GENOMIC AND PHYSIOLOGICAL DIFFERENCES FOR GHRELIN AND LEPTIN RECEPTOR IN LINES OF CHICKENS SELECTED FOR HIGH AND LOW BODY WEIGHT

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Autonomic nervous system (ANS) activity is related to body weight regulation. Based on the hypothesis that Most Obesities kNown Are Low In Sympathetic Activity (MONA LISA), it has been suggested that most obese subjects and animals have low sympathetic nervous system activity. Leptin, leptin receptor, and ghrelin genes influence the ANS regulation of body weight and food intake. The aim of this study was to investigate whether there are differences in leptin, the leptin receptor, or ghrelin regulation between lines of chickens selected for high (HWS) or low body weight (LWS).

Intraperitoneal injections of reserpine were administrated to chickens from the HWS and LWS lines. Body weight and food intake were then compared to evaluate ANS regulation. While reserpine caused a transitory decrease in food intake and body weight in both lines, the magnitude of the change was greater in the HWS than in the LWS chickens. However, chickens from the LWS line exhibited greater catecholamine and indoleamine level changes in response to reserpine than those from the HWS line. Therefore, HWS chickens were more sensitive to the body weight–reducing effects of reserpine than LWS lines, while LWS chickens appeared to have greater sympathetic nervous system activity.
Food and water intakes were differentially affected in HWS and LWS chickens in response to intracerebroventricular administration of human recombinant leptin. Leptin caused a linear decrease in food intake in the LWS line, but no effect on food intake in the HWS lines. The HWS chickens tended to have reduced water intake following leptin administration. These results suggest that the leptin receptor, or the down-stream neuropeptide regulation pathway mediating the effect of leptin; may be different between chickens from the HWS and LWS lines.

Leptin, insulin like growth factor (IGF)-1, and IGF-2 concentrations in the plasma of HWS and LWS lines of chickens were evaluated. Leptin, IGF-1 and IGF-2 levels were significantly higher in the LWS than HWS chickens. The HWS female leptin concentrations were significantly lower than in HWS males or LWS females. Male chickens had greater IGF-1 concentrations in the plasma than female chickens. However, the concentration of IGF-2 did not differ between sexes. The difference in leptin concentrations in these lines and sexes may explain the differences in age of sexual maturity. Different IGF-1 and IGF-2 concentrations may be involved in the obese and anorexic conditions, fast and slow growth, and high and low food consumption found in these two lines of chickens.

Differences in the gene sequence of the leptin receptor were observed in HWS and LWS lines of chickens. A single nucleotide polymorphism (SNP) in the intron between exon 8 and 9 introduced a restriction site for the enzyme \( Sel \ I \) in the HWS, but not the LWS line. Two SNP were detected in the leptin receptor cDNA region at nucleotides 189 and 234. At nucleotide 189, the LWS line has both a homozygous (T-T) and heterozygous (C-T), whereas the HWS line has only homozygous (T-T) form. The
SNP found in nucleotide 234 introduces a restriction site *Mse I* in the HWS, but not the LWS line. These specific changes may be directly involved or closely linked to differences between the two lines in either the coding or regulatory domains of the leptin receptor.

Differences in the leptin receptor gene expression between HWS and LWS lines of chickens in various organs and ages were observed. Leptin receptor expression in the whole brain was significantly different between sexes at 28 days-of-age in the HWS and LWS lines. The LWS line had higher leptin receptor gene expression in the liver at 2 days-of-age than at 56 and 363 days-of-age, but no differences were observed in the HWS line. In addition, at 2 days-of-age, liver leptin receptor gene expression was higher in LWS than HWS chickens, but the reverse was observed at 363 days-of-age. In adipose tissue, leptin receptor expression was higher in the LWS than HWS line. Leptin receptor expression in adipose tissue was greater at 363, than 28 and 56 days-of-ages. Our results showed that changes in the regulation of leptin and the leptin receptor were associated with sex, age, and growth.

Differences in the ghrelin gene in the HWS and LWS lines under different feeding conditions were investigated. Both HWS and LWS chickens have six extra base pairs in the 5’-untranslated region. The LWS male ghrelin gene expression was significantly lower than in the LWS female and HWS male. The 84 day-old males had lower gene expression than 84 day-old females and 363 day-old males. When comparing different feeding methods, females allowed *ad libitum* feed consumption had a lower cycle threshold cycle number (CT) ratio than males allowed *ad libitum* feeding or fasted females. However, the inflection point cycle number of *ad libitum* fed females was lower
than that of the *ad libitum* fed males, but greater than the fasted females. Ghrelin gene
expression was different between the two lines of chickens, and the expression of ghrelin
in chickens was influenced by body weight selection, sex, age, and feeding condition.

Key words: chicken, gene expression, ghrelin, leptin receptor, sequence, single
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CHAPTER I

INTRODUCTION

It has been known for years that endogenous lipid stores are well regulated, and that it is unlikely that such regulation can be explained by a single gene. Obesity involves a disproportional increase of body fat mass, and is often accompanied by alterations in both sympathetic and parasympathetic nervous system function.

Many mechanisms involved in controlling body weight have been described. Hypothalamic, genetic, dietary, and adrenoendocrine forms of experimental obesity are all associated with reduced activity of the sympathetic nervous system. The MONA LISA hypothesis, an acronym for Most Obesities Known Are Low In Sympathetic Activity, restates the autonomic hypothesis in memorable terms, associated with food intake regulation, synaptosomal uptake of norepinephrine, local concentration of monoamine neurotransmitters, and adrenal dependence of hypothalamic obesity.

The sympathoadrenal system represents a major contributor to physiological regulation. Catecholamines have an effect on glucose and protein metabolism. Epinephrine is secreted by both sympathetic neurons and the adrenal medulla. The sympathetic nervous system and adrenal function may have different effects on the body weight and food intake regulation.

Leptin is a 167 amino acid polypeptide hormone with an amino-terminal secretory signal sequence of 21 amino acids, and is produced by adipocytes. Discovery of leptin, the product of the ob gene, has shed some light on mechanisms regulating lipid deposition. In mammals, it appears that a mutation in either leptin or its receptor results in changes in lipid stores and food intake. In animals, catecholamine agonists can reduce
ob mRNA expression and leptin secretion in vitro and in vivo, suggesting that the sympathetic nervous system exerts an inhibitory effect on leptin production.

Ghrelin is a 28 amino acid peptide originally identified as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R). Ghrelin is produced predominately by the stomach, with lower concentrations found in other parts of the gastrointestinal tract, kidney, and hypothalamus. Stimulating the GHS-R increases GH release and feed intake. In studying ghrelin’s effects on GH release in humans, feelings of hunger were noted as a side effect in the majority of test subjects. Serum ghrelin is reduced in obese humans, and following acute overfeeding, whereas circulating ghrelin is increased with fasting. Intracerebroventricular (ICV) administration of ghrelin to rodents induced obesity by increasing feed intake and reducing fat utilization. Ghrelin stimulated food intake by binding to a specific receptor in the hypothalamus and activating arcuate nucleus neurons which produced neuropeptide Y (NPY) and agouti-related peptide (AGRP).

The leptin receptor gene influences the autonomic nervous system (ANS) regulation of body weight and food intake. Preliminary research has indicated high body weight selected (HWS) and low body weight selected (LWS) lines of chickens have differences in the ANS’s control of the cardiovascular system. Several animal models, selected on the basis of a single gene mutation, have been utilized to study obesity. However, most obesities are not the result of a single gene defect or factor. The lines of chickens used in this study provide a unique genetic model for studying the regulation of body weight since they were developed using long-term divergent selection for high- or low- juvenile body weight. The aim of this study was to investigate leptin regulation,
leptin receptor polymorphism determination, leptin receptor gene expression, ghrelin polymorphism detection, and ghrelin gene expression in HWS and LWS chickens.
CHAPTER II
LITERATURE REVIEW

Central Nervous System Dependence of Hypothalamic Obesity

Autonomic function is controlled by the central nervous system, and damage to various hypothalamic areas can cause either an increase or decrease in body weight. Bernardis and Bellinger (1996) reported that the lateral hypothalamus (LH) is involved in catecholaminergic and serotonergic feeding systems. Furthermore, bilateral LH dopaminergic cell transplants, which presumably resulted in chronically and persistently enhanced dopaminergic activity in the LH, were associated with overeating and weight gain (Yang et al., 1996). Decreased turnover of norepinephrine, dopamine, and serotonin in the lateral hypothalamus (LH) stimulated sympathetic nervous system activity in brown adipose tissue, and LH stimulation of sympathetic activity may have occurred by suppression of norepinephrine, dopamine and serotonin turnover. Yang and Meguid (1995) reported that mean basal dopamine levels were significantly higher in obese those lean Zucker rats.

Meguid et al. (1999) studied the role of LH dopamine and serotonin in the regulation of the feeding patterns during obesity by grafting the lean rat’s embryonic dopaminergic and serotonergic neurons into the LH of obese Zucker rats. Results showed that both serotonin-grafted and dopamine-grafted rats decreased the number of meals and feed intake, indicating that dopamine and serotonin in the LH are involved in the regulation of feeding patterns associated with obesity.

Injections of norepinephrine into the ventromedial hypothalamic nucleus (VMH) and paraventricular nucleus (PVN) increased food intake and body weight gain
(Leibowitz and Brown, 1980). Corticotrophin releasing factor (CRF) inhibited norepinephrine release in the LH and increased sympathetic activity, decreased parasympathetic outflow, and decreased appetite behavior (Egawa et al., 1990; Osumi, 1990; Bray, 1991; Spina et al., 1996). Furthermore, increasing levels of neuropeptide Y in the PVN induced by exercise or fasting were followed by increased appetite and food intake, and increased parasympathetic activity but suppressed sympathetic activity (Kokot and Ficek, 1999).

**Peripheral Dependence of Hypothalamic Obesity**

An important role for adrenal hormones in the short- and long-term regulation of food intake and body weight has been strongly suggested (Bray, 1984; Leibowitz, 1986). Adrenalectomy reverses hypothalamic obesity, and injection of corticosterone restores the hyperphagia and obesity in such animals (Tokunaga et al., 1989). Adrenal glucocorticoids have an effect on the development of hypothalamic obesity and appear essential for the development of all forms of genetic obesity (Satio and Bray, 1984; Shimizu et al., 1988; Bray et al., 1989). Genetic and lesion-induced obesity are strongly related to high levels of corticosterone which greatly enhance fat deposit (King and Smith, 1985; Kumar and Libowitz, 1988). Adrenalectomy decreased the secretion of corticosteroids, increased the release of CRF, decreased food intake, and stimulated the activity of the sympathetic nervous system (Brown et al., 1982). Adrenalectomy decreased food intake to normal levels in obese mice, increased energy expenditure, and restored catecholamine turnover to normal in brown adipose tissue, and returned sympathetic nervous system activity to normal (Satio and Bray, 1984; Vander Tuig et al., 1984; York et al., 1985).
Neuropeptides involved food intake regulation

There are several distinct hypothalamic neuropeptide-containing pathways that have emerged as mediators of leptin’s action in the central nervous system (Schwartz et al., 2000). Schwartz et al. (2000) reviewed the orexigenic peptides including NPY, agouti related peptide (AGRP), melanin-concentrating hormone (MCH), Orexin A and B, and Galanin. The anorexigenic peptides are alpha melanocyte-stimulating hormone, CRF, thyroid releasing hormone (TRH), cocaine and amphetamine-regulated transcript (CART), interleukin 1, urocortin, glucagon-like peptide 1, oxytocin, and neurotensin.

Neuropeptide Y

Neuropeptide Y (NPY) is widely expressed throughout the central nervous system (Clark et al., 1984). Neuropeptide Y is involved with the anabolic pathway by increasing the depletion of body fat stores in response to reduced leptin signaling to the brain (Stanley et al., 1986; Wilding et al., 1993). The central administration of NPY stimulated feeding in rats (Stanley et al., 1985; Stanley and Leibowitz, 1985). In addition, NPY decreased energy expenditure, and induced lipogenic enzymes in the liver and white adipose tissue (Billington et al., 1991). Continuous or repeated central administration of NPY lead to obesity (Kokot et al., 1999; Zarjevski et al., 1993). It is known that leptin receptor and NPY gene expression are coordinately regulated in response to fasting in obese rats, cold-exposure in lean mice, and leptin-treatment in ob/ob mice (Baskin et al., 1999; Mercer et al., 1997; Woods et al., 1998). The leptin receptor is found on NPY neurons in the arcuate nucleus of the hypothalamus (Bask et al., 1999), suggesting that leptin receptor downstream signaling proteins may regulate the NPY gene.
**Melanocortins**

Alpha melanocyte-stimulating hormone (α-MSH) belongs to the class of melanocortin peptides (Cone et al., 1996). It is produced as a cleavage product from pro-opiomelanocortin (POMC), a precursor molecule, and exerts its effects by binding to melanocortin receptors (Cone et al., 1996). The discovery of α-MSH was followed by evidence that a synthetic antagonist had the opposite effect (Fan et al., 1997). Mice that lack the melanocortin 4 (MC4) receptor are hyperphagic and obese (Huszar et al., 1997), indicating that activation of the MC4 receptor could decrease food intake and fat mass. Mice with a deleted MC4 allele become obese (Huszar et al., 1997). Humans with the MC4 receptor mutation, which lack a full complement of central MC4 receptors, have pathological weight gain and hyperphagia (Chemelli et al., 1999; Vaisse et al., 1998; Yeo et al., 1998).

The agouti mice is an important autosomal dominant model of genetic obesity characterized by yellow coat color and an obese phenotype (Miller et al., 1993). The agouti protein is an antagonist of the melanocortin 1 (MC1) receptor that is encoded in the agouti gene, and is expressed by hair follicles (Cone et al., 1996). By reducing MC1 receptor activity, light coat color and obesity in mice are induced (Cone et al., 1996).

The AGRP was subsequently cloned from the Agrp gene, and was shown to also act as an antagonist of melanocortin 3 (MC3) and MC4 receptors (Ollmann et al., 1997; Shutter et al., 1997). The AGRP is expressed in the arcuate nucleus during fasting and leptin deficiency (Broberger et al., 1998; Hahn et al., 1998; Ollmann et al., 1998), which is close to where NPY and POMC neurons are located. Intracerebroventricular (ICV) injection of AGRP increased food intake (Rossi et al., 1998). These results indicated that
the central melanocortin receptors are one of the major pathways regulating food intake (Broberger et al., 1998; Hahn et al., 1998; Ollmann et al., 1998).

**Leptin and leptin receptor**

**Leptin**

Leptin is a 167 amino acid polypeptide hormone with an amino-terminal secretory signal sequence of 21 amino acids, and is produced by adipocytes (Zhang et al., 1994). The translocation of microsomes is followed by the subsequent removal of the signal peptide and secretion into the blood stream (Considine and Caro, 1997). Leptin is involved with regulation of body weight through metabolism control, feeding behavior, energy balance, and neuroendocrine responses (Friedman and Halaas, 1998; Ahima et al., 2000). Circulating leptin is a 146 amino acid peptide. Human leptin is 84% homologous to mouse leptin (Zhang et al., 1994). The crystal structure of leptin is a four helix bundle similar to the long-chain helical cytokine family (Prolo et al., 1998). Leptin is encoded in the gene located in human chromosome 7q31.3, with 3 exons and 2 introns. Leptin gene transcription and translation occurs in adipose tissue, placenta and the gastrointestinal tract; the leptin production rate depends on the fat mass (Prolo et al., 1998). At normal body weight, leptin is correlated with absolute fat mass rather than with body mass index or percent body fat (Margetic et al., 2002).

**Leptin Receptor**

The leptin receptor is a single membrane-spanning receptor that is similar to the class I cytokine receptor family (Tartaglia et al., 1995). The majority of transcripts in most tissues encoding either the long (Ob-Rl) and short (Ob-Rs) isoforms. The transcript for Ob-Rl is less abundant, except in the hypothalamus (Ghilardi et al., 1996). Leptin
receptor gene mRNA and protein, as well as leptin-binding sites, are concentrated in hypothalamic neuronal areas including the arcuate (Satoh et al., 1997), VMN, and dorsomedial nuclei (Madiehe et al., 2001). Evidence suggests that the Ob-R can be expressed at different levels of the neuroaxis (Elmquist et al., 1998). Several observations suggest that the adipocyte hormone leptin inhibits food intake by activating the Ob-R1 isoform in the hypothalamus (Ahima and Flier, 2000). Campfield et al. (1996) reviewed several studies and concluded that leptin receptor appears to play a major role in the control of body fat stores through coordinated regulation of feeding behavior and the ANS in rodents, primates and humans. Mice with a mutation in the leptin receptor are hyperphagic and severely obese (Chen et al., 1996; Lee et al., 1996).

