ghrelin on leptin production by cultured rat white adipocytes. Obesity 14:19-27.

Gnanapavan S, Kola B, Bustin A, Morris D, McGee P, Fairclough P, Bhattacharya S, Carpender R, Grossman A, Korbonits M. 2002. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. The Journal of Clinical Endocrinology and Metabolism 87:2988-2991.

Gomez G, Englander E, Greeley G. 2004. Nutrient inhibition of ghrelin secretion in the fasted rat. Regulatory Peptides 117:33-36.

Goto M, Arima H, Watanabe M, Hayashi H, Banno R, Sato I, Nagasaki H, Oiso Y. 2006. Ghrelin increases neuropeptide Y and agouti-related peptide gene expression in the arcuate nucleus in rat hypothalamic organotype cultures. Endocrinology 147:5102-5109.

Gourcerol G, Million M, Adelson D, Wang Y, Wang L, Rivier J, St-Pierre H, Tache Y. 2006. Lack of interaction between peripheral injection of CCK and obestatin in regulation of gastic satiety signaling in rodents. Peptides 27:2811-2819.

Grinspoon S, Miller K, Herzog D, Grieco K, Klibanski A. 2004. Effects of estrogen and recombinant human insulin-like growth factor-I on ghrelin secretion in severe undernutrition. The Journal of Clinical Endocrinology and Metabolism 89:3988-3993.

Greenman Y, Golani N, Gilad S, Yaron M, Limor R, Stern N. 2004. Ghrelin secretion in modulated in a nutrient- and gender-specific manner. Clinical Endocrinology 60:382-388.

Gualillo O, Caminos J, Blancos M, Garcia-Caballero T, Kojima M, Kangawa K, Dieguez C, Casanueva F. 2001a. Ghrelin, a novel derived placental hormone. Endocrinology 142:788-794.

Gualillo O, Caminos J, Kojima M, Kangawa K, Arvat E, Ghigo E, Casanueva F, Dieguez C. 2001b. Gender and gonadal influences on ghrelin mRNA levels in the rat stomach. European Journal of Endocrinology 144:687-690.

Gualillo O., Lago F., Gomez-Reino J., Casanueva F., Dieguez C. 2003. Ghrelin, a widespread hormone: insights into molecular and cellular regulation of its expression and mechanisms of action. FEBS Letters 552:105-109.

Gualillo O, Lago F, Casanueva F, Dieguez C. 2006. One ancestor, several peptides: Post-translational modifications of proghrelin generate several peptides with antithectical effects. Molecular and Cellular Endocrinology 256:1-8.

Guan X, Yu H, Palyha O, McKee K, Feighner S, Sirinathsinghji D, Smith R, Van der Ploeg L, Howard A. 1997. Distribution of mRNA encoding growth hormone secretagogue receptor in the brain and in peripheral tissues. Brain Research: Molecular Brain Research 48:23-29.

Hakovirta H, Yan W, Kaleva M, Zhang F, Vanttinen K, Morris P, Soder M, Parvinen M, Toppari J. 1999. Function of stem cell factor as a survival factor of spermatogonia and localization of messenger ribonucleic acid in the rat seminiferous epithelium. Endocrinology 140:1492-1498.

Hansen T, Dall R, Hosoda H, Kojima M, Kangawa K, Christiansen J, Jorgenson J. 2002. Weight loss increases circulating levels of ghrelin in human obesity. Clinical Endocrinology 56:2003-2006.

Hataya Y, Akamizu T, Takaya K, Ariyasu N, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K, Nakao K. 2001. A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. The Journal of Clinical Endocrinology and Metabolism 86:4552-4555.

Hayashida T, Nakahara K, Mondal M, Date Y, Nakazato M, Kojima M, Kangawa K, Murakami N. 2002. Ghrelin in neonatal rats: distribution in stomach and its possible roles. Journal of Endocrinology 173:239-245.

Heck A, Onagbesan O, Tona K, Metayer S, Putterflam J, Jego Y, Trevidy J, Decuypere E, Williams J, Picard M, Bruggeman V. 2004. Effects of *ad libitum* feeding on performance of different strains of broiler breeders. British Poultry Science. 45:695-703.

Hirsh D, Heinrichs C, Leenders B, Wong A, Cummings D, Chanoine J. 2005. Ghrelin is suppressed by glucagon and does not mediate glucagon related growth hormone release. Hormone Research 63:111-118.

Hosoda H, Kojima M, Matsuo H, Kangawa K. 2000. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochemical and Biophysical Research Communications 279:909-913.

Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. 2003. Structural divergence of human ghrelin. The Journal of Biological Chemistry 278:64-70.

Hosoda H, Doi K, Nagaya N, Okumura H, Nakagawa E, Enomoto M, Ono F, Kangawa K. 2004. Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. Clinical Chemistry 50:1077-1080.

Howard A, Feighner S, Cully D, Arena J, Liberator P, Rosenblum C, Hamelin M, Hreniuk D, Palyha O, Anderson J, Paress P, Diaz C, Chou M, Lui K, McKee K, Pong S, Chaung L, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsingji D, Dean D, Melillo D, Patchett A, Nargund R, Griffin P, DeMartino J, Gupta S, Schaeffer J, Smith R, Van der Ploeg L. 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974-977.

Huang E and NalbandovA. 1979. Steroidogenesis of chicken granulosa and theca cells: *in vitro* incubation system. Biology of Reproduction 20:442-453.

Iqbal J, Kurose Y, Canny B, Clarke I. 2006. Effect of central infusion of ghrelin on food intake and plasma levels of growth hormone, luteinizing hormone, prolactin, and cortisol secretion in sheep. Endocrinology 147:510-519.

Johnson A, Bridgham J, Witty J, Tilly J. 1996. Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene expression. Endocrinology 137:2059-2066.

Johnson A, Bridgham J, Jensen T. 1999. Bcl-XLONG protein expression and phosphorylation in granulosa cells. Endocrinology 140:4521-4529.

Johnson A, Bridgham J. 2001. Regulation of steroidogenic acute regulatory protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. Endocrinology 142:3116-3124.

Johnson A, Bridgham J, Woods D. 2004. Cellular mechanisms and modulation of activin A- and transforming growth factor □-mediated differentiation in cultured hen granulosa cells. Biology of Reproduction 71:1844-1851.

Kaiya H, van der Geyten S, Kojima M, Hosoda H, Kitajima Y, Matsumoto M, Geelissen S, Darras V, Kangawa K. 2002. Chicken ghrelin: purification, cDNA cloning, and biological activity. Endocrinology 143:3454-3463.

Kaiya H, Saito E, Tachibana T, Furuse M, Kangawa K. 2007. Changes in ghrelin levels of plasma and proventriculus and ghrelin mRNA in fasted and refed layer chicks. Domestic Animal Endocrinology 32:247-259.

Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. 2000. Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. Endocrinology 141:4797-4800.

Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. 2001. Chronic infusion of ghrelin increases hypothalamic neuropeptide Y and agouti-related protein mRNA levels and body weight in rats. Diabetes 50:2438-2443.

Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Oikawa S. 2004. Effects of insulin, leptin and glucagon on ghrelin secretion from isolated perfuse rat stomach. Regulatory Peptides 119:77-81.

Katayama T, Shimamoto S, Oda H, Nakahara K, Kangawa K, Murakami N. 2007. Glucagon receptor expression and glucagon stimulation of ghrelin secretion in the rat stomach. Biochemical and Biophysical Research Communications 357:865-870.

Kato M, Shimada K, Saito N, Noda K, Ohta M. 1995. Expression of P450 17 alpha-hydroxylase and P450 aromatase genes in isolated granulosa, theca interna, and theca externa layers of chicken ovarian follicles during follicular growth. Biology of Reproduction 52:405-410.

Kawamura K, Sato N, Fukuda J, Kodama H, Kumagai J, Tanikawa H, Nakamura A, Honda Y, Sato T, Tanaka T. 2003. Ghrelin inhibits the development of mouse preimplantation embryos in vitro. Endocrinology 144:2623-2633.

Katsuki A, Urakawa H, Gabazza E, Murashima S, Nakatani K, Togashi K, Yano Y, Adachi Y, Sumida Y. 2004. Circulating levels of active ghrelin is associated with abdominal adiposity, hyperinsulinemia and insulin resistance in patients with type 2 diabetes mellitus. European Journal of Endocrinology 151:573-577.