Alternative splicing of leptin receptor

The leptin receptor has at least 6 alternatively spliced forms (Lee et al., 1996). Mouse cDNAs predicted proteins with differing cytoplasmic portions, designated Ob-Ra, Ob-Rb, Ob-Rc, and Ob-Rd. The Ob-Re has a different predicted amino-acid sequence after His 796. (Lee et al., 1996). All isoforms of mouse leptin receptors have an identical extracellular, ligand-binding domain while the intracellular domain at the C-terminal is different (Tartaglia et al., 1995). The Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rc, Ob-Rd and Ob-Rf contain transmembrane domains (Figure 2-1), where Ob-Re is truncated before the membrane-spanning domain and is therefore to be secreted (Margetic et al., 2002). Leptin receptors have short intracellular forms, but their functions have not been fully chartreuse (Tartaglia, 1997). Short isoform mRNAs are found in areas where leptin protein might cross the blood-brain-barrier, which include the choroid plexus and brain microvessels (Bjorbaek et al., 1998; Golden et al., 1997). Long isoforms ware found in
the hypothalamus (Mercer et al., 1996), and the region of the hindbrain which is involved with feeding and energy balance (Mercer et al., 1998). The Ob-Ra can immediately activate early gene expression and stimulate mitogen-activated protein kinase (MAPK) phosphorylation, but not phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3) (Baumann et al., 1996; Murakami et al., 1997; Yamashita et al., 1998). Bennett et al. (1998) reported the total leptin receptor mRNA decreased in response to an increase in Ob-Rb mRNA. The data suggest that neurons can switch the type of leptin receptor in response to various signals.

Leptin receptor induced signal transduction

The Ob-R lacks intrinsic tyrosine kinase activity, but requires activation of receptor-associated kinases of the Janus family (JAKs) (Ghilardi and Skoda., 1997),

Figure 2-1. Six different isoforms of leptin receptors in the cytoplasm membrane. The Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Re are short forms, Ob-Rb is the long isoform. The Ob-Rb is located in the hypothalamus, and when leptin binds to the Ob-Rb, induction of the intracellular signal JAK/STAT pathway occurs to activate the action of leptin and leptin receptors may occur (Tartaglia et al., 1995).
which activate downstream signaling including the STAT family (Figure 2-2) (Bumann et al., 1996; Schwartz et al., 1996). When leptin binds to its receptor, JAKs autophosphorylate and tyrosine phosphorylates various STATs. Activated STAT in the hypothalamus dimerize and translocate to the nucleus, where specific gene responses are elicited (Vaisse et al., 1996; Sanchez-Margalet and Martin-Romero, 2001). After the JAK/STAT is activated, it also activates theSuppressor-of-Cytokine-Signalling-3 (SOCS3) (Starr et al., 1997; Endo et al., 1997; Naka et al., 1997; Bjorbaek et al., 1998; Banks et al., 2000), which is part of an intracellular negative-feedback loop inhibiting JAK activity, and turning off or inhibiting cytokine signal transduction (Bjorbaek et al., 1998).

The SOCS3 serves as a negative regulatory pathway, acting the downstream of the leptin receptor by affecting activated JAK/STAT proteins (Starr et al., 1997; Zhang et al., 1999). The SOCS3 protein is expressed in the arcuate nucleus (Bjorbaek et al., 1999), and its mRNA levels are increased by exogenous leptin administration (Bjorbaek et al., 1998). The action between the leptin receptor and SOCS3 in response to leptin suggests that the leptin receptor promoter may be one of the target genes of activated STAT transcription factors (Good, 2000).
Figure 2-2. The regulation of signal transductions through the leptin receptor. Leptin binds the leptin receptor on hypothalamic neurons. The intracellular domain of leptin receptor contains a JAK binding site, so JAK protein binds to this region causing phosphorylation. The SOCS and SHP-2 proteins help regulate the JAK activity. Phosphorylation of STAT transcription factors by JAKs allows them to dimerize and move to the nucleus where then can stimulate the transcription of leptin receptor regulated genes. And the binding of STAT could be inhibited by protein inhibitors of activated STAT (PIAS) (Banks et al., 2000).

**Leptin, leptin receptor secondary signaling pathway**

The first order of the neuronal signaling pathway is in the arcuate nucleus. The arcuate nucleus is a collection of neuronal cell bodies on the floor of the third ventricle (Hahn et al., 1998). The NPY and AGRP-containing neuron are localized in the arcuate nucleus (Hahn et al., 1998), demonstrating that a single neuronal cell type can contain
multiple anabolic effector molecules. The POMC and CART neurons are also located in
the arcuate nucleus next to the NPY/AGRP neurons (Elías et al., 1998), indicating the
circuits originating in this brain area have highly specialized roles in energy homeostasis
(Figure 2-3) (Schwatz et al., 2000). Leptin receptors are expressed on both NPY/AGRP
and POMC/CART neurons, and both neurons are regulated by leptin, but in an opposite
manner (Cheung et al., 1997). The NPY/AGRP neurons are inhibited by leptin, and thus
increase their activity when plasma leptin is low (Schwatz et al., 1996).

The second order of the neuronal pathway is in the PVN, zona incerta,
perifornical area, and lateral thalamus area. In these regions, there are axons from
arcuate nucleus NPY/AGRP and POMC/CART neurons (Elmquist et al., 1998; Elmquist
et al., 1999). Lesion of the paraventricular nucleus caused hyperphagic obesity, and
lesioning of LH area caused anorexia and weight loss (Bray et al., 1990; Steller, 1954).
Stimulation of the PVN inhibits food intake, whereas the stimulates the LH area and
adjacent perifornical area (Stanley et al., 1993). These observations indicate
anorexigenic and orexigenic signaling molecules might be synthesized in these regions.

Several neuropeptides are synthesized in the PVN, and caused reduced food
intake and body weight when given ICV (Dunbar et al., 1997). The CRF caused anorexia
and activated the sympathetic nervous system, it also regulates the hypothalamic-
pituitary-adrenal axis (Dallman et al., 1993). Thyroid releasing hormone reduced food
intake in addition to stimulating the thyroid axis (Kow et al., 1991). If these neurons are
second order catabolic effectors located downstream of the arcuate nucleus, they should
be stimulated by melanocortin or CART signaling, and inhibited by NPY. Further
studies are needed to test these predictions.
The second order neuronal pathway is involved with anabolic signaling which is supported by melanocortin-concentrating hormone located in these areas of the brain (Qu et al., 1996). Melanocortin–concentrating hormone synthesis is elevated by energy restriction and leptin deficiency (Qu et al., 1996), and melanocortin-concentrating hormone-knockout mice have reduced food intake and lowered body weight (Shimada et al., 1998). Intraventricular injection of orexins A and B caused increased food intake, which suggested that reduced orexin signaling has the potential to control food intake (Sakurai et al., 1998). It is predicted that melanocortin-concentrating hormone and orexin-containing neurons are inhibited by melanocortin or CART input and stimulated by NPY signaling from the arcuate nucleus.

**Figure 2-3.** The role of the arcuate nucleus neurons in adiposity signaling. Decreased leptin signaling stimulates NPY/AGRP neurons and inhibits POMC neurons, resulting in increased food intake. Conversely, high leptin concentration inhibits NPY neurons and stimulate POMC neurons, and to decreasing food intake. (Schwartz et al., 2000)
Leptin and leptin receptor mutation

Sympathetic nervous system activity is decreased in most forms of obesity, even in various genetic models. It has been reported that \textit{ob/ob} mice have a leptin deficiency, and leptin regulates body weight and fat deposition through effects on metabolism and appetite (Pellemounter et al., 1995). Because leptin released from adipose tissue effects the autonomic nervous system acting within the hypothalamus, it can act as a feedback signal from adipose tissue to modulate neuronal and hormonal systems involved in the regulation of body weight and food intake (Blum, 1997; Mistry et al., 1997). Harris et al. (1998) reported that giving \textit{ob/ob} mice high concentrations of leptin caused increased sympathetic activity. The Pima Indians appear similar to the \textit{ob/ob} mouse in that they lack leptin and are prone to obesity and have low sympathetic nervous system activity (Ravussin and Gautier, 1999; Steppan and Swick, 1998).

Leptin and leptin receptors are involved with both orexigenic and anorexigenic pathways in the hypothalamus (Baskin et al., 1999). While \textit{db/db} mice produce normal concentrations of leptin, a mutation in both alleles of the leptin receptor gene in these mice produces a truncated protein lacking the intracellular signaling domain (Tartaglia, 1995). Based on the result of parabiosis studies of \textit{ob/ob} and \textit{db/db} mice, the \textit{ob/ob} mice have decreased body fat, but not \textit{db/db} mice (Coleman, 1973). Since the \textit{db/db} mice have mutated leptin receptors, the binding of leptin does not activate the JAK/STAT signaling pathway and no leptin-induced gene regulatory changes occurred (Vaisse et al., 1996).

The \textit{fa/fa} mice contain a missense mutation in the leptin receptor gene, which allows activation of the STAT 1 and 3 pathway, but not STAT 5, thus leading to obesity (Takaya et al., 1996; Good, 2000). The leptin receptor mutation animal model \textit{db3J/db3J}
mice exhibit a frameshift in the leptin receptor protein affecting all splicing forms (Lee et al., 1997). The soluble leptin receptor protein is absent in the db3J/db3J mice, which resulted in truncation of the leptin receptor at amino acid 625 and induced obesity (Li et al., 1998). Brown et al. (2000) reported a mutation, with deleted a nucleotide G in the leptin receptor exon 12, which induced obesity and diabetes. This mutation causes a frameshift of the reading frame, which substitutes 11 amino acids followed by a premature termination of translation before the membrane-spanning domain (Brown et al., 2000).

In human studies, Clement et al. (1998) reported a homozygous mutation in the human leptin receptor gene that resulted in a truncated leptin receptor lacking both the transmembrane and intracellular domains causing early-onset morbid obesity. Patients homozygous for this mutation have no pubertal development and their secretion of growth hormone and thyrotropin is reduced. Mammes et al. (2001) reported that genotype and allele frequencies of the Ser (T) 343 Ser (C) polymorphism (exon 9) were significantly different between the normal and overweight women, with the T allele being more frequent in the overweight than the normal weight group, and overweight women carrying the C allele of this polymorphism lost more weight in response to a low calorie diet than the non-carriers (Mammes et al., 2001). These results indicate that variations at the leptin receptor locus are associated with common obesity phenotypes and are a part of the polygenic influences on the response to nutritional environment (Mammes et al., 2001). Quinton et al. (2001) detected a single nucleotide polymorphism that induces a Gln223Arg in the leptin receptor extracellular region, and changes the amino acid sequence. They found that A allele homozygous human had increased body
mass index and leptin concentration, but not humans with the G allele (Quinton et al., 2001).

**Ghrelin**

Ghrelin was identified in the stomach as an endogenous ligand specific for the GHS-R. Ghrelin is a 28-amino acid (human and rat) gastrointestinal peptide that is highly conserved across species (Figure 2-4). This highly conserved acylated peptide is produced primarily by cells in the oxyntic glands of the stomach (Date et al., 2000). As a natural ligand for the GHS-R, it is expected to stimulate growth hormone (GH) release. Kojima et al. (1999) reported that ghrelin stimulates GH release from pituitary cells in a dose-dependent manner. Ghrelin also functions as a blood-born orexignenic signal from the gut to the brain (Pinkney and Williams, 2002).

Figure 2-4. Structure of ghrelin (Kojima et al., 1999).

**Ghrelin receptor**

Growth hormone secreted from the anterior pituitary is a multifunctional hormone that regulates overall body and cell growth (Kellendonk et al., 1999). Growth hormone release is controlled by three mechanisms: stimulation by hypothalamic GH-releasing hormone, inhibition by somatostatin, and stimulation by GH secretagogues (GHS). Many GHS are synthetic compounds that are potent stimulators of GH release working through
the GHS-R (Kojima et al., 2001). Growth hormone secretagogue receptor is a G-protein-coupled seven-transmembrane receptor that has been identified in the hypothalamus, pituitary, and hippocampus. (Howard et al.,1996; McKee et al.,1997; Smith et al.,1999). Stimulation of the GHS-R causes calcium release through stimulation of the G protein subunit $G_\alpha$ (Figure 2-5) (Bodart et al., 1999). The GHS-R can be activated by a group of synthetic peptides (GHRP-2, and GHRP-6) and nonpeptide GHS (L-692, 429, MK-0677) (Pong et al., 1996; Smith et al., 1996).

Stimulation of the GHS-R with growth hormone releasing peptide (GHRP) increased GH release and feed intake in rats (Pandya et al., 1998; Shibasaki et al., 1998; Kuriyama et al., 2000). Shuto et al. (2002) compared feed intake, body weight, adipose tissue size, and GH levels in plasma of normal and GHS-R-impaired rats. The GHS-R-impaired rats had lower body weight, reduced daily feed intake, less adipose tissue, and lower GH levels than control rats. The natural ligand for GHS-R was not known until ghrelin was found. Therefore, the GHS-R had been considered an orphan receptor.

![Figure 2-5. Interaction between ghrelin, and growth hormone receptor (GHS-R) (Bodart et al., 1999).](image)

*Effects of ghrelin on feed intake*
The first published evidence for involvement of ghrelin in the regulation of appetite was provided by Ghigo and co-workers (Arvat et al., 2000). Three of four healthy volunteers spontaneously reported hunger following ghrelin administration in a clinical trial analyzing GH release. Tschop et al. (2000) observed ghrelin concentration changes in the plasma of mice before and after fasting. Ghrelin concentrations were increased by fasting and reduced by re-feeding or oral glucose administration, but not water ingestion. Date et al. (2001) found that ICV administration of ghrelin increased gastric acid output in a dose-dependent manner in urethane-anesthetized rats. This indicated that ghrelin participates in the central regulation of gastric acid secretion by activating the vagus system.

Nakazato et al. (2001) studied the role of ghrelin in central nervous system regulation of feeding. Ghrelin and anti-ghrelin immunoglobulin G (IgG) were both injected ICV into the lateral ventricles of rats. Ghrelin increased feed intake in both satiated and fasted rats, whereas anti-ghrelin IgG injection caused a dose-dependent suppression of feed intake in free feeding rats.

The effect of ghrelin on appetite and food intake has been investigated in humans (Wren et al., 2002). Although subjects receiving ghrelin increased food intake compared to those receiving saline, ghrelin had no effect on gastric emptying. They suggested that ghrelin is a newly discovered peripheral signal that acts in the brain to stimulate food intake. Subsequent studies established the role of ghrelin in the pathogenesis of obesity and anorexia nervosa by comparing plasma ghrelin levels of lean and obese humans (Shiiya et al., 2002). Ghrelin levels were significantly lower in obese subjects than in those with normal body weight and anorexia nervosa patients. Therefore,
nutritional state is a determinant of plasma ghrelin levels in humans; ghrelin level is up-regulated under conditions of negative energy balance and down-regulated during positive energy balance.

Ukkola et al. (2001) studied mutations in the ghrelin gene in obese humans. They found that 6.3% of obese subjects had a mutation in exon two of the encoding region of ghrelin. These obese subjects had lower body weight until 40 years-of-age. The results indicate that sequence variations in the ghrelin genes may play a role in the etiology of obesity.

Ghrelin and domestic livestocks

Hayashida et al. (2001) studied ghrelin levels before and after a meal in cattle, and the effect on plasma GH after a single injection of ghrelin in goats. Ghrelin increased before feeding and decreased after a meal in Shiba goats. Plasma GH levels increased after the injection of ghrelin. Most studies have shown that ghrelin increased food intake and body weight in mammals (Date et al., 2001; Hayashida et al., 2001; Nakazato et al., 2001). However, ICV injection of rat ghrelin decreased feed intake of day-old chicks, suggesting that the mechanisms controlling feeding of the neonatal chick through GH release are different than those in mammals (Furuse et al., 2001). Ahmed and Harvey (2002) used immunohistochemistry to locate ghrelin expression in chickens. Ghrelin was not detect in gastrointestinal organs, but was found in the hypothalamus. The use of rat and human ghrelin as the probe for the experiment may explain their inability to detect ghrelin in gastrointestinal organs. Further studies have been conducted to purify and clone chicken ghrelin (Kaiya et al., 2002). Chicken ghrelin sequence has 54% homology to the human ghrelin, and is strongly expressed in the proventriculus and corpus striatum.
Recently, the feed intake response to ICV injection of chicken ghrelin, rat ghrelin, bullfrog ghrelin, and GHRP-2 to neonatal chicks was reported (Saito et al., 2002). All varieties of ghrelin and growth hormone-releasing peptide-2 decreased feed intake. It is possible that in birds, the control of GH secretion differs from that in mammals, and that chicken GHRH has minimal GH releasing activity in chickens (Harvey, 1999). Chicken GH release is more likely induced TRH (Ahmed and Harvey, 2002).