Kellokoski E, Poykko S, Karjalainen A, Ukkola O, Heikkinen J, Kesaniemi Y, Horkko S. 2005. Estrogen replacement therapy increases plasma ghrelin levels. The Journal of Clinical Endocrinology and Metabolism 90:2954-2963.

Kim M, Yoon C, Park K, Shin C, Park K, Kim S, Cho B, Lee H. 2003. Changes in ghrelin and ghrelin receptor expression according to feeding status. Neuroendocrinology 14:1317-1320.

Kluge M, Schusslet P, Uhr M, Yassouridis A, Steiger A. 2007. Ghrelin suppresses secretion of luteinizing hormone in humans. The Journal of Clinical Endocrinology and Metabolism 92:3202-3205.

Kohno D, Nakata M, Maekawa F, Fujiwara K, Maejima Y, Kuramochi M, Shimazaki T, Okana H, Onaka T, Yada T. 2007. Leptin suppresses ghrelin-induced activation of neuropeptide Y neurons in the arcuate nucleus via phosphatidylinositol 3-kinase- and phosphodiesterase 3-mediated pathway. Endocrinology 148:2251-2263.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. 1999. Ghrelin is a growth hormone releasing acylated peptide from stomach. Nature 402:656-660.

Kojima M, Hosoda H, Kangawa K. 2001. Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. Hormone Research 56 (supplement 1):93-97.

Kojima M, Kangawa K. 2005. Ghrelin: structure and function. Physiological Reviews 25:426-457.

Korbonits M, Bustin S, Kojima M, Jordan S, Adams E, Lowe D, Kangawa K, Grossman A. 2001. The expression of the growth hormone secretagogue receptor ligand in normal and abnormal human pituitary and other neuroendocrine tumors. The Journal of Clinical Endocrinology and Metabolism 86:881-887.

Kurose Y, Iqbal J, Rao A, Murata Y, Hasegawa Y, Terashime Y, Kojima M, Kangawa K, Clarke I. 2005. Changes in expression of the genes for the leptin receptor and the growth hormone-releasing peptide/ghrelin receptor in the hypothalamic arcuate nucleus with long term manipulation of adiposity by dietary means. Journal of Neuroendocrinology 17:331-340.

Lagaud G, Young A, Acena A, Morton M, Barrett T, Shankley N. 2007. Obestatin reduces food intake and suppresses body weight gain in rodents. Biochemical and Biophysical Research Communications 357:264-269.

Lauwers E, Landuyt B, Arckens L, Schoofs L, Luytan W. 2006. Obestatin does not activate orphan G protein-coupled receptor GPC39. Biochemical and Biophysical Research Communications 351:21-25.

Lebenthal Y, Gat-Yablonski G, Shtaif B, Padoa A, Phillip M, Lazar L. 2006. Effect of sex hormone administration on circulating ghrelin levels in peripubertal children. The Journal of Clinical Endocrinology and Metabolism 91:328-331.

Lebrethon M, Aganina A, Fournier M, Gerard A, Parent A, Bourguignon J. 2006. Effect of in vivo and in vitro administration of ghrelin, leptin and neuropeptide mediators on the pulsatile gonadotrophin-releasing hormone secretion from the male rat hypothalamus before and after puberty. Journal of Neuroendocrinology 19:181-188.

Lee H, Bahr J. 1989. Inhibitory sites of androgens and estradiol in progesterone biosynthesis in granulosa cells of the domestic hen. Endocrinology 125:760-765.

Lee H, Wang G, Englander E, Kojima M, Greeley G. 2002. Ghrelin, a new gastrointestinal peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. Endocrinology 143:185-190.

Li Z, Johnson A. 1993. Regulation of P450 cholesterol side-chain cleavage messenger ribonucleic acid expression and progesterone production in hen granulosa cells. Biology of Reproduction 49:463-469.

Lippl F, Kircher F, Erdmann J, Allescher H, Schusdziarra V. 2004. Effects of GIP, GPL-1, insulin and gastrin in the isolated rat stomach. Regulatory Peptides 119:93-98.

Livak K, Schmittgen T. 2001. Analysis of relative gene expression data using real-time quantative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402-408.

Lui Y, Yakar S, Otero-Corchon V, Low M, Liu J. 2002. Ghrelin gene expression is age-dependant and influenced by gender and the level of circulating IFG-I. Molecular and Cellular Endocrinology 189:97-103.

Luque R, Kineman R, Park S, Peng X, Gracia-Navarro F, Castana J, Malagon M. 2004. Homologous and heterologous regulation of pituitary receptors for ghrelin and growth hormone releasing hormone. Endocrinology 145:3182-3189.

Martini A, Fernandez- Fernandez R, Tovar S, Navarro V, Vigo E, Vazquez M, Davies J, Thompson N, Aguilar E, Pinella L, Wells T, Dieguez C, Tena-Sempere M. 2006. Comparative analysis of the effects of ghrelin and unacylated ghrelin on luteinizing hormone secretion in male rats. Endocrinology 147:2374-2382.

Matsubara M, Sakata I, Wada R, Yamazaki M, Inoue K, Sakai T. 2004. Estrogen modulates ghrelin expression in the female rat stomach. Peptides 25:289-297.

Matsumoto M, Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, Matsuo H, Kojima M, Hayashi Y, Kangawa K. 2001. Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochemical and Biophysical Research Communications 287:142-146.

Monteleone P, Bencivenga R, Longobardi N, Serritella C, Maj M. 2003. Differential responses of circulating ghrelin to a high-fat or high-carbohydrate meal in healthy women. The Journal of Clinical Endocrinology and Metabolism 88:5510-5514.

Morris T, Nalbandov A. 1961. Induction of ovulation in starving pullets using mammalian and avian gonadotropins. Endocrinology 68:687-697.

Moyle W, Ramachandran J. 1973. Effect of LH on steriodogenesis and cyclic AMP accumulation in rat Laydig cell preparations and mouse tumor Leydig cells. Endocrinology 93:137-134.

Muccioli G, Tschop M, Papotti M, Deghenghi R, Heiman M, Ghigo E. 2002. Neuroendocrinology and peripheral activities of ghrelin: implications in metabolism and obesity. European Journal of Pharmacology 440:235-254.

Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal M, Nakazato M, Kojima M, Kangawa K. 2002. Role for central ghrelin on food intake and secretion profile of stomach ghrelin in rats. Journal of Endocrinology 174:283-288.

Murdolo G, Lucidi P, Loreto C, Parlanti N, De Cicco A, Fatone C, Fanelli C, Bolli G, Santeusanio F, De Feo P. 2003. Insulin is required for prandial ghrelin suppression in humans. Diabetes 52:2923-2927.

Nakahara K, Nakagawa M, Baba Y, Sato M, Toshnai K, Date Y, Nakazato M, Kojima M, Miyazato M, Kaiya H, Hosoda H, Kangawa K, Murakami N. 2006. Maternal ghrelin plays an important role in rat fetal development during pregnancy. Endocrinology 147:1333-1342.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa S. 2001. A role for ghrelin in the central regulation of feeding. Nature 409:194-198.

Neter, J., W. Wassermann, and M. H. Kutner. 1990. Pages 519-561 in Applied Linear Statistical Models. 3rd ed. Richard D. Irwin Inc., Boston, MA.

Nogueiras R, Tovar S, Mitchell S, Rayner D, Archer Z, Dieguez C, Williams L. 2004. Regulation of growth hormone secretagogue receptor gene expression in the arcuate nuclei of the rat by leptin and ghrelin. Diabetes 53:2552-2558.

Ohkubo T, Tanaka M, Nakashima K. 2000. Structure and tissue distribution of chicken leptin receptor (cOb-R) mRNA. Biochimica et Biophysica Acta. 1491:303-308.

Onagbesan O, Metayer S, Tona K, Williams J, Decuypere E, Bruggeman V. 2006. Effect of genotype and feed allowance on plasma luteinizing hormones, follicle-stimulating hormones, progesterone, estradiol levels, follicle differentiation, and egg production rates of broiler breeder hens. Poultry Science. 85:1245-1258.

Otto B, Cunzt U, Fruehauf E, Wawarta R, Folwaczny C, Riepl R, Heiman M, Lehnert P, Fichter M, Tschop M. 2001. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. European Journal of Endocrinology 145:669-673.