Ghrelin and leptin

It is known that leptin acts as part of a feedback loop to maintain constant body fat stores and that GHS-R and leptin receptors are located in the hypothalamus. The similarities of leptin and ghrelin are that they are both secreted from the peripheral system, cross the blood brain barrier, and bind to receptors in the central nervous system. It has been suggested that leptin and ghrelin co-regulate the hypothalamic peptidergic systems controlling food intakes in opposite ways (Horvath et al., 2001). Nakazato et al. (2001) reported that anti-NPY IgG and anti-AGRP IgG block the action of ghrelin and decrease feed intake in rats. The ICV administration of leptin after ghrelin also suppressed the action of ghrelin and caused a decrease in feed intake. These results suggest that ghrelin may antagonize leptin action in the regulation of the NPY system (Figure 2-6).
Figure 2-6. Stimulation of leptin and ghrelin through neuropeptide Y neurons.

High and Low Body Weight Selected Lines of Chickens

Foundation stock for the high (HWS) and low body weight selected (LWS) lines chickens consisted of crosses of seven inbred lines of White Plymouth Rocks (Siegel, 1962). From this segregating gene pool, two lines were selected on the basis of body weight at 8 weeks of age. Chickens with heavier body weights formed the parent stock for the HWS line whereas those with the lighter body weights were used as parents for the LWS line. Thereafter, individual phenotypic selection was practiced within each line for the single characteristic, body weight at 8 weeks of age. After forty years of selection for body weight, the differences of body weight between HWS and LWS chickens was 8
fold. Some females form the LWS line do not consume enough feed *ad libitum* to attain a minimum threshold of body weight, body composition, or both, which are essential to commence egg production (Zelenka et al., 1987). They will, however, commence egg production when forced-feed. The LWS males still produced semen. In the HWS line, a feed restriction program is used after 8 weeks of age. If the feed restriction is not applied, egg production is impaired. Because feed consumption exceeds skeletal and lean body weight needs, fat deposition increases in the HWS. The resulting obesity is accompanied by undesirable reproductive complications (Katanbaf et al., 1989; Liu et al., 1994). This indicates that the HWS natural puritanical results in obesity.


Good DJ. How tight are your genes? Transcriptional and posttranscriptional regulation of the leptin receptor, NPY, and POMC genes. Horm Behav 2000;37:284-98.


CHAPTER III

THE EFFECT OF RESERPINE ON BODY WEIGHT GAIN, FEED EFFICIENCY, CATECHOLAMINE AND INDOLEAMINE LEVELS IN HIGH AND LOW BODY WEIGHT SELECTED CHICKENS
ABSTRACT

The effect of reserpine on body weight, feed intake, brain, and plasma neurotransmitter concentrations in high- (HWS) and low- (LWS) weight selected lines of chickens was investigated. Three treatment groups from each line were injected intraperitoneally with saline, 1.25, or 2.50 mg/kg of reserpine at hatch, and again at 5 weeks-of-age. At 7 weeks-of-age, 12 males and 12 females from each group were sacrificed. The levels of catecholamines and indoleamine in the whole brain were determined. Body weight of the HWS chickens was decreased dose-dependently through 7 weeks-of-age whereas body weight was decreased only through the first 2 weeks-of-age in the LWS line. Reserpine also decreased weekly body weight gain, feed consumption, and feed efficiency in a linear manner in the HWS, but not the LWS chickens. After determining the sex of each bird at 5 weeks-of-age, it was noted that reserpine caused a dose-dependent decrease in body weight in both HWS male and female chickens, but not in LWS chickens from 5-7 weeks-of-age. In the central nervous system, norepinephrine concentration in the whole brain of HWS birds was higher than in LWS birds, while epinephrine, dopamine, 3,4-dihydroxyphenylacetic acid, and 5-hydroxyindoleacetic acid were higher in LWS than HWS birds. In the LWS birds, norepinephrine concentration decreased in a quadratic manner in response to the various doses of reserpine, but not in the HWS birds. The epinephrine concentration decreased in a linear and quadratic manner in LWS, but not HWS line. Dopamine levels decreased in a linear manner in response to reserpine in both lines. The 3,4-dihydroxy-L-phenylalanine decreased in a linear manner in the LWS line. Levels of serotonin were not affected by reserpine. The HWS chickens has greater norepinephrine, and lower 5-hydroxyindoleacetic acid in the
plasma than LWS chickens. In conclusion, reserpine had less effect on body weight and feed intake in LWS than HWS chickens. The HWS birds were more sensitive to the body weight–reducing effects of reserpine than LWS birds, with the latter appearing to have greater sympathetic nervous system activity.

Key Words: body weight, chicken, catecholamine, food intake, reserpine
INTRODUCTION

The sympathetic nervous system has a role in the etiology of body weight gain through its impact on food intake (Bray, 1991b). Two opposite hypotheses concerning these mechanisms have been proposed. The first is that obesity results from low sympathetic nervous system activity (Bray, 1991b; Ravussin and Gautier, 1999). The second theory suggests that sympathetic nervous system activation occurs as a result of chronic overeating (Rumantir et al., 1999). Both hypotheses suggest that the sympathetic nervous system can alter food intake and body weight through the central and peripheral nervous system (Bray, 1991a, Ravussin, 1995).

It is known that various neurotransmitters including norepinephrine (NE), epinephrine (Epi), dopamine (DA), and serotonin (5HT) in the hypothalamus have an impact on the regulation of body weight and food intake in rats (Leibowitz and Brown, 1980; Robert et al., 1990). Early studies suggest that DA is involved in maintaining normal feeding (Heffner et al., 1980), and there is increased DA release in the lateral hypothalamus (LH) associated with feeding (Meguid et al., 1995, Meguid et al., 1997). Furthermore, Cincotta (1997) demonstrated that obese \((ob/ob)\) mice treated with DA, D1, and D2 receptor agonists reduce food intake, body weight and fat mass.

Injecting NE into the ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN) of rats enhanced food intake, and ultimately, body weight gain (Leibowitz and Brown, 1980; Leibowitz et al., 1984; Lichtenstein et al., 1984). The 5HT also acts in the central nervous system to regulate food intake and body weight gain (Samanin and Garattini, 1988). Increasing 5HT in the medial hypothalamus reduced ingestion of carbohydrate while sparing protein intake (Leibowitz and Shor-Posner, 1986).
of 5HT or its agonists into the perifornical hypothalamus (Goldman et al., 1971), LH or medial PVN of rats decreased food intake (Gardier et al., 1989). Denbow et al. (1983) reported that intracerebroventricular injection of 5HT decreased food intake in fasted Leghorns. Many circulating hormones also impact serotonergic function and satiety (Leibowitz and Alexander, 1998).

Reserpine impairs the storage of biogenic amines, and results in depletion of NE, DA, and 5HT in both the central and peripheral nervous systems, and disturbs the digestive system and decreases food intake (Ahmed et al., 1997). The aim of this study was to compare the response of HWS and LWS of chickens to reserpine.

**MATERIALS AND METHODS**

Day–old male and female HWS and LWS line chicks were vaccinated for Marek’s disease and placed in electric heated batteries with raised wire floors. Thirty-six pens were randomly assigned to each of three treatment groups. The groups were injected with 5 ml/ kg intraperitoneal (i.p.) of either 1.25 or 2.50 mg/kg reserpine, or 0.85% saline which served as the control at 1 day-of-age, and 5 weeks-of-age (Ahmed et al., 1986). Birds were provided a starter (20% crude protein, 2684 kcal/ kg) diet and water available for *ad libitum* consumption. The birds were exposed to continuous fluorescent lighting. At 4 weeks-of-age, the birds were transferred to grower batteries (75.12 cm W × 38.10 cm L × 38.10 cm H).

Body weight and feed intake were measured at weekly intervals and feed efficiency determined. Data were analyzed using the General Linear Model of the Statistical Analysis System with line, sex, and treatment, and the interactions between them in the model (SAS Institute Inc., 1999-2001). The orthogonal contrast was used
when line by treatment interaction was significant, with treatment effects separated into linear and quadratic contrasts to determine dose-dependent responses in each line at each week. Significant differences imply \( P \leq .05 \).

At 8 weeks-of-age, 12 males and 12 females from each group were sacrificed for catecholamine and indoleamine assays. Blood was collected via cardiac puncture using a syringe coated with heparin. The blood was centrifuged at 300 x g for 20 min, and the plasma collected and stored at \(-80 \, ^\circ\text{C}\) until analysis. The brain was removed, quickly frozen in liquid nitrogen, and stored at \(-80 \, ^\circ\text{C}\) until analysis.

Neurotransmitter levels were analyzed using high performance liquid chromatography with electrochemical detection (HPLC-EC). Brain samples were homogenized in ice cold homogenization buffer which contained 0.1 M sodium acetate, 25mM citric acid, and 134 \( \mu \text{M} \) ethylenediaminetetraacetic acid (ETDA), 6\% methanol and 230 \( \mu \text{M} \) octanesulfonic acid (Jussofie et al., 1993). The homogenized mixtures were centrifuged (4 \(^\circ\text{C}\)) at 11,000 g for 15 min and the homogenate collected. The homogenate was then centrifuged (4 \(^\circ\text{C}\)) at 1,600 g for 5 min and the supernatant filtered using a 0.2 um membrane filter (Millipore, Dreieich, Germany).

Concentrations of NE, Epi, DA, 3,4-dihydroxy-L-phenylalanine (DOPAC), 5-hydroxyindole, 3-acetic acid (5HIAA), and 5 HT were analyzed using the General Linear Model of the Statistical Analysis System with line, treatment, and the interactions between them in the model (SAS Institute Inc., 1999-2001). A polynomial contrast was used when line by treatment interactions were significant, line by treatment interactions were tested using linear and quadratic contrasts. If interactions occurred, trends were observed within lines. Significant differences imply \( P \leq .05 \).
RESULTS

Body Weight

Body weight of the HWS chicken showed a linear decrease in response to the treatments through 7 weeks, but LWS chickens only displayed a linear response in the first and second weeks (Figure 3-1). From 5 though 7 weeks-of-age, the HWS line males and females showed linear decreases in body weight in response to the treatments, but not in the LWS chickens (Figure 3-4).

Feed Consumption

In the HWS birds, there was linear decrease in feed consumption from week 1 to week 3 in response to the 1st injection of reserpine. There was also a linear decrease observed at 7 weeks-of-age in response to reserpine injected at 5 weeks-of-age. The LWS chickens had a linear decrease in feed consumption in response to the 1st injection at week 1, but did not respond to reserpine injected at 5 weeks-of-age (Figure 3-2).

Feed Efficiency

The HWS chickens had a linear decrease in feed efficiency in response to the 1st injection from week 1. There was a linear decrease at 6 weeks-of-age in response to reserpine injected at 5 weeks-of-age (Figure 3-3).

Catecholamine and indoleamine levels

Brain

In the brain, the concentration of Epi, DOPAC, DA, 5HIAA, DOPAC/DA, and 5HIAA/5-HT were greater in LWS than HWS chickens. However, NE concentration displayed the opposite trend. The plasma concentrations of NE were significantly higher in males than in females.
The NE concentration in the brain of LWS chickens was affected by reserpine in a quadratic manner (Table 3-1), while DOPAC brain concentrations displayed a linear response (Table 3-1), and Epi brain concentration was affected in both linear and quadratic manners (Table 3-1). Both HWS and LWS chicken’s DA concentrations in the central nervous system are affected by reserpine in the linear manner (Table 3-1).

**Plasma**

The LWS line had higher NE concentrations in the plasma than the HWS line, and the HWS line had greater 5HIAA concentration in the plasma than the LWS line. No difference was observed between the lines in the plasma Epi and DOPAC concentrations (Table 3-2).

**DISCUSSION**

Reserpine treatment caused linear decreases in weekly body weight of HWS chickens through 7 weeks. However, reserpine only affected LWS body weight in the 1 and 2 weeks after treatment, and no response after the second injection. Feed consumption and feed efficiency similarly showed that HWS chicken had a stronger response to reserpine than LWS chickens. The regulation of energy balance and energy expenditure could be limited when the sympathetic nervous system activity ability is suppressed by reserpine (Saad et al., 1991; Chaloupka et al., 1998). The HWS birds had less sympathetic nervous system activity and higher parasympathetic nervous system activity in the cardiovascular action (Kuo et al., 2001). Therefore, they would have less ability to regulate energy balance and expenditure and less ability to recover from the depression caused by the reserpine. The opposite response was found in LWS birds and could be related to greater sympathetic tone.
Studies report that infusing NE or Epi into the VMH and PVN increased food intake, body weight gain and reduced sympathetic nervous system activity in rats (Leibowitz and Brown, 1980; Cransac et al., 1996). The HWS had higher NE concentration in the brain, which may stimulate feed intake and body weight in this line of chicken.

Meguid et al. (1999) reported that transplanting embryonic dopaminergic and serotonergic neurons of lean rats into the lateral hypothalamus of obese Zucker rats decreased food intake, indicating involvement of lateral hypothalamus DA and 5HT in the regulation of feeding during obesity. In the central nervous system, the LWS had greater Epi, DOPAC, and DA concentrations, and these compounds showed linear or quadratic decreases in response to reserpine in LWS, but not HWS birds. Leibowitz (1990) observed the anorexic patients had lower concentrations of 5HIAA and 5 HT in the plasma. Chickens from the LWS line exhibited higher NE and lower 5 HIAA in the plasma than those from the HWS line. Reserpine had a greater effect on brain catecholamine and indoleamine levels in LWS than HWS chickens, while having less affect on body weight and feed intake in LWS, indicating greater sympathetic tone in the LWS chickens. This may result in a greater ability to cope with the effect of reserpine.

Since reserpine caused an overall depletion of catecholamines and indoleamine in both the central and peripheral nervous system, and body weight and food intake are controlled by multiple mechanisms, it is difficult to identify the actual site of depletion that caused the differences between HWS and LWS chickens. Kuo et al. (2001) present study suggest differences in autonomic nervous system activity among HWS and LWS chickens. Obese Zucker rats have a higher alpha 2 receptor activity and less beta 3
activity which causes increased food intake and body weight (Jhanwar-Uniyal et al., 1991; Tsujii and Bray, 1992; Tsujii and Bray, 1998). Christin et al. (1993) compared the sympathetic nervous system activity in the Pima Indians who have a higher prevalence of obesity than white Caucasians. Pima Indians and white Caucasians have similar plasma NE appearance rates, but Pima Indians tend to be more resistant to beta-adrenergic stimulation. Tataranni et al. (1998) compared the sympathetic nervous system beta-adrenergic receptor’s sensitivity in Pima Indian and Caucasians. Pima Indian males had lower beta-adrenergic sensitivity than Caucasians. Therefore, increased body weight may be associated with lower beta-adrenergic sensitivity which can cause low sympathetic nervous system activity (Tataranni et al., 1998).

Denbow et al. (1984) reported that intracerebroventricular injection of 5HT decreased food intake, and that the HWS birds were more sensitive to the effect of serotonin as evidence by its longer action than in LWS birds. Moreover, VMN serotonergic activities were reduced in obese Zucker rats (Routh et al., 1994). These results suggest that animals differing in body weight may have differing numbers of receptors, and rates of metabolism of 5HT.

In conclusion, reserpine depressed overall sympathetic nervous system activity, which decreased body weight and feed intake in chickens from both HWS and LWS lines. The LWS birds however recovered from the depression faster than HWS birds, showing that LWS birds have greater sympathetic nervous system activity in order to maintain homeostasis.