Pagotto U, Gambineri A, Pelusi C, Genghini S, Cacciari M, Otto B, Castaneda T, Tschop M, Pasquali R. 2003. Testosterone replacement therapy restores normal ghrelin in hypogonadal men. The Journal of Clinical Endocrinology and Metabolism 88:4139-4143.

Palmer S, Bahr J. 1992. Follicle-stimulating-hormone increases serum oestradiol-17-beta concentrations, number of growing follicles and yolk deposition in aging hen (*Gallus gallus domesticus*) with decreased egg production. British Poultry Science 33:403-414.

Peino R, Baldelli R, Rodriguez-Garcia J, Rodriguez-Segade S, Kojima M, Kangawa K, Arvat E, Ghigo E, Dieguez C, Casanueva F. 2000. Ghrelin induced growth hormone secretion in humans. European Journal of Endocrinology 143:R11-R14.

Petersenn S. 2002. Growth hormone secretagogues and ghrelin: an update of physiology and clinical relevance. Hormone Research 58 (suppliment 3):56-61.

Purnell J, Weigle D, Breen P, Cummings D. 2003. Ghrelin levels correlate with insulin levels, insulin resistance, and high-density lipoprotein cholesterol, but not with gender, menopausal status, or cortisol levels in humans. The Journal of Clinical Endocrinology and Metabolism 88:5747-5752.

Qader S, Salehi A, Håkanson R, Lundquist I, Ekelund M. 2005. Long-term infusion of nutrients suppresses circulating ghrelin in food-deprived rats. Regulatory Peptides 131:82-88.

Renema R, Robinson F, Proudman J, Newcombe M, McKay R. 1999. Effects of body weight and feed allocation during sexual maturation in broiler breeder hens. 2. Ovarian morphology and plasma hormone profiles. Poultry Science. 78:629-639.

Richards M, Poch S, Coon C, Rosebrough R, Ashwell C, McMurty J. 2003. Feed restriction significantly alters lipogenic gene expression in broiler breeder chickens. Journal of Nutrition. 133:707-715.

Richards M, Poch S, McMurtry J. 2005. Characterization of turkey and chicken ghrelin genes, and regulation of ghrelin and ghrelin receptor mRNA levels in the broiler chicken. General and Comparative Endocrinology 145:298-310.

Riis A, Hansen T, Moller N, Weeke J, Jorgensen J. 2003. Hyperthyroidism is associated with suppressed circulating levels of ghrelin. The Journal of Clinical Endocrinology and Metabolism 88:853-857.

Robbins K, McGhee G, Osei P, Beauchene R. 1986. Effect of feed restriction on growth, body composition, and egg production of broiler females through 68 weeks of age. Poultry Science. 65:2226-2231.

Robinson F, Etches R, Anderson-Langmuir C, Burke W, Cheng K, Cunningham F, Ishii S, Sharp P, Talbot R. 1988. Steroidogenic relationships of gonadotropins hormones in the ovary of the hen. General and Comparative Endocrinology 69:455-466.

Salehi A, de la Cour C, Hakanson R, Lundquist I. 2004. Effects of ghrelin on insulin and glucagon secretion: a study of isolated pancreatic islets and intact mice. Regulatory Peptides 118:143-150.

Saito E, Kaiya H, Takagi T, Yamasaki I, Denbow D, Kangawa K, Furuse M. 2002. Chicken ghrelin and growth-hormone releasing peptide-2 inhibit food intake of neonatal chicks. European Journal of Pharmacology 453:75-79.

Saito E, Kaiya H, Tachibana T, Tomonaga S, Denbow D, Kangawa K, Furuse M. 2005. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regulatory Peptides 125:201-2008.

Sanchez J, Oliver P, Palou A, Pico C. 2004. The inhibition of gastric ghrelin production by food intake in rats is dependent on the type of macronutrient. Endocrinology 145:5049-5055.

Scanes, C. G., S. Harvey, and A. Chadwick. 1976. Plasma luteinizing hormone and follicle stimulating hormone concentration in fasted immature male chickens. IRCS medical science: Clinical pharmacology and therapeutics 4: 371.

Schaller G, Schmidt A, Pleiner J, Woloszczuk W, Woltz M, Luger A. 2003. Plasma ghrelin concentrations are not regulated by glucose or insulin. Diabetes 52:16-20.

Senior B, Furr B. 1975. Preliminary assessment of source of estrogen within the ovary of domestic fowl, Gallus domesticus. Journal of Reproduction and Fertility 43:241-247.

Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal M, Tanaka M, Nozie S, Hosoda H, Kangawa K, Matsukura S. 2002. Plasma ghrelin levels in lean and obese humans and the effects of glucose on ghrelin secretion. The Journal of Clinical Endocrinology and Metabolism 87:240-244.

Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K. 2001. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227-232.

Shousha S, Nakahara K, Kojima M, Miyazato M, Hosoda H, Kangawa K, Murakami N. 2005. Different effect of peripheral and central ghrelin on regulation of food intake in the Japanese quail. General and Comparative Endocrinology 141:178-183.

Sirotkin A, Grossman R, Maria-Peon M, Roa J, Tena-Sempere M, Klein S. 2006. Novel expression and functional role of ghrelin in the chicken ovary. Molecular and Cellular Endocrinology 257-258:15-25.

Sirotkin A, Grossman R. 2007a. Effects of ghrelin and its analogs on chicken ovarian granulosa cells. Domestic Animal Endocrinology. In Press.

Sirotkin A, Grossman R. 2007b. The role of ghrelin and some intracellular mechanisms in controlling the secretory activity of chicken ovarian cells. Comparative Biochemistry and Physiology 147:239-246.

Spradley J. 2007. Influence of twice a day feeding regimen after photostimulation on the reproductive performance of broiler breeder hens. Master's thesis 1-94. University of Georgia, Athens GA.

Sun Y, Ahmed S, Smith R. 2003. Deletion of ghrelin impairs neither growth or appetite. Molecular and Cellular Biology 23:7973-7981.

Sun Y, Garcia J, Smith R. 2007. Ghrelin and growth hormone secretagogue receptor expression in mice during aging. Endocrinology 148:1323-1329.

Tanaka M, Naruo T, Muranaga T, Yasuhara D, Shiiya T, Nakazato M, Matsukura S, Nozoe S. 2002. Increased fasting ghrelin levels in patients with bulemia nervosa. European Journal of Endocrinology 146:R1-R3.

Tanaka M, Miyazaki T, Yamamota I, Nakai N, Ohta Y, Tsushima N, Wakita M, Shimada K. 2003. Molecular characterization of chicken growth hormone secretagogue receptor gene. General and Comparative Endocrinology 134:198-202.

Tanabe Y, Ogawa T, Nakamura T. 1981. The effect of short-term starvation on pituitary and plasma LH, plasma estradiol and progesterone, and on pituitary response to LH-RH in the laying hen (*Gallus domesticus*). General and Comparative Endocrinology. 43:392-398.

Tena-Sempere M, Barreiro M, Gonzaez L, Gaytan F, Zhang F, Caminnos J, Pinilla L, Casanueva F, Dieguez C, Aguilar E. 2002. Novel expression and functional role of ghrelin in rat testis. Endocrinology 143:717-725.

Tena-Sempere M. 2005. Exploring the role of ghrelin as a novel regulator of gonadal function. Growth Hormone and IGF Reseach 15:83-88.

Theander-Carrillo C, Wiedmer P, Cettour-Rose P, Nogeiras R, Perez-Tilve D, Pfluger P, Castaneda T, Muzzin P, Schurmann A, Szanto I, Tschops M, Rohner-Jeanrenaud F. 2006. Ghrelin action in the brain controls adipocyte metabolism. The Journal of Clinical Investigation 116:1983-1993.

Thompson N, Gill D, Davies R, Loveridge N, Houston P, Robinson I, Wells T. 2004. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretatagogue receptor. Endocrinology 145: 234-242.

Tilly J, Kowalski K, Johnson A, Hsueh A. 1991. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 129:2799-2801.

Tolle V, Kadem M, Bluet-Pajot MT, Frere D, Foulon C, Bossu C, Dardennes R, Mounier C, Zizzari P, Lang F, Epelbaum J, Estour B. 2003. Balance in ghrelin and leptin plasma levels in *anorexia* nervosa patients and constitutionally thin women. Journal of Clinical Endocrinology and Metabolism 88:109-116.