Jussofie A. Anew highly sensitive procedure-a prerequisite to evaluate the density of GABA A receptors and to study their allostERIC properties in distinct areas of rat brain. Biol Chem Hoppe Seyler 1993;374:61-8


Figure 3-1. Effect of intraperitoneal injections of reserpine on body weight of high and low body weight selected chickens. HWS, high body weight selected line; LWS, low body weight selected line; * significant linear response to the treatments (P ≤ .05).
Figure 3-2. Effect of intraperitoneal injections of reserpine on feed consumption of high and low body weight selected chickens. HWS, high body weight selected line; LWS, low body weight selected line; * significant linear response to the treatments (P ≤ .05).
Figure 3-3. Effect of intraperitoneal injections of reserpine on feed efficiency of high and low body weight selected chickens. HWS, high body weight selected line; LWS, low body weight selected line; * significant linear response to the treatments (P ≤ .05).
Figure 3-4. Effect of intraperitoneal injections of reserpine on body weight of male and female, high and low body weight selected chickens. HWS, high body weight selected line; LWS, low body weight selected line; * linear respond to the treatments (P $\leq$ .05).
Table 3-1. Effect of intraperitoneal injections of reserpine on brain norepinephrine, epinephrine, dopamine, 3,4-dihydroxy-L-phenylalanine, 5-hydroxyindole, 3-acetic acid, and serotonin concentrations of high and low body weight selected lines of chickens. NE, norepinephrine; Epi, epinephrine; DA, dopamine; DOPAC, 3,4-dihydroxy-L-phenylalanine; 5 HIAA, 5-hydroxyindole, 3-acetic acid, and 5 HT, serotonin.

<table>
<thead>
<tr>
<th>Line</th>
<th>Treatment (mg/Kg)</th>
<th>Line × treatments Interaction</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1.25</td>
<td>2.50</td>
</tr>
<tr>
<td>NE (µg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>2.58 ± 0.16</td>
<td>2.57 ± 0.17</td>
<td>2.39 ± 0.17</td>
</tr>
<tr>
<td>LWS</td>
<td>2.31 ± 0.17</td>
<td>1.70 ± 0.17</td>
<td>2.27 ± 0.16</td>
</tr>
<tr>
<td>Epi (µg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>0.042 ± 0.002</td>
<td>0.032 ± 0.002</td>
<td>0.035 ± 0.003</td>
</tr>
<tr>
<td>LWS</td>
<td>0.062 ± 0.002</td>
<td>0.031 ± 0.002</td>
<td>0.032 ± 0.003</td>
</tr>
<tr>
<td>DA (µg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>0.188 ± 0.013</td>
<td>0.082 ± 0.014</td>
<td>0.088 ± 0.014</td>
</tr>
<tr>
<td>LWS</td>
<td>0.285 ± 0.014</td>
<td>0.139 ± 0.014</td>
<td>0.096 ± 0.013</td>
</tr>
<tr>
<td>DOPAC (µg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>0.043 ± 0.003</td>
<td>0.038 ± 0.003</td>
<td>0.044 ± 0.003</td>
</tr>
<tr>
<td>LWS</td>
<td>0.060 ± 0.003</td>
<td>0.049 ± 0.003</td>
<td>0.042 ± 0.003</td>
</tr>
<tr>
<td>5HIAA (µg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>0.092 ± 0.006</td>
<td>0.097 ± 0.006</td>
<td>0.101 ± 0.006</td>
</tr>
<tr>
<td>LWS</td>
<td>0.113 ± 0.006</td>
<td>0.126 ± 0.006</td>
<td>0.115 ± 0.006</td>
</tr>
<tr>
<td>5HT (µg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>0.407 ± 0.019</td>
<td>0.316 ± 0.022</td>
<td>0.310 ± 0.021</td>
</tr>
<tr>
<td>LWS</td>
<td>0.390 ± 0.021</td>
<td>0.295 ± 0.020</td>
<td>0.265 ± 0.020</td>
</tr>
<tr>
<td>DOPAC/DA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>0.238 ± 0.039</td>
<td>0.522 ± 0.043</td>
<td>0.661 ± 0.042</td>
</tr>
<tr>
<td>LWS</td>
<td>0.225 ± 0.042</td>
<td>0.366 ± 0.042</td>
<td>0.557 ± 0.041</td>
</tr>
<tr>
<td>5HIAA/5HT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>0.236 ± 0.030</td>
<td>0.318 ± 0.032</td>
<td>0.349 ± 0.032</td>
</tr>
<tr>
<td>LWS</td>
<td>0.299 ± 0.031</td>
<td>0.450 ± 0.030</td>
<td>0.505 ± 0.030</td>
</tr>
</tbody>
</table>

1Value represents Mean ± SE. Where line by sex, sex by age interaction were significant.

* = Means when line, was significant. And linear or quadratic respond to the treatments.
Table 3-2. Effect of intraperitoneal injections of reserpine on plasma norepinephrine, epinephrine, dopamine, 3,4-dihydroxy-L-phenylalanine, 5-hydroxyindole, 3-acetic acid, and serotonin concentrations of high and low body weight selected lines of chickens. NE, norepinephrine; Epi, epinephrine; DA, dopamine; DOPAC, 3,4-dihydroxy-L-phenylalanine; 5 HIAA, 5-hydroxyindole, 3-acetic acid, and 5 HT, serotonin.

<table>
<thead>
<tr>
<th>Line</th>
<th>Treatment (mg/Kg)</th>
<th>Line different</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1.25</td>
</tr>
<tr>
<td>NE (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>25 ± 6</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>LWS</td>
<td>40 ± 7</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>Epi (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>7 ± 2</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>LWS</td>
<td>7 ± 1</td>
<td>7 ± 0.9</td>
</tr>
<tr>
<td>DOPAC (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>9 ± 0.4</td>
<td>10 ± 0.4</td>
</tr>
<tr>
<td>LWS</td>
<td>10 ± 0.7</td>
<td>8 ± 0.7</td>
</tr>
<tr>
<td>5HIAA (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>1.7 ± 1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>LWS</td>
<td>1.3 ± 0.2</td>
<td>1 ± 0.1</td>
</tr>
</tbody>
</table>

1Value represents Mean ± SE. Where line by sex, sex by age interaction were significant.

* = Means when line, was significant. And linear or quadratic respond to the treatments.
CHAPTER IV

EFFECT OF HUMAN RECOMBINANT LEPTIN ON FOOD AND WATER INTAKE IN LINES OF CHICKENS SELECTED FOR HIGH AND LOW BODY WEIGHT
ABSTRACT

There is an association between autonomic nervous system output and obesity. The sympathetic nervous system stimulates lipid metabolism and regulates food intake and, hence body weight. Leptin, produced by adipocytes in proportion to their size, has been shown to directly stimulate the satiety center. We compared food and water intake in lines of chickens selected for high (HWS) or low (LWS) body weight at 8 weeks-of-age after central administration of human recombinant leptin. LWS chickens responded to treatment with a linear decrease in food intake; but not the HWS. The HWS chickens tended to have reduced water intake post leptin administration. Others reported that leptin decreased food intake in both broiler and Leghorn chickens. Leptin concentration in the central nervous system may not contribute directly to the difference of body weight between HWS and LWS chickens. Studies reported that obesity may also be influenced by leptin receptor binding, leptin receptor concentration, STAT 5 signaling pathway, and neuropeptides related to the pathway. Central administration of human recombinant leptin to LWS chickens increases the production of alpha- melanocyte-stimulating hormone (α-MSH), and over stimulates the melanocortin receptor. Since LWS birds are anorexic and it is possible that they are hypersensitive to α-MSH. These factors could be explain in party why the response to the leptin is different in chickens of the HWSand LWS lines.

Key Words: chicken, food intake, intracerebroventricular, human recombinant leptin, water intake
INTRODUCTION

Obesity is a prevalent nutritional disorder in affluent societies. It is believed that obesity is caused by multiple factors. Studies have shown that the autonomic nervous system (ANS) regulates energy uptake, food intake, and body weight (Bray, 1991; Bray and York, 1979; Yang et al., 1996). The sympathetic nervous system is pivotal in the regulation of lipolysis. Lipogenesis and lipolysis in adipose tissue provides large energy reserves (Hales et al., 1978). Stimulation of the sympathetic nervous system causes fatty acid release and decreases adipose tissue weight. If sympathetic or ganglionic pathways are blocked, mobilization of lipid is inhibited (Rebuffe-Scrive, 1991).

Part of the ANS control of food intake is mediated by a network of leptin, bioamines, and orexigenic neuropeptides (Ingvartsen and Boisclari, 2001). Studies using mature adipocytes in vitro or using adipocytes in primary cell culture have shown that catecholamines inhibit leptin production (Mitchell et al., 1997; Gettys et al., 1996; Hardie et al, 1996).

Leptin, a 16kDa polypeptide hormone encoded by the obese (ob) gene, is produced by adipocytes (Zhang et al., 1994). Leptin has various functions which include regulation of body weight, food intake, and neuroendocrine responses through the ANS pathway (Friedman and Halaas, 1998; Ahima et al., 2000). Fox et al. (2000) showed that intracerebroventricular (ICV) injection of leptin into lean and obese female Zucker rats caused decreased food intake and body weight gain.

Various animal models have been used to study obesity. Obese rodents (fa/fa, ob/ob, db/db mutants) and humans have a decreased sympathetic nervous system response and down-regulated beta-adrenoceptors in white adipose tissue compared to
lean counterparts (Collins et al., 1997; Collin et al., 1999; Breslow et al., 1997). This is manifested by both decreased fatty acid mobilization and decreased responsiveness of the leptin system to sympathetic stimulation (Rayner and Trayhurn, 2001).

The present study was conducted using lines of chickens selected for more than forty generations for either high (HWS) or low (LWS) body weight (Siegel 1970). The body weight difference is caused by multiple genes, thus making these lines of chickens a good model for human obesity. Most studies investigating body weight regulation use ob/ob mice or other animal models possessing a single gene alteration affecting body weight. However, human obesity is not due to such defects. Therefore, knockout or transgenic mice are limited in their ability to model human obesity. Recently, it has been reported that the chicken is genetically closer to that of humans than are mice (Burt et al., 1999; Hedges and Kumar, 2002). Chickens selected for altered body weight may provide an additional model for studying obesity.

Pervious research from our laboratory showed that HWS chickens have a higher parasympathetic and lower sympathetic nervous system tone than LWS chickens (Kuo et al., 2001). Intracerebroventricular administration of human recombinant leptin decreased food intake in Leghorn chickens (Denbow et al., 2000). However, Bungo et al. (1999) reported that mouse leptin did not alter the food intake in the chickens. The aim of this study was to compare food and water intake of HWS and LWS chickens in response to human recombinant leptin.
MATERIALS AND METHODS

Animals

Day-old male HWS and LWS chicks were vaccinated for Marek’s disease and placed in electric heated batteries with raised wire floors. Birds were provided a starter (20% crude protein, 2,684 kcal/ kg of metabolizable energy) diet and water ad libitum. The birds were exposed to continuous fluorescent lighting. At 12 weeks-of-age the birds were transferred to individual cages measuring 17.6×26.4×17.6 cm. Each cage was supplied with individual feeders and waterers.

Surgical Preparation

At 12 weeks-of-age, chickens from the HWS and LWS lines were anesthetized with sodium pentobarbital (25 mg/kg body weight iv) and a 23-gauge thin-walled stainless steel guide cannula was stereotaxically implanted into the right lateral cerebral ventricle (Denbow and Van Krey, 1987). Placement of the cannula into the ventricle was verified by the presence of cerebrospinal fluid in the guide cannula. Birds were allowed a minimum of 3 days recovery prior to injection.

Experiment Design

Male HWS (n=36) and LWS (n=36) chickens were assigned to treatments in a replicated Latin Square design in which birds and days were the blocking factors. All treatments were administered with artificial cerebrospinal fluid (aCSF) as the vehicle (Anderson et al., 1972). Birds were injected with 0, 5, 10, 20 µg of human recombinant leptin (Calbiochem, La Jolla, CA) in total volume of 10 µL using a Hamilton syringe with a 60-cm length of PE-20 tubing (Clay Adams). Feed and water intake was monitored at 15-min intervals from 0 to 180 min postinjection.
**Statistical Analysis**

Cumulative feed and water intake was analyzed using analysis of variance at each time period. Individual bird body weight was used as a covariant. Treatment effects were partitioned into linear and quadratic contrasts to determine the dose-response relationships at each time period (SAS, 1999-2001).

**RESULTS AND DISCUSSION**

Intracerebroventricular injection of human recombinant leptin to chickens from the LWS line caused a linear decrease in food consumption from 60 to 180 min postinjection. Results are summarized in Figure 4-1 and 4-2. However, leptin did not affect food intake in HWS chickens, but there was a tendency to have reduced water intake post leptin administration (P ≤ 0.1).

Previous experiments conducted in our laboratory showed that ICV injection of human recombinant leptin decreased food intake in broiler and Leghorn chickens (Denbow et al., 2000). Broilers have been genetically selected for rapid muscle and body weight gain (Gous, 1986), while Leghorns have been indirectly selected for slow growth and low body weight as a result of selection for high egg production (Kinney, 1969). The central administration of leptin has been shown to decrease food intake in both lean and obese Zucker rats (Wang et al., 1998). While both broilers and Leghorns, lean and obese Zucker rats, and the LWS line of chickens responded to leptin, the HWS chickens did not. This phenomenon may be attributed to several reasons.

According to Dridi and Taouis (2001), chickens of higher body weight have higher leptin concentrations in the plasma than chickens with lower body weight. Results from an unpublished experiment (USDA) demonstrated HWS and LWS birds did not
differ in leptin mRNA expression. Therefore, plasma leptin concentration may be not the cause for differences in body weight and food intake between HWS and LWS chickens.

Leptin is involved with both orexigenic and anorexigenic pathways in the hypothalamus (Baskin et al., 1999). While \(db/db\) mice produce normal concentrations of leptin, a mutation in both alleles of the leptin receptor gene in these mice produces a truncated protein lacking the intracellular signaling domain (Tartaglia, 1995). Based on the result of parabiosis studies of \(ob/ob\) and \(db/db\) mice, the \(ob/ob\) mice have decreased body fat, but not \(db/db\) mice (Coleman, 1973). Since the \(db/db\) mice have mutated leptin receptors, the binding of leptin does not activate of JAK/STAT signaling pathway and no leptin-induced gene regulatory changes occur (Vaisse et al., 1996). The \(fa/fa\) mouse contain a missense mutation in its leptin receptor gene, which allows activation of the STAT 1 and 3 pathway, but not STAT 5 and thus leading to obesity (Takaya et al., 1996; Good, 2000).

The binding of leptin and its leptin receptor in HWS chickens may be similar to that in \(db/db\) and \(fa/fa\) mice. Leptin receptors are located on both Neuropeptie Y (NPY) and pro-opiomelanocortin (POMC) secreting neurons. Binding of leptin up-regulates POMC, and down-regulates NPY mRNA expression (Baskin et al., 1999). Leptin receptor downstream signaling proteins have been suggested to converge on and regulate NPY gene expression. Like the leptin receptor, NPY could potentially be regulated at a number of levels (Good, 2000).

Alpha- melanocyte-stimulating hormone (\(\alpha\)-MSH) is produced from POMC neurons. Stimulation of the \(\alpha\)-MSH receptor could lead to anorexia (Ollmann et al., 1997), which supports the role of POMC and \(\alpha\)-MSH in body weight regulation. Levels
of POMC mRNA are increased by leptin treatment (Schwartz et al., 1997). It is likely that the POMC gene is a direct target of leptin receptor signals. The binding of α-MSH to the melanocortin (MC) receptor activates anorexic pathways which reduce food intake (Blevins et al., 2002). Schioth et al. (2003) reported that stimulation of the MC-4 receptor induced an anorexic condition in rats. Dunnington and Siegel (1997) showed that some chickens from the LWS lines exhibit anorexia symptoms. It is possible that LWS chickens are either more sensitive to leptin or have a greater concentration of leptin receptors than HWS birds. This may produce a greater amount of α-MSH and over-stimulate the MC receptor which decreases food intake during the central administration of leptin and further induces anorexia.

The leptin regulation pathway involves several different neuropeptides, and several complex intracellular pathways. It is know that human obesity is caused by multiple factors. The HWS lines of chicken did not decrease food intake after the central administration of human recombinant leptin. This suggests the possibility that the leptin receptor, down stream neuropeptides regulation, or the JAK-STAT pathway may be involved in the differences between chickens from the HWS and LWS lines.
LITERATURE CITED


Good DJ. How tight are your genes? Transcriptional and posttranscriptional regulatin of the leptin receptor, NPY, and POMC genes. Horm Behav 2000;37:284-98.


Figure 4-1. Effect of intracerebroventricular injection of human recombinant leptin on food intake of low body weight selected and high body weight selected lines of chickens; aCSF, artificial cerebrospinal fluid; HWS, high body weight selected; LWS, low body weight selected; MIN, minutes, PSE, treatment standard error; LIN, linear respond; QUAD, quadratic respond; +, P ≤0.05.
Figure 4-2. Effect of intracerebroventricular injection of human recombinant leptin on water intake of low body weight selected and high body weight selected lines of chickens; aCSF, artificial cerebrospinal fluid; HWS, high body weight selected; LWS, low body weight selected; MIN, minutes, PSE, treatment standard error; LIN, linear respond; QUAD, quadratic respond; +, $P \leq 0.05$. 
CHAPTER V

LEPTIN, INSULIN-LIKE GROWTH FACTOR 1 AND 2 CONCENTRATION IN PLASMA OF CHICKENS SELECTED FOR HIGH AND LOW BODY WEIGHT
ABSTRACT

Leptin, IGF (insulin like growth hormone)-1 and IGF-2 regulate growth, body development, nutrition metabolism, and sexual maturity. High-(HWS) and low-(LWS) body weight selected lines of chickens which were developal from a common base serve as obese and anorexic models respectively due to body weight differences, and other physiological and endocrine differences after 44 generations of selection. The aim of this study was to determine the leptin, IGF-1 and IGF-2 concentrations in the plasma of these chickens. Leptin, IGF-1 and IGF-2 levels were significantly higher in the LWS than HWS chickens at the 225 days-of-age. A line by sex interaction was detected for leptin concentration. The HWS female leptin concentrations were significantly lower than HWS males and LWS females. Males had greater IGF-1 concentrations in the plasma than female chickens. However, the concentration of IGF-2 did not differ between sexes. A comparison of plasma concentrations of these hormones at the 29th and 44th generations was made. The IGF-1 concentrations were similar. However, in the present study, the IGF-1 concentration was different between sexes, an effect was not observed in the 29th generation. The difference in leptin concentrations in these lines and sexes may explain in present, the differences between the age of sexual maturity. Male chickens had greater IGF-1 concentrations, which is similar to others species. The obesity and anorexia condition may also be related to the regulation of IGF-1 and IGF-2 in circulation. High IGF-2 concentration is due to greater sympathetic tone and better coupling mechanisms for stress. Differences in the IGF-1 and IGF-2 may explain obese and anorexic conditions, fast and slow growth, high and low food consumption and differences in feed efficiency between these two lines of chickens.
INTRODUCTION

Leptin is a 16 kDa polypeptide secreted from adipocytes, which binds to the leptin receptor in the acurate nucleus of the hypothalamus and its serves in a negative feedback to control food intake and energy expenditure (Ashwell et al., 1999; Denbow et al., 2000). Growth hormone (GH) is secreted from the anterior pituitary, and mediates development in the organism. It was reported that administration of GH decrease food intake in various animals (Florkowski et al., 1996; Nam et al., 1995, Vasilatos-Younken et al., 1998; Wray-Cahen et al., 1991). Intracerebroventricular (ICV) injection of leptin stimulated GH production, thus showing a relationship between GH and leptin (Considine, 1997).

The effects of growth hormone are mediated by somatomedins, also known as insulin like growth factor (IGF)-1 and 2 (Schoenle et al., 1982). When GH is released or GH receptor concentration in the liver increases, the stimulation of the IGF-1 secretion occurs from the liver (Vasilatos-Younken et al., 1999). The IGF-1 and 2 are structurally related proteins with multiple effects on growth, development and metabolism in mammals and birds (Rotwein 1991; Jones and Clemmons, 1995; and McMurtry et al., 1997).

During puberty, IGF-1 concentration is high, leading to stimulation of muscle and bone growth (Coleman et al., 1994). The IGF-1 inhibits the function of insulin, and causes fat metabolism for tissue growth. Vickers et al. (2001) reported that IGF-1 treatment reduced hyperphagia and obesity in rats, similar to results using GH. The IGF-2 is needed for embryo and fetal development, and the function is similar to IGF-1.
(Stewart and Rotwein, 1996). During adulthood, IGF-2 is also an indicator of stress (Kluge et al., 1995). It has been demonstrated that the beta-adrenergic receptor could mediate IGF-2 expression to reduce stress (Tritos et al., 1998).

Leptin, and IGF-1 and 2 are involved in growth. Indirect interactions between these compounds have been studied. The ICV injection of leptin increased the GHRH and GH gene expression levels in the central nervous system (Carro et al., 1997; LaPaglia et al 1998). Hardie et al. (1996) reported that incubation of isolated rat adipocytes with GH or IGF-1 in culture had no effect on leptin synthesis or secretion. However, Reul et al. (1997) reported that IGF-1 inhibited dexamethasone-induced leptin expression in rat adipose tissue. Combined these results indicate that leptin regulates GH synthesis in the pituitary, and is further related to IGF-1 and 2 regulation.

In avians, leptin is expressed in both adipose and liver tissue, unlike in mammalian in which it is expressed only in white adipose tissue (Ashwell et al., 1999). This difference is attributable to differing avian lipid metabolism where the liver is the primary source of lipogenesis, and is the major organ from which IGF-1 and IGF-2 are secreted (Ashwell et al., 1999).

In lines of chickens selected for high (HWS) or low (LWS) body weight for over 44 generations, growth rate, food consumption and body weight differ significantly (Dunnington and Siegel, 1996). Our interest is to determine changes in leptin and IGF-1 and 2 in these two lines of chickens, and to compare their concentrations with previous generations.

MATERIALS AND METHODS

Animals
Day-old male HWS and LWS chicks were vaccinated for Marek’s disease and placed in electric heated batteries with raised wire floors. Birds were provided a starter (20% crude protein, 2,684 kcal/kg of metabolizable energy) diet and water *ad libitum*. The birds were exposed to continuous fluorescent lighting. At 8 weeks-of-age the birds were transferred to individual cages measuring 17.6×26.4×17.6 cm. Each caged was supplied with an individual feeder and waterer.

*Sample Collection*

At 225 days-of-age, whole blood was collected from 30 HWS males, 61 HWS females, 30 LWS males and 90 LWS females. Blood was collected from the brachial vein using a syringe coated with EDTA (0.1mM). The blood was centrifuged at 3000 xg for 20 min, and the plasma collected and stored at −80 °C until analysis.

Leptin, IGF-1, and IGF-2 concentrations in the plasma were determined using the radioimmunoassays (McMurtry et al., 1994; McMurtry et al., 1998). Intra assay coefficients of variations were 2.9%, 2.3%, and 3.5% for leptin, IGF-1 and IGF-2, respectively.

*Statistical Analysis*

Data were analyzed using ANOVA with line, sex, and the interaction between them, as the main effects (SAS, 1999). When the interactions between line and sex was significant, comparisons were made within each line and sex. All values are reported as means ± SE. Significant differences imply P ≤ .05.

**RESULTS AND DISCUSSION**

Leptin, IGF-1 and IGF-2 concentrations were significantly higher in LWS chickens. The HWS females had lower plasma leptin concentrations than LWS female
and HWS male chickens (Figure 5-1). Leptin and IGF-1 concentrations were greater in males than females (Table 5-1.)

Elevated leptin concentrations in the central and peripheral systems, which inhibited the neuropeptide Y neuron, further decreased neuropeptide secretion, and decreased the food intake (Denbow et al., 2000; Coleman, 1973). Therefore, it appears that LWS chickens have greater leptin concentration in circulation, and over inhibit neuropeptide Y neurons, and causing an anorexia condition.

HWS chickens are obese (Liu et al., 1994). They have increased abdominal fat (3% of the total empty body weight) (Katanbaf et al., 1988), feed consumption, and body weight (Dunnington et al., 1994), and need to be placed on feed to prevent morbid obesity resulting in decreased egg production (Liu et al., 1994). Kaseki et al. (2003) indicated that low concentrations of leptin in blood inhibit ovulation in humans. Obese Zucker rats have a mutated leptin receptor and show reproductive dysfunction (Fox and Olster, 2000). These results may relate to the obesity and low egg production found in HWS chickens.

Obese Zucker females are infertile and hyporesponsive to the inductive effects of ovarian hormones on sexual behaviors (Marin-Bivens et al., 1998). Increasing leptin concentration in the plasma reverses reproductive dysfunction due to perturbations in energy balance in other animal models (Fox and Olster, 2000). In our study, HWS females had the lowest plasma leptin concentration and exhibited lower egg production than other chickens. This might be similar to the condition found in the obese Zucker female rats.
Previous studies (USDA, unpublished data) showed that at 5 weeks-of-age, leptin concentrations were similar for HWS and LWS chickens. However, at 225 days-of-age, the leptin concentrations were different between lines (LWS>HWS) and sexes (Male>Female). It was found in humans that women have different leptin levels in the serum before and after ovulation, indicating that the change was due to the sexual maturity (Kaseki et al., 2003). Because of differing sexual maturity ages, and obese and anorexia condition in these two lines of chickens, the difference in plasma leptin concentration could be greater between lines and sex after the birds reached sexual maturity (Dunnington and Siegel, 1997).

In the present study, the IGF-1 and IGF-2 concentrations of HWS and LWS chickens were measured in chickens from the 44th generation while Scanes et al. (1989) reported the values for chickens from the 29th generation. Although in both studies found that IGF-2 concentrations were similar for both sexes, several differences are seen between these two studies. First, in the 44th generation LWS chickens at 225 days-of-age had greater IGF-1 levels than HWS line. However, in the 29th generation, no difference between these lines at 325 and 1,054 days-of ages in IGF-1 were detected (Table 5-2). Second, in the 44th generation HWS and LWS male chickens had greater IGF-1 concentrations in the plasma than females, however, no sex difference was found in the 29th generation study (Scanes et al., 1989). Third, the 44th generation LWS had greater plasma IGF-2 concentrations than HWS chickens, a result opposite that observed in the 29th generation birds (Table 5-2). Therefore, continuous divergent selection for juvenile body weight has resulted in different physiological mechanism altering leptin, IGF-1 and IGF-2 levels and of feeding behavior between these lines.
The HWS females reach sexual maturity before the LWS line (Dunnington and Siegel, 1996). It is possible that the HWS chicken IGF-1 and IGF-2 concentrations started to decrease before the LWS, but this response was not measured. Perhaps selection shifts the terminal rate of change diminished concentration of these peptides.

Nir et al. (1987) measured the concentration of GH in HWS and LWS lines of chickens at 25 and 61 days-of-age. The GH levels were significantly lower in the HWS than LWS chickens at both ages. Gianotti et al. (1998) reported that humans with anorexia nervosa have hypersecretion of GH while obese humans have a lower GH level. Maccario et al. (1999) reported that obese humans have lower plasma IGF-1 concentrations. Van Dam et al., (2002) indicate that obese women have reduced plasma GH concentrations. It is known that IGF-1 and GH concentrations correlate to each other (Coleman et al., 1994). Our results support these findings since LWS chickens have higher IGF-1 levels than HWS chickens.

Male turkeys have greater IGF-1 levels than females (Bacon et al., 1993). Viswanadha et al. (2002) also reported that mature male mice have significantly higher IGF-1 concentrations in the plasma. We also found that male chickens exhibit greater plasma IGF-1 concentrations. Male animals tend to have greater muscle and bone mass than females, which may be associated with elevated levels of IGF-1 in the system.

McMurtry et al. (1998) reported that chicken IGF-2 level decreased after food withdrawal, which responds differentially to the level of nutrient deprivation, and could relate to an anorexic condition in LWS chickens. The IGF-2 concentration decreased after sexual maturity. The IGF-2 has also been implicated as a stress response (Kluge et al., 1995). Kuo et al. (2001) reported that LWS chickens have greater cardiovascular
sympathetic tone, indicated that greater stress tone. The higher IGF-2 level could be another indicator of the higher sympathetic activity in the LWS chickens.

The MONA LISA hypothesis (Most Obesities kNown Are Low In Sympathetic Activity) describes that obesity is associated with low sympathetic tone resulting in higher food intake (Bray, 1991). The interaction between the MONA LISA hypothesis and leptin has been reviewed, and leptin is perceived as a key afferent signal and the sympathetic nervous system, acting through beta receptors, is an essential element of this control system (Bray and York, 1998). Harris et al. (1998) reported that leptin increased sympathetic nervous system activity and decreased body weight in ob/ob mice (Harris et al., 1998). In our study, LWS chickens had higher plasma leptin and IGF-2 concentrations that could result in greater sympathetic tone, further inducing less body weight and food consumption in these lines of birds. Phenotypically, these two lines of chickens are diverging each generation, and endocrine regulation also has been changing through selection. It is possible that the gradual change of body weight in each generation is associated with changes in endocrine regulation.
LITURATURE CITED


Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. Endocrine Reviews 1995;16:3-34.

Katanbaf MN, Dunnington EA, Siegel PB. Allomorphic relationships from hatching to 56 days in parental lines and F1 crosses of chickens selected 27 generations for high or low body weight. Growth Dev Aging 1988;52:11-21.


Marin-Bivens CL, Kalra SP, Olster DH. Intraventricular injection of neuropeptide Y antisera curbs weight gain and feeding, and increases the display of sexual behaviors in obese Zucker female rats. Regul Pept 1998;75-76:327-34.


Viswanadha S, Wark WA, Loor JJ, Herbein JH. Alterations in tissue weights and carcass composition of growing mice fed diets containing 0, 0.15 and 0.30% t10, c12-conjugated linoleic acid. FASEB 2002;16:A1025.

Table 5-1. Plasma concentrations of leptin, IGF-1 and IGF-2 (ng/ml) concentrations in HWS and LWS, and male and female chickens at 363 days-of-age.; *, P ≤ 0.05.

<table>
<thead>
<tr>
<th>LINE</th>
<th>HWS</th>
<th>LWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>7.28 ± 0.27</td>
<td>*</td>
</tr>
<tr>
<td>IGF-1</td>
<td>4.14 ± 0.19</td>
<td>*</td>
</tr>
<tr>
<td>IGF-2</td>
<td>24.81 ± 0.65</td>
<td>*</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>SEX</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>8.83 ± 0.29</td>
<td>* 7.15 ± 0.19</td>
</tr>
<tr>
<td>IGF-1</td>
<td>7.50 ± 0.54</td>
<td>* 5.53 ± 0.27</td>
</tr>
<tr>
<td>IGF-2</td>
<td>27.07 ± 1.44</td>
<td>NS 25.89 ± 0.50</td>
</tr>
</tbody>
</table>

NS = No significant difference between lines or sexes.  
* Significant lines or sex effect
Figure 5-1. Plasma concentration of leptin in HWS and LWS chickens at 363 days-of-age; *, P ≤0.05.
Table 5-2. Plasma concentrations of IGF-1 and IGF-2 in young and adult chickens of high or low weight selected lines (Scanes, 1989); *, P ≤ 0.05.

<table>
<thead>
<tr>
<th>Population</th>
<th>HWS</th>
<th>LWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (units/ml)</td>
<td>902 ± 46</td>
<td>*</td>
</tr>
<tr>
<td>IGF-2 (ng/ml)</td>
<td>20.1 ± 0.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = No significant difference between lines or sexes.
※ Significant lines or sex effect
Table 5-3. Plasma concentrations of IGF-1 and IGF-2 in young and adult chickens of high or low weight selected lines at 51, 325, and 1054 days-of-age (Scanes, 1989); *, P ≤0.05.

<table>
<thead>
<tr>
<th>IGF-1 Age (days)</th>
<th>Population</th>
<th>HWS</th>
<th>LWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td></td>
<td>946(^{a}) ± 76</td>
<td>533(^{a}) ± 78</td>
</tr>
<tr>
<td>325</td>
<td></td>
<td>181(^{b}) ± 19</td>
<td>253(^{b}) ± 46</td>
</tr>
<tr>
<td>1054</td>
<td></td>
<td>170(^{b}) ± 21</td>
<td>207(^{b}) ± 49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IGF-2 Lines</th>
<th>Population</th>
<th>HWS</th>
<th>LWS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16.5 ± 0.8</td>
<td>15.7 ± 0.8</td>
</tr>
</tbody>
</table>

NS = No significant difference between lines or sexes.

* Significant lines or sex effect
CHAPTER VI

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN THE LEPTIN RECEPTOR GENE OF CHICKEN LINES DIVERGENTLY SELECTED FOR JUVENILE BODY WEIGHT
ABSTRACT

Divergent selection for body weight at 8 weeks-of-age for 44 generations has resulted in dramatic phenotypic differences between high weight-(HWS) and low (LWS) weight-selected lines. The primary phenotypic difference between the two lines, in addition to the selected trait, is in feed intake. The peptide hormone leptin has been implicated as a regulator of feed intake in several species, including chickens. Circulating leptin levels do not differ between the HWS and LWS lines at 5 weeks-of-age (Unpublished data). The role of the leptin receptor, however, has not been investigated and contribute to differences between these lines in food intake. Utilizing the reported chicken genomic sequence for the leptin receptor (Genbank AF222783), primers were designed to amplify the intron sequence between exon 8 and exon 9 by PCR. The cDNA primers were designed base on chicken leptin receptor cDNA sequence (Genbank AB033383.1) to amplify cDNA sequences between 916 and 1522 by Real time PCR. The genomic DNA PCR products were subjected to sequence analysis for the presence of single nucleotide polymorphisms between the HWS and LWS lines. This analysis revealed a single polymorphism at nucleotide 182 within the intron in which C was replaced by T as compared to the previously reported sequence. The T-type allele is present in the LWS line in both the homozygous (T-T) and heterozygous (T-C) forms. This single base change introduces a restriction site for the enzyme Sel I, which can be utilized for characterizing the genotype of individual birds at this locus. In cDNA sequence analysis, two single nucleotide polymorphisms were detected at nucleotide 189 and 234. At nucleotide 189, there was an extra base in the HWS and LWS lines, which was not reported in broilers or Leghorns, and a single nucleotide polymorphism was
detected in both HWS and LWS lines. A T-type allele is present in the LWS line in both homozygous (T-T) and heterozygous (C-T) form. However, only the homozygous (T-T) form was observed in the HWS line. In the predicted protein sequences, the extra base could cause a change in the protein sequences, and introduce a stop codon in both lines.

The single nucleotide polymorphism at nucleotide 234, is present in the LWS line in the homozygous (A-A) form and HWS with homozygous (T-T) form. This change introduces a restriction site *Mse I* in the HWS, but not LWS lines. These specific changes may be directly involved or closely linked to any difference in either the coding or regulatory domains of the leptin receptor that may exist between the two lines.

Key Words: DNA, heterozygous, homozygous, sequence, single nucleotide polymorphism
INTRODUCTION

The leptin receptor is a single membrane-spanning receptor that is similar to the class I cytokine receptor family (Tartaglia et al., 1995). The transcript for the leptin receptor long form is less abundant. Leptin expressed in the hypothalamus inhibits food intake by activating the leptin receptor long isoform in the brain (Ghilardi et al., 1996; Ahima and Flier, 2000). When leptin binds to its receptor, the signal activates the JAK/STAT pathway, and further inhibits feed intake (Baumann et al., 1996). Most of the long isoform leptin receptors are expressed in various locations of the brain, often being found on neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurons in the hypothalamus (Baskin et al., 1999). The concentration of the leptin receptor influences the action of the leptin on body weight and food intake regulation (Baskin et al., 1999).

Alterations in the leptin receptor were identified and found to be responsible for the phenotype of the db/db mouse (Tartaglia et al., 1995). The db/db mice carry a mutation in both alleles of the leptin receptor gene resulting in a truncated protein. Leptin can not bind to the receptor, and does not activate the JAK/STAT signaling pathway. Because of the lack of an inhibitory signal from leptin, there is increased feed intake and obesity (Vaisse et al., 1996). The fa/fa mouse contains a missense mutation in the leptin receptor gene that results in the deactivation of STAT5 activity (Takaya et al., 1996). Udy et al. (1997) found that deletion of the gene coding STAT5B caused increase fat deposition in adult mice, which decreased expression of the leptin receptor.

Leptin receptor polymorphisms could be either directly or indirectly related to obesity. Clement et al. (1998) demonstrated that a homozygous mutation in the human leptin receptor gene resulted in a truncated leptin receptor lacking both the
transmembrane and intracellular domains which caused early-onset morbid obesity. Wauters et al. (2001) identified three leptin receptor gene polymorphisms in women, and concluded that they influenced fat disposition and increased body weight. Van Rossum et al. (2003) reported a polymorphism in the leptin receptor gene between obese and lean humans. An Arg229 was found in a group of obese humans which had greater leptin concentrations in the body, but not in the lean group (van Rossum et al., 2003). Mammes et al., (2001) reported that two genetic variants, T70C (exon 1) and Asp (A) 96 Asp (G) (exon 4), were related to obesity. Zhang et al. (1997) reported that the leptin receptor mediated autocrine regulation, and the concentration of leptin and leptin mRNA expression are related to the level of leptin receptor expression. Decreasing concentration of the leptin receptor leads to decreased action of leptin in adipose tissue (Zhang et al., 1997).

Horev et al. (2000) first cloned the leptin receptor in chickens. The chicken leptin receptor gene shares an average of 60% nucleotide sequence identity with mammalian leptin receptors. Comparisons with predicted protein sequences demonstrates a tight conservation of previously characterized leptin receptor motifs and essential tyrosine residues (Horev et al., 2000). Similarities between the chicken and the mammalian leptin receptor genes were also observed in the pattern of mRNA expression (Horev et al., 2000).

The HWS and LWS lines of chickens have been selected for body weight for over 44 generations. Selection was based on body weight at 8 weeks-of-age (Siegel, 1962). Although a prior study noted (USDA, unpublished data) that leptin concentration was similar in these two lines of chickens, leptin receptor regulation has not been studied in
these lines. The purpose of this study was to determine if there are polymorphisms in the leptin receptor gene in both genomic and cDNA of these two lines of chickens.

MATERIALS AND METHODS

Genomic DNA

Whole blood were collected from 30 HWS male, 61 HWS female, 30 LWS male and 90 LWS female chickens at 225 days-of-age (Siegel, 1962). Genomic DNA was isolated using a DNA purification system (Gentra Systems). Utilizing reported chicken genomic sequence information for the leptin receptor (Genbank AF222783), primers were designed to amplify the intron sequence between exon 8 and exon 9 by PCR (Dunn et al., 2000).

cDNA

Total RNA from brain, liver, heart, lung, kidney, and fat of HWS and LWS chickens were isolated using the Tri-Reagent® protocol (Molecular Research Center, Inc. Cincinnati, OH). Synthesis of single-strand cDNA, and reverse transcriptase (RT) reactions were carried out using random primers (Promega, Madison WI), MMLV reverse transcriptase (Promega, Madison WI), and 1 µg of total RNA in 50 µl reactions.

Specific chicken leptin receptor cDNA sequence (AB033383.1) was obtained from the public GenBank sequence database of the National Center for Biotechnology Information, and primers were designed to amplify the chicken leptin receptor cDNA. Fourteen primers were designed to amplify the full length of the chicken leptin receptor cDNA. Primer 9 (ATCTGCTGGTCTGAGCCTGT) and 10 (GTTGGGTTTGCAGACCCTGT) were chosen for Real time PCR to amplify the chicken
leptin receptor cDNA, and amplification products of all tissues from both lines and sexes were analyzed.

All samples were sequenced by fluorescent BigDye-terminator chemistry using an ABI377 automated DNA sequencer (Perkin-Elmer, Foster City, CA).

RESULTS

Genome DNA

SNP-1

A single polymorphism (SNP) was found between HWS and LWS lines of chickens. The HWS line exhibited only the homozygous (C-C) form; LWS had both homozygous (T-T) and heterozygous (T-C) forms. The single base change introduced a restriction site for the enzyme Sel I (Figure 6-1.).

cDNA

SNP-2

At nucleotide 234, a SNP was found between the HWS and LWS line leptin receptor cDNA sequences. The HWS exhibited a homozygous (A-A) form, while the LWS line had a homozygous (T-T) form. This single base change introduced a restriction site for the enzyme Mse I in HWS (Figure 6-2), but not in the LWS line of chickens.

SNP-3

An extra base was found at nucleotide 189 in both the HWS and LWS lines compared to broiler and Leghorn leptin receptor cDNA sequences. The HWS line exhibited only the homozygous (T-T) form, but the LWS line had both the homozygous (T-T) and heterozygous (T-C) forms. Protein sequences of HWS and LWS chickens were predicted and compared with broiler and Leghorn leptin receptor protein sequences.
(Figure 3). This extra base and its polymorphism between HWS and LWS lines changed the protein sequences in the leptin receptor (Figure 3).

DISCUSSION

Seven polymorphisms were found between lean and obese Pima Indian’s leptin receptor sequences. These polymorphisms were related to the amino acid change in the extracellular region, silent substitution, and three of the polymorphisms were in the non-coding region (Thompson et al., 1997). This study demonstrated that non-coding region polymorphisms found only in the obese Pima Indian may not directly alter the leptin receptor, but can serve as a marker to identify leptin receptor function or other functions that are related to the leptin receptor transcription, mRNA stability or translational differences (Thompson et al., 1997). These results are similar to our study where we found a SNP (SNP-1) in the intron between exon 8 and 9 of the chicken leptin receptor. The single base change introduces a restriction site, and is possible to use as a marker to determine a difference in leptin receptor between HWS and LWS lines, or further serve as a marker to identify the phenotypic difference in feed intake and body weight.

Clement et al. (1998) demonstrated a homozygous mutation in the human leptin receptor gene that results in a truncated protein lacking both the transmembrane and the intracellular domains, which cause early-onset morbid obesity. Patients homozygous for this mutation have no pubertal development, and their secretion of growth hormone and thyrotropin is reduced. Mammes et al. (2001) reported that genotype and allele frequencies of the Ser (T) 343 Ser (C) polymorphism (exon 9) were significantly different between normal and overweight women. The T allele is more frequently observed in overweight than normal weight group, and the overweight women carrying
the C allele polymorphism lost more weight in response to low calorie diet than the non-carriers (Mammes et al., 2001). These results indicate that variation at the leptin receptor locus is associated with common obesity phenotypes and is a part of the polygenic influences on the response to the nutritional environment (Mammes et al., 2001). Quinton et al. (2001) detected a SNP that induces the Gln223Arg in the leptin receptor extracellular region in humans. Homozygous humans had an increased body mass index and leptin concentration compared with those having the G allele (Quinton et al., 2001). In our study, the SNP-2 showing different A and T alleles introduced a restriction site Mse I in the HWS, not LWS lines. This polymorphism was detected in the exon region, and it may cause a greater impact in the activity of the leptin receptor. In the predicted protein sequences, this polymorphism introduced a stop codon in the HWS, but not LWS chickens. If the prediction is correct, this polymorphism may induce differences in leptin receptor protein sequences and structure between HWS and LWS lines of chickens, relating to their differences in body weight and feed intake.

The leptin receptor mutation animal model db3J/db3J mice exhibits a frameshift in the leptin receptor protein, and it affects all of the splicing forms (Lee et al., 1997). The soluble leptin receptor protein was absent in the db3J/db3J mice, which resulted in truncation of the leptin receptor at amino acid 625 resulting in obesity (Li et al., 1998). Brown et al. (2000) discovered a mutation, which deleted a nucleotide G in the leptin receptor exon 12, that induced obesity and diabetes. This mutation caused the frameshift of the reading frame, which substitutes 11 amino acids followed by premature termination of translation before the membrane-spanning domain (Brown et al., 2000). Based on the protein sequence prediction, we found the HWS and LWS lines exhibit an extra base
(SNP-3) in the leptin receptor not observed in broilers and Leghorns. This mutation may cause a frameshift and mutation in the protein sequence. It was also predicted that the SNP-3 introduces a stop codon in both HWS and LWS chickens compared to Leghorn and broiler protein sequences. The mutations in both mRNA and predicted protein sequences may possibly cause different leptin receptor gene expression levels, and are further related to phenotypic differences between these two lines of chickens.

The HWS line exhibits obesity while some LWS line chickens are anorexic (Dunnington and Siegel, 1997). Although mutations in the leptin receptor gene induce obesity in animals (Brown et al., 2000; Lee et al., 1997), however, there is no evidence that a mutated leptin receptor induces anorexia (Hinney et al., 1998). Adan et al. (2003) reported a mutation in the melanocortin gene causing anorexia, and Kipman et al. (2002) reported a mutation in the serotonin receptor causing anorexia in rats. Based on this evidence, a mutation in the leptin receptor may be associated with anorexia.

Single nucleotide polymorphisms were detected using restriction enzymes to identify the presence or absence of cutting sites and scored by observing the resulting fragment length variation (Botstein et al., 1980). The studies demonstrated that SNP could be used to test the association with a disease trait or as a marker for linkage disequilibrium, which is a measure of the degree of association of two genetic markers, or they could be used to identify those regions of the genome associated with disease in a population (Kwok and Chen, 2003; Gray et al., 2000). In animals, the advantage of SNP is that they are usually linked to the gene of interest, and the association of the SNP with traits of economic importance can be analyzed using candidate gene approaches (Emara
and Kim et al., 2003). Similar to human research, SNP has been used to identify disease resistance genes in chickens (Emara and Kim, 2003).

In a chicken genetic mapping study, it was found that loci have homology with known human or mammalian genes (Emara and Kim, 2003). Recently, it was reported that the chicken is genetically closer to humans than mice (Burt et al., 1999; Hedges and Kumar, 2001). The SNP that in leptin receptor sequences in the HWS and LWS lines may be similar to mutations in the human leptin receptor gene. This may provide some explanation between obesity and the regulation of the leptin receptor in both human and animals.


Figure 6-1. Leptin receptor exon 8 to 9 partial cDNA and genomic DNA sequences of HWS and LWS lines of chickens.

Figure 6-2. Polymorphism detection of liver leptin receptor cDNA sequences of HWS and LWS chickens.
LWS 1---TTNNNAGNCCTTTTGGGGAACCCCGCCGGGTTTTGACGTCTACCCAAATTCAGATCAA  60
HWS 53  ATGACTGGCAGGTGGTTCTTGTTGCTTTAAATACCTCATTAGACATAGACAATATGCTG  111
LWS 61  ATGACTGGCAGGTGGTTCTTGTTGCTTTAAATACCTCNTTAGACATAGACAATATGCTG  119
HWS 112 CTTGATTCTTCCTCCTTTGCTCAAGTGAGGTGCAAGAGTCATTGTGGTCCCGGGTTCTGG  171
LWS 120 CTTGATTCTTCCTCCTTTGCTCAAGTGAGGTCCAAGAGTCATTGTGGTCCCGGGTTCTGG  179
HWS 172 AGTGAATGGAGACACACTTGTATAATCTGAATGTGGGAGCTGAAGTGCTGGTACTTCCCT  230
LWS 180 AGTGAATGGAGACACMYGTATAATCTGAATGTGGGAGCTGAAGTGCTGGTACTTCCCT  238

Mse I
HWS 231 TTTTAAAGATACTGACCAGTGGTGTGTTCTAACCCTTTTCGTTTCTATTGCATCTATAAAAACAA  289
LWS 239 TTTTAGATACTGACCAGTGGTGTGTTCTAACCCTTTTCGTTTCTATTGCATCTATAAAAACAA  297
HWS 290 AACCCAGAGCTACGCTCAAGAAGATTGTTTGCTGAATTTAGCAGAAGAATCC  349
LWS 298 AACCCAGAGCTACGCTCAAGAAGATTGTTTGCTGAATTTAGCAGAAGAATCC  357
HWS 350 CAGAAAGTCAGTATACTCTGGTGAACCGCTCGCCTGGGGATCCCTATCNAACTGAA  410
LWS 358 CAGAAAGTCAGTATACTCTGGTGAACCGCTCGCCTGGGGATCCCTATCNAACTGAA  418
HWS 411 CCCGGGAGGGGGACTTTAACTAAAAAGGACTTTGCAGATGCGCCCTGCAACCCCAAAACCGGC  470
LWS 419 CCCGGGAGGGGGACTTTAACTAAAAAGGACTTTGCAGATGCGCCCTGCAACCCCAAAACCGGC  478
HWS 471 AAAAAATAGGGGATTTTTCTCTATAGGATCCGCTGTTTTTTTTTGGGGGGAGGGGGCTGC  529
LWS 479 AAAAAATAGGGGATTTTTCTCTATAGGATCCGCTGTTTTTTTTTGGGGGGAGGGGGCTGC  537
HWS 530 AACCCCAAAATGGG------------------825
LWS 538 AACCCCAAAATGGG------------------800
Figure 6-3. Leptin receptor cDNA and predicted protein sequences of broiler, Leghorn, HWS and LWS chickens.

<table>
<thead>
<tr>
<th></th>
<th>HWS</th>
<th>LWS</th>
<th>Leghorn</th>
<th>Broiler</th>
</tr>
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<tbody>
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<td>67</td>
<td>853</td>
<td>1001</td>
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<td>CACCGCGCGCTGTTGGAATCTACCCAAATTCAGAT</td>
<td>CGCTCCGGTGTGAAGTGGAACATCTTCTCTGGAATTACGTTACAAAATGACTG</td>
<td>CGCTCCGGTGTGAAGTGGAACATCTTCTCTGGAATTACGTTACAAAATGACTG</td>
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<tr>
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<td>902</td>
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<tr>
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<td>GCAG---GTGGTTATTGTTGCTTTAATACCTCATTAGACATAGACAAAT</td>
<td>GCAG---GTGGTTATTGTTGCTTTAATACCTCATTAGACATAGACAAAT</td>
<td>GCAG---GTGGTTATTGTTGCTTTAATACCTCATTAGACATAGACAAAT</td>
</tr>
<tr>
<td>LWS</td>
<td>Q V V I V A L N T S L D I D N</td>
<td>Q V V I V A L N T S L D I D N</td>
<td>Q V V I V A L N T S L D I D N</td>
<td>Q V V I V A L N T S L D I D N</td>
</tr>
<tr>
<td>Broiler</td>
<td>Q V V V A L N T S L D I D N</td>
<td>Q V V V A L N T S L D I D N</td>
<td>Q V V V A L N T S L D I D N</td>
<td>Q V V V A L N T S L D I D N</td>
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<td>1097</td>
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<td>Length</td>
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<td>213</td>
<td>213</td>
<td>1097</td>
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<tr>
<td>HWS</td>
<td>TGGTCCCAGGTGTCTCTGATGATGGAGTGCAAGAGACACTTGTATAATCTGAAATGTTG</td>
<td>TGGTCCCAGGTGTCTCTGATGATGGAGTGCAAGAGACACTTGTATAATCTGAAATGTTG</td>
<td>TGGTCCCAGGTGTCTCTGATGATGGAGTGCAAGAGACACTTGTATAATCTGAAATGTTG</td>
<td>TGGTCCCAGGTGTCTCTGATGATGGAGTGCAAGAGACACTTGTATAATCTGAAATGTTG</td>
</tr>
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CHAPTER VII

QUANTITATIVE ANALYSIS OF LEPTIN RECEPTOR AND ACETYL-COA mRNA IN HIGH AND LOW BODY WEIGHT SELECTED CHICKENS USING REAL TIME POLYMERASE CHAIN REACTION
ABSTRACT

Several observations in mammals suggest that leptin inhibits food intake by activating the leptin receptor long isoform in the hypothalamus. Using lines of chickens divergently selected for high or low body weight at 8 weeks-of-age, the role of the leptin receptor long isoform on body weight regulation was investigated. Based on leptin/beta actin CT ratio analysis, the leptin receptor expression in the whole brain was significantly different between sexes at 28 days-of-age in the high body weight selected (HWS) and low body weight selected (LWS) lines of chickens. There was a line by age interaction in leptin receptor expression of the liver. The LWS birds had higher leptin receptor gene expression in the liver at 2 days-of-age, than at 56 and 363 days-of-age. However, this pattern was not observed in the HWS line. In addition, at 2 days-of age, leptin receptor gene expression was higher in LWS than HWS chickens while the opposite was found at 363 days-of age. In the adipose tissue, leptin receptor expression was higher than in the LWS than HWS line. At 363 days-of-age, the leptin receptor expression in the fat tissue was greater than at 28 and 56 days-of-ages. The acetyl-CoA enzyme gene expression was higher at 56 days-of-age than at 2, 28, and 363 days-of-age. The results from the inflection point cycle number analysis were different than the CT ratio analysis. Our results show that the regulation of leptin and leptin receptor are involved with body weight, sex, and age.

Key Words: acetyl-CoA enzyme cycle threshold cycle number, gene expression, leptin receptor, inflection point cycle number
INTRODUCTION

Autonomic nervous system activity is related to body weight regulation. Based on the MONA LISA hypothesis (Bray, 1991), it has been suggested that obesity is generally associated with low sympathetic nervous system activity. Campfield et al. (1996) reviewed several studies and concluded that leptin appears to play a major role in the control of body fat stores through coordinated regulation of feeding behavior and the autonomic nervous system in rodents, primates and humans.

The leptin receptor is a single membrane-spanning receptor that is similar to the class I cytokine receptor family (Tartaglia et al., 1995). The majority of transcripts in most tissues are encoded either in long or short forms (Tartaglia et al., 1995). The transcript for the leptin receptor long form is less abundant; leptin expressed in the hypothalamus may inhibit food intake by activating the leptin receptor long isoform in the brain (Ghilardi et al., 1996; Ahima and Flier, 2000). When leptin binds to its receptor, the signals activate the JAK/STAT pathway, and further inhibit feed intake (Baumann et al., 1996). The leptin receptor was identified, and was found to be responsible for the phenotype of the db/db mouse (Tartaglia et al., 1995). Most of the long isoform leptin receptors are expressed in various locations of the brain, including on neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurons in the hypothalamus (Baskin et al., 1999). The concentration of the leptin receptor influences the action of the leptin to body weight and food intake regulation (Baskin et al., 1999). Leptin receptor gene mRNA and protein, as well as leptin-binding sites, are concentrated in the arcuate, ventromedial, and dorsomedial nuclei (Madiehe et al., 2001). Evidence suggests that the leptin receptor can be expressed at different levels of the neuroaxis (Elmquist et al.,...
Mice with a mutation in the leptin receptor are hyperphagic and severely obese (Chen et al., 1996; Lee et al., 1997).

Studies using animals with a mutated leptin receptor indicated that the leptin receptor is necessary for maintaining normal body weight (Chen et al., 1996; Takaya et al., 1996). Furthermore, leptin receptor expression is influenced by diet, fasting, and hormone balance (Bennett, 1998; Bennett et al., 1999).

Acetyl-CoA carboxylase (ACC) catalyzes the formation of malonyl-CoA from acetyl-CoA in the formation of the long chain fatty acid (Wakil et al., 1983). The ACC enzyme involves covalent modification and allosteric mechanisms, and the enzyme is interconverted by phosphorylation-dephosphorylation mechanisms (Wakil et al., 1983). Birds can store large quantities of excess energy in the form of triglycerides in the liver and adipose tissue (Hermier, 1997). The avian liver is the primary source of avian lipogenesis, and is able to secrete leptin (Ashwell et al., 1999; O’Hea and Leveille, 1968). Leptin administration to rats fed fat-free diets significantly suppressed ACC and fatty acid synthase mRNA levels; indicating that leptin mediates fatty acid biosynthesis by changing lipogenic enzymes mRNA levels in vivo (Iritani et al., 2000).

Mouse models containing either a leptin mutation (ob/ob) or leptin receptor gene mutation (db/db) have been used to study obesity (Harris et al., 1998). However, most obesities are not caused by a single gene or factor. The lines of chickens used in the study reported here provide a unique genetic model for studying the regulation of body weight since they have undergone long-term divergent selection for high- or low-juvenile body weight and involved multigenetic changes.
The foundation stock for high-(HWS) and low-body weight selected (LWS) lines of chickens consisted of crosses of seven inbred lines of White Plymouth Rocks (Siegel, 1962). From this segregating gene pool, two lines were selected on the basis of body weight at 8 weeks-of-age. After forty generations of selection for body weight, the differences of body weight between HWS and LWS lines is 8-fold at selection age. The LWS females do not consume enough feed *ad libitum* to attain a threshold of body weight, body composition, or both, which are essential to commence egg production (Zelenka et al., 1987). In the HWS line, a feed restriction program is used after 8 weeks-of-age to prevent the impaired egg production. Since feed consumption exceeds maintenance skeletal and lean body weight needs, fat deposition increases in the HWS birds. The resulting obesity is accompanied by undesirable reproductive complications (Katanbaf et al., 1998; Liu et al., 1994). These results indicate that selection for high-body weight has resulted in obesity.

A preliminary study indicated that HWS have a higher parasympathetic and lower sympathetic nervous system tone than the LWS counterparts (Kuo et al., 2001). Further research is needed to characterize the relationship between the leptin receptor, body weight and food intake regulation at different body weights. The ACC gene expression between these two lines of chicken was also determined.

**MATERIALS AND METHODS**

*Animals*

Day-old male and female HWS and LWS chicks were vaccinated for Marek’s disease and placed in electric heated batteries with raised wire floors. Birds were provided a starter (20% crude protein, 2684 kcal/ kg of metabolizable energy) diet and
water available for *ad libitum* consumption. The birds were exposed to continuous fluorescent lighting. At 28 days-of-age, the birds were transferred to grower batteries (75.12 cm W × 38.10 cm L × 38.10 cm H).

Brain, liver, heart, lung, kidney, and adipose tissues were collected from 12 birds per line per sex at 2, 28, 56 and 363 days-of-ages. Tissues were collected and quick frozen in liquid nitrogen and stored at –80 °C until analysis.

*Total RNA Isolation and cDNA Synthesis*

Total RNA was isolated using the Tri-Reagent® protocol (Molecular Research Center, Inc. Cincinnati, OH). The integrity of RNA was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density at 260 and 280 nm. Synthesis of single-strand cDNA, and reverse transcriptase (RT) reactions were carried out using random primers (Promega, Madison WI), MMLV reverse transcriptase (Promega, Madison WI), and 1 µg of total RNA in a 50 µl reaction. PCR amplifications were performed using oligonucleotide primers specific for chicken beta actin that produced a product of 612 bp.

*PCR Primers Design and Sequencing Analysis*

Specific chicken leptin receptor cDNA sequences (AB033383.1) were obtained from the public GenBank sequence database of the National Center for Biotechnology Information, and primers were designed. Fourteen primers were designed to amply the full length chicken leptin receptor cDNA. Primer 9 (ATCTGCTGGTCTGAGCCTGT) and 10 (GTTGGGTTTGAGCAGACCATC) were chosen for Real time PCR to detect chickens leptin receptor gene expression.
SYBR Green Real-time PCR

Real time PCR was performed (DNA Engine Opticon, MJ Research, Inc, Waltham, MA) to analyze chicken leptin receptor gene expression, using QuantiTect® SYBR® Green PCR kit (Qiagen, La Jolla, CA). Amplification was carried out in a total volume of 25 µl containing 1× SYBR Green master mix, 0.3 µM of each primers, RNase-free water and 1 µl of cDNA. Each sample was amplified with chicken leptin receptor primers or beta actin primers which served as an external control.

Data Analysis

Cycle Threshold Ratio Analysis

Cycle threshold (CT) is defined as the cycle number at which fluorescence is distinguishable from noise. This point appears during the exponential phase of the PCR reaction and is inversely proportional to the initial number of template molecules in the sample. Results were expressed as relative expression. The cycle number for expression of leptin receptor CT was calculated and normalized with the beta actin CT (Meijerink et al., 2001).

Inflection Point Cycle Number Analysis

The fluorescence level of each cycle was determined to plot symmetrical lines and equations for each reaction. The inflection point cycle number of leptin receptor and beta actin was determined and beta actin served as co-variance.

Data were analyzed using ANOVA with line, sex, age, and interactions among here as the main effects (SAS, 1999-2001). When the interactions were significant, comparisons were made within line×sex, line×age, and age×sex. All values are reported as means ± SE. Significant differences imply P ≤ .05.
RESULTS

Leptin Receptor/Beta Actin CT Ratio

An age by sex interaction for the leptin receptor was detected in brain tissue. Females had a lower CT ratio than males in the brain at 28 days-of-age. At this age, both males and females had a lower CT ratio than other ages (Table 7-1). There was a line by age interaction in the liver. At 2 days-of-age, the LWS chickens had a lower CT ratio than these in the HWS line; however, the opposite pattern occurred at 363 days-of-age. The lowest CT ratio was detected in the LWS chickens at 2 days-of-age, which was significantly lower than at 56 and 363 days-of-ages, but not 28 days-of-age.

In the adipose tissue, the leptin receptor CT ratio was lower in LWS than HWS lines (Table 7-2). At 56 days-of-age, the CT ratio was greater than at 28, and 363 days-of-ages (Table 7-2). There were no differences in the CT ratio of the leptin receptor in heart, kidney, or lung tissue.

The ACC/Beta Actin CT ratio

The ACC expression CT ratio was significantly different between ages. Chickens 56 days-of-age had the greatest ACC CT ratio compared to other ages (Table 7-3).

Leptin Receptor Inflection Point Cycle Number

In the adipose tissue, the LWS leptin receptor inflection point cycle number was greater than in the HWS chickens. The inflection point cycle number was greater in male than female liver. Age differences were detected in the brain; at 28 days-of-age, the leptin receptor inflection point cycle number was greater than other ages (Table 7-4).
DISCUSSION

The leptin receptor long isoform is primarily located in the hypothalamus and influences food intake (Mercer et al., 1996; Mercer et al., 1997; Fei et al., 1997; Levin et al., 2003). In this study, LWS chickens exhibited higher leptin gene expression than HWS line chickens according to the CT ratio analysis, but not using the inflection point cycle number analysis. The higher expression of the leptin receptor gene might increase the concentration of the leptin receptor, and may be related to the anorexic condition in the LWS line (Dunnington and Siegel., 1997).

Cohen et al. (2001) found that mice with disruption of leptin receptor in the brain exhibit obesity, but hepatocyte-specific disruption of leptin receptors did not affect body weight. These results indicate that the brain is a direct target for the weight-reducing and neuroendocrine effects of leptin while liver leptin receptor abnormalities of db/db mice are secondary to defective leptin signaling in the brain.

Leptin can also serve as a cytokine. Yamauchi et al. (2003) reported the db/db mice exhibit steatosis and delayed liver regeneration caused by a mutation of the leptin receptor. However, in avians, the adipocyte and liver are the two major tissues that produce leptin, which is different than mammals that only produce leptin in adipocytes (Margetic et al., 2002). Birds also could store larger quantities of excess energy in the form of triglycerides in the liver, as well as in adipose tissue (Hermier, 1997). Based on this evidence, avians exhibit greater leptin receptor activity and levels in the liver, which is involved with lipogenesis activity. In this study, the liver also exhibited high leptin receptor expression levels, similar to the brain. This indicates that the avian liver is another major organ exhibiting leptin receptor activity.
The leptin receptor has been reported to be present in various tissues, but some organs have very low long isoform levels (Chen et al., 1999; De Matteis et al., 1998; Ghilardi et al.; 1996; Kieffer et al., 1996). This might relate to the low expression we found in the heart, kidney, and lung.

Van Dielen et al. (2002) demonstrated that in obese humans leptin receptor levels decreased when leptin levels increased. Ogier et al. (2002) reported that lean humans had greater leptin receptor level than obese humans, and indicated that high leptin receptor levels enhance leptin action in lean humans. Anorexic humans exhibited higher leptin receptor levels, which decreased after weight gain (Kratzsch et al., 2002). These results are similar to our findings that LWS chicken liver leptin receptor expression (based on CT ratio analysis) was higher than in HWS chickens at 2 days-of-age. However, at 363 days-of-age, the results were opposite. Rats under long-term feed restriction had increased leptin receptor levels in the hypothalamus and decreased leptin levels in circulation (Fernandez-Galaz et al., 2002). Since the HWS line was placed on feed restriction after 56 days-of-age to prolong and regulate egg production (Dunnington and Siegel, 1997), it is possible that long-term feed restriction induced the leptin receptor expression in the HWS birds.

When body weight and fat stores reach a certain level, adipocytes increase the release of leptin into the bloodstream, and induce puberty in female rats (Cheung et al., 1997). However, conflicting results were found in male Rhesus monkeys where puberty is not triggered by increasing leptin concentrations in the circulation (Cunningham et al., 1999; Mann et al., 2000). The interactions between leptin, estrogens and androgens indicate that leptin’s role in the regulation of puberty is mediated by different
mechanisms in males and females. Mann et al. (2003) demonstrated that the leptin receptor and gonadal hormones change during puberty in adult humans. They reported that at puberty, leptin receptor expression is greater in males than females, due to increasing circulating testosterone concentrations in the former (Mann et al., 2003). Leptin acts on hypothalamic cells to stimulate the release of LHRH, triggering gonadotropin release (Ahima et al., 1997). In our study, at 28 days-of-age leptin receptor expression in the brain was greater in female than male chickens. Increased circulating leptin and leptin receptor may activate signals in the central nervous system that metabolic conditions are adequate to support pubertal development (Margetic et al., 2002). It is possible the leptin related regulation of puberty is different between sexes, which further induces different leptin receptor expression between male and female chickens.

The leptin receptor expression in rats was decreased in the paraventriculus nucleus from birth through sucking, compared to adults, which gradually decreased with age increased (Fernandez-Galaz et al., 2001; Kratzsch et al. 2002; Matsuda et al., 1999). These findings suggest that high levels of leptin receptor may reflect an up-regulation of the leptin receptor to suppress leptin action during energy deficiency. We found that the leptin receptor is expressed differently among ages in brain, liver and adipose tissues, and most likely the leptin receptor is expressed higher at younger ages or under long-term feed restriction. These results suggest a relationship between the need for energy and leptin receptor expression.

Obese rats had greater ACC mRNA expression in the liver, but no difference between ACC protein expression (Jang et al., 2003). Kouba et al. (1992) found that
chickens had more fat in the body than the turkey, and the ACC concentration was lower at 12, than at 4, 8 weeks-of-age. Increased muscle mass decreases ACC concentration in chickens (Kouba et al., 1992). Takai et al. (1988) found that ACC expression in chickens was much greater after hatch than before hatch, and lower in adult chicken than young chicks. They concluded that the developmental regulation of ACC in the post-hatching period of chicks is tissue specific and occurs primarily at a pretranslational step (Takai et al., 1988).

Calabotta et al. (1985) studied the ACC concentrations in liver and adipose tissue in the HWS and LWS lines of chickens. The ACC concentration was higher in the LWS than HWS line in the liver, while no differences were found in the bone. We found ACC expression in liver was greater at 2 days-of-age than 56 days-of-age. It is possible that as birds are growing, energy is focused on making muscle and bone mass, which decreases the ACC concentration.

We observed several single nucleotide polymorphisms in the leptin receptor gene in HWS and LWS lines of chickens in both intron and exon regions (unpublished data). The mutation could induce differences in gene expression (Lee et al., 1997). We had observed differences of leptin receptor gene expression levels between line, sex, age, and line by age interactions. The polymorphisms are the possible cause of those differences.

Real time PCR was used to quantify leptin receptor and ACC gene expressions. Increased gene expression levels are based on the assumption that fewer thermo-cycles are required for samples to reach cycle threshold. It is also assumed that the slope and shape of each reaction profile are similar. However, in the present study, we found that slope and shape were unique for each sample. Thus, a secondary method was used to
determine gene expression. Each reaction’s inflection point cycle number was
determined and used this for analysis. This point indicates the cycle numbers at which
the reaction reached a maximal velocity. This appears to be a more precise and
repeatable model of analysis.

Significant effects were dependent upon the specific parameter analyzed from
Real Time PCR data. The CT and inflection point cycle numbers were not correlated
among samples. Our data demonstrate that samples can have similar inflection points
with dissimilar CT. The factors contributing to these differences are unknown. Problems
associated with interpreting Real Time PCR data have been reviewed by Bustin (2002).
Muller et al. (2002) indicated the major problem with real time PCR is not the technique
itself, but rather the statistical analysis of the data. Several statistical methods have been
developed to fit this new technology (Muller et al., 2002; Pfaffl et al., 2002). Since the
accepted statistical analysis for this new equipment has not been agreed upon, the two
aforementioned modes of analysis were explored here.


Fei H, Okano HJ, Li C, lee GH, Zhao C, Darnell r, Friedman JM. Anatomical localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proc Natl Acad Sci 1997;94:7001-5.


Levin BE, Dunn-Meynell AA, Banks WA. Obesity-prone rats have normal blood brain barrier transport but defective central leptin signaling prior to obesity onset. Am J Physiol Regul Integr Comp Physiol 2003;Epub ahead of print.


Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 2002;32:1372-9


Figure 7-1. Experimental design.

Age: 2 day, 28 days, 56 days, 363 days old.
Animals: HWS and LWS chicken, 12/line/sex/age (Siegel, 1970).

RNA extraction - Total RNA

Reverse Transcriptase PCR - cDNA

Amplify first-strand cDNA with β-actin primers - determine the quality of cDNA in samples
Real Time PCR - Leptin receptor - gene of interest, β-actin standard
Table 7-1. Mean ± SE of leptin receptor/ beta actin CT ratio of brain and liver tissues where line × age and age × sex interactions were significant in brain and liver tissues (P ≤ .05).  

<table>
<thead>
<tr>
<th>Brain</th>
<th>Age (day)</th>
<th>2</th>
<th>28</th>
<th>56</th>
<th>363</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.96 ± 0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.01 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.90 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.86 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.96 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.94 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.92 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>1.99 ± 0.02</td>
<td>0.99 ± 0.02</td>
<td>1.92 ± 0.01</td>
<td>1.89 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LWS</td>
<td>1.93 ± 0.01</td>
<td>0.96 ± 0.02</td>
<td>1.91 ± 0.04</td>
<td>1.89 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Liver</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.94 ± 0.02</td>
<td>0.99 ± 0.03</td>
<td>1.03 ± 0.01</td>
<td>1.09 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>0.99 ± 0.04</td>
<td>0.99 ± 0.05</td>
<td>1.14 ± 0.06</td>
<td>1.06 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWS</td>
<td>1.03 ± 0.03</td>
<td>0.95 ± 0.05</td>
<td>1.04 ± 0.04</td>
<td>1.04 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LWS</td>
<td>0.94 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.13 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.11 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Value represents Mean ± SE. Where line by age, sex by age interactions were significant.

* = Means when line, and sex was significant.

<sup>ab</sup> = Means when age was significant.

NS = Means when line, sex, age was not significant.
Table 7-2. Mean ± SE of leptin receptor/ beta actin CT ratio of adipose tissue where line and age were significant (P ≤ .05)\(^1\).

<table>
<thead>
<tr>
<th>Adipose</th>
<th>Line</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HWS</td>
<td>LWS</td>
</tr>
<tr>
<td></td>
<td>1.59 ± 0.06</td>
<td>* 1.42 ± 0.09</td>
</tr>
<tr>
<td>Age</td>
<td>28</td>
<td>56</td>
</tr>
</tbody>
</table>

\(^1\)Value represents Mean ± SE. Where line and age was significant.

* = Means when line was significant.

\(^a\) = Means when age was significant.

NS = Means when line, and age was not significant.
Table 7-3. Mean ± SE of ACC/ beta actin CT ratio of liver tissue where age was significant (P ≤ .05).  

<table>
<thead>
<tr>
<th>ACC liver</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>ACC liver</td>
<td>1.12 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Value represents Mean ± SE. Where age was significant.
<sup>ab</sup> = Means when age was significant.
NS = Means when line, sex, age was not significant.
Table 7-4. Mean ± SE of leptin receptor inflection point of adipose, liver, and brain tissues where line, sex, and age were significant (P ≤ .05).

<table>
<thead>
<tr>
<th>Line</th>
<th>HWS</th>
<th>LWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose</td>
<td>15.91 ± 0.60</td>
<td>* 24.09 ± 1.29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>29.70 ± 0.56</td>
<td>* 28.44 ± 0.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age</th>
<th>2</th>
<th>28</th>
<th>56</th>
<th>363</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>27.95 ± 0.38^b</td>
<td>32.77 ± 1.57^a</td>
<td>27.89 ± 0.63^b</td>
<td>28.40 ± 0.91^b</td>
</tr>
<tr>
<td>Liver</td>
<td>28.09 ± 0.45^b</td>
<td>30.88 ± 0.91^a</td>
<td>28.61 ± 0.66^b</td>
<td>28.98 ± 0.55^b</td>
</tr>
</tbody>
</table>

^1Value represents Mean ± SE. Where line, sex, and age were significant.

*= Means when line, and sex was significant.

^ab = Means when age was significant.
CHAPTER VIII

GHRELIN GENE SEQUENCE AND EXPRESSION IN LINES OF CHICKENS
SELECTED FOR HIGH AND LOW BODY WEIGHT
ABSTRACT

Ghrelin, a recently discovered neuropeptide, is a natural ligand for the growth hormone secretagogue receptor. It is secreted primarily from the stomach and stimulates food intake in mammalian species. This study investigated potential differences in the gene sequence and expression of ghrelin in lines of chickens that had undergone 46 consecutive generations of selection for high (HWS) or low juvenile body weight (LWS). Utilizing the reported chicken ghrelin mRNA sequence from the broiler proventriculus (GenebankAB075215), primers were designed to amplify proventriculus cDNA from non-weight selected Leghorns by PCR. Using these PCR products, we found that Leghorn ghrelin contained 8 extra base pairs in the 5’-untranslated region. Then primers were designed based on broiler and Leghorn ghrelin sequences to amplify proventriculus cDNA from HWS and LWS chicken. Both HWS and LWS chickens have 6 extra base pairs in the 5’-untranslated region, similar to that in Leghorns. Ghrelin gene expression levels in HWS and LWS chickens at 84 and 363 days-of-age, in both ad libitum or fasted birds were determined using Real time-RT PCR. Ghrelin/beta actin cycle threshold cycle number (CT) ratio and ghrelin inflection point cycle numbers were both analyzed. The LWS male CT ratio was higher than the LWS female and HWS male. At 84 days-of-age, the male CT ratio was also greater than in 84 day-old females and 363 day-old males. Female and 363 day-old chickens have lower inflection point cycle numbers than males and 84 day-old chickens. When comparing different feeding conditions, ad libitum females had a lower CT ratio than ad libitum males or fasted females. However, the ad libitum inflection point cycle number was lower for females than males, and greater than for fasted females. In contrast to mammals, intracerebroventricular injection of ghrelin
decreases feed intake in chickens. Therefore, it appears that ghrelin has a different affect on feed intake in avians than mammals. In conclusion, there is a difference in the ghrelin gene sequence between various types of chickens. The expression of ghrelin in chickens is influenced by body weight selection, sex, age, and feeding condition.

Key Words: gene expression, ghrelin, sequence, polymorphism, real time PCR
INTRODUCTION

Ghrelin is a 28-amino acid gastrointestinal peptide that was identified in the stomach as an endogenous ligand specific for the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). The GHS-R is located in the pituitary and hypothalamus. Ghrelin is a highly conserved acylated peptide that is produced primarily by cells in the oxyntic glands of the stomach (Date et al., 2000). As a natural ligand for the GHS-R, it stimulates GH release. Kojima et al. (1999) reported that ghrelin stimulates GH release from pituitary cells in a dose-dependent manner. Ghrelin also functions as a blood-born orexigenic signal from the gut to the brain (Pinkney and Williams, 2002).

The first published evidence for the involvement of ghrelin in the regulation of appetite demonstrated that humans spontaneously reported hunger following i.v. ghrelin administration (Arvat et al., 2000). Tschop et al. (2000) observed that ghrelin concentrations were increased by fasting and reduced by re-feeding or oral glucose administration, but not after water ingestion. Date et al. (2001) found that intracerebroventricular (ICV) administration of ghrelin rapidly increased gastric acid output in a dose-dependent manner in urethane-anesthetized rats. This indicated that ghrelin participates in the central regulation of gastric acid secretion by activating the vagus system (Date et al., 2001).

Nakazato et al. (2001) demonstrated a role for ghrelin in the central nervous system control of feeding behavior. The ICV administration of ghrelin increased feed intake in both satiated and FA rats, whereas anti-ghrelin IgG caused a dose-dependent suppression of feed intake in free feeding rats. Shiiya et al. (2002) described a role of ghrelin in the pathogenesis of obesity and anorexia nervosa. Ghrelin concentrations were
significantly lower in obese subjects compared to normal weight subjects or patients with anorexia nervosa. Therefore, nutritional state is a determinant of plasma ghrelin levels in humans; ghrelin concentration is up-regulated by negative energy balance and down-regulated during positive energy balance.

Leptin receptors and GHS-R are located in the hypothalamus. Leptin and ghrelin may inversely co-regulate the hypothalamic peptidergic systems that control food intake through modulation of neuropeptide Y neuron activity (Horvath et al., 2001). Nakazato et al. (2001) reported that antibodies against neuropeptide Y and agouti-related peptide block the physiological affect on ghrelin and result in a decrease in feed intake in rats. The ICV administration of leptin after ghrelin also suppressed the action of ghrelin and caused a decrease in feed intake (Nakazato et al., 2001). These results indicated that ghrelin may antagonize the action of leptin for the regulation of the neuropeptide Y feeding behavior system.

This study was conducted to determine if differences exist in the chicken ghrelin gene sequence and if differences exist in gene expression levels from chickens selected for high (HWS) or low juvenile body weight (LWS) for 46 consecutive generations. Ghrelin expression in HWS and LWS chickens under ad libitum and fasting conditions was also compared.

MATERIALS AND METHODS

Animals

Day-old male and female HWS and LWS chicks were vaccinated for Marek’s disease and placed in electric heated batteries with raised wire floors. Birds were provided a starter (20% crude protein, 2,684 kcal/ kg of metabolizable energy) diet and
water available for ad libitum consumption. The birds were exposed to continuous fluorescent lighting. At 28 day-of-age, birds were transferred to grower batteries (75.1 cm W × 38.1 cm L × 38.1 cm H).

Experiment 1, proventriculus samples were collected from 12 birds per line per sex at 84 and 363 days-of-age. Experiment 2, proventriculus samples were collected from 12 birds per line per sex at 84 days-of-age that had been fed AL or FA for 48 hours. Proventriculus samples were frozen in liquid nitrogen immediately after collection, then stored at –80 °C until gene expression analysis.

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated using the Tri-Reagent® protocol (Molecular Research Center, Inc. Cincinnati, OH). The integrity of RNA was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density at 260 and 280 nm. Synthesis of single-strand cDNA, and reverse transcriptase (RT) reactions were carried out using random primers (Promega, Madison WI), MMLV reverse transcriptase (Promega, Madison WI), and 1 µg of total RNA in a 50 µl reaction.

PCR Primers Design and Sequencing Analysis

The broiler ghrelin cDNA sequence (AB075215) was obtained from the public GenBank sequence database of the National Center for Biotechnology Information, and primers were designed (Fig. 8-1) to isolate Leghorn ghrelin cDNA (Genebank AY299454). Leghorn ghrelin cDNA was cloned and compared with broiler ghrelin sequences. Based on Leghorn and broiler ghrelin cDNA sequences, primers 1S (CCTTGGGACAGAAACTGCTC), and 1AS (CACCAATTTCAAAAAGGAACG) were
designed to detect HWS and LWS line of chicken ghrelin gene expression. Primers 2S (CAGCAGTTTGGTGAAGCAAC), and 2AS (TCAGGCCATCTAGTGCTGTG) were designed for amplify HWS and LWS ghrelin cDNA for polymorphism determination.

**SYBR Green Real-time PCR**

Real-time PCR was performed (DNA Engine Opticon, MJ Research, Inc, Waltham, MA) to analyze ghrelin gene expression, using QuantiTect™ SYBR® Green PCR kit (Qiagen, La Jolla, CA). Amplification was carried out in a total volume 25 µl containing 1× SYBR Green master mix, 0.3 µM of each primers, RNase-free water and 1 µl of cDNA. Each sample was amplified with chicken ghrelin primers or beta actin primers, which served as external control. All determinations were performed in duplicates.

**Data Analysis**

**Cycle Threshold Ratio Analysis**

Cycle threshold (CT) is defined as the cycle number at which fluorescence is distinguishable from noise. This point appears during the exponential phase of the PCR reaction and is inversely proportional to the initial number of template molecules in the sample. Results were expressed as relative expression. The cycle number for expression of ghrelin CT was calculated and normalized with the beta actin CT (Meijerink et al., 2001).

**Inflection Point Cycle Number Analysis**

The fluorescence level at each cycle was determined using to plot a symmetrical line and equations for each reaction was determine. The inflection point cycle number of ghrelin and beta actin was determined and beta actin served as a co-variant.
Data were analyzed using ANOVA with line, sex, age, feeding condition, and interactions among these the main effects (SAS, 1999-2001). When the interactions were significant, comparisons were made within line by sex, line by age, age by sex