Tschop M, Smiley D, Meiman M. 2000. Ghrelin induces adiposity in rodents. Nature 407:908-913.

Tschop M, Weyer C, Tataranni P, Devanarayan V, Ravussin E, Heiman M. 2001. Circulating ghrelin levels are decreased in human obesity. Diabetes 50:707-709.

Toshinai K, Mondal M, Nakazato M, Date Y, Murakami N, Kojima M, Kangawa K, Matsukura S. 2001. Upregulation of ghrelin expression in the stomach upon fasting, insulin induced hypoglycemia, and leptin administration. Biochemical and Biophysical Research Communications 281:1220-1225.

Traebert M, Riediger T, Whitebread S, Scharret E, Schmid H. 2002. Ghrelin acts on leptin responsive neurons in the rat arcuate nucleus. Journal of Neuroendocrinology 14:580-586.

van der Lely A, Tschop M, Heiman ML, Ghigo E. 2004. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocrine Reviews 25:426-457.

Vulliemoz N, Xiao E, Xia-Zhang L, Germond M, Rivier J, Ferin M. 2004. Decrease in luteinizing hormone pulse frequency during a five-hour peripheral ghrelin infusion in the ovariectomized rhesus monkey. The Journal of Clinical Endocrinology and Metabolism 89:5718-5723.

Wada R, Sakata I, Kaiya H, Nakamura K, Hayashi Y, Kangawa K, Sakai T. 2003. Existance of ghrelin-immunopostive and –expressing cells in the proventriculus of the hatching and adult chicken. Regulatory Peptides 111:123-128.

Whatmore A, Hall C, Jones J, Westwood M, Clayton P. 2003. Ghrelin concentration in healthy children and adolescents. Clinical Endocrinology 59:649-654.

Williams D, Cummings D, Grill H, Kaplan J. 2003. Meal-related ghrelin suppression requires postgastric feedback. Endocrinology 144: 2765-2767

Woods D, Johnson A. 2005. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biology of Reproduction 72:643-650.

Wren A, Small C, Ward H, Murphy K, Dakin C, Taheri S , Kennedy A, Roberts A, Morgan D, Ghatei M, Bloom S. 2000. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141: 4325-4328.

Yamamoto D, Ikeshita N, Daito R, Herningtyas E, Toda K, Takahashi K, Iida K, Takahashi Y, Kaji H, Chihara K, Okimura Y. 2007. Neither intravenous or intracerebroventricular administration of obestatin affects the secretion of GH, PRL, TSH, and ACTH in rats. Regulatory Peptides 138:141-144.

Yamato M, Sakata I, Wada R, Kaiya H, Sakai T. 2005. Exogenous administration of octanoic acid accelerates octanoylated ghrelin production in the proventriculus of neonatal chicks. Biochemical and Biophysical Research Communications 333:583-589.

Yan W, Linderborg J, Suominen J, Toppari J. 2000a. Stage-specific regulation of stem cell factor gene expression in the rat seminiferous epithelium. Endocrinology 140:1499-1504.

Yan W, Suominen J, Toppari J. 2000b. Stem cell factor protects germ cells from apoptosis in vitro. Journal of Cell Science 113:161-168.

Yoshimura Y, Nagano K, Subedi K, Kaiya H. 2005. Identification of immunoreactive ghrelin and its mRNA in the oviduct of laying Japanese quail, *Coturnix japonica*. The Journal of Poultry Science 42:291-300.

Yu M, Robinson F, Charles R, Weingardt R. 1992. Effect of feed allowance during rearing and breeding on female broiler breeders. 2. Ovarian morphology and production. Poultry Science. 71:1750-1761.

Yuan J, Zhou J, Hu X, Li N. 2007. Molecular cloning and comparison of avian preproghrelin genes. Biochemical Genetics 45:185-194.

Zhang W, Zhao L, Lin T, Chai B, Fan Y, Gantz I, Mulholland M. 2004. Inhibition of adipogensis by ghrelin. Molecular Biology of the Cell 15:2484-2491.

Zhang J, Ren P, Avsian-Kretchmer O, Luo C, Klein C, Hsueh A. 2005. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 310:996-999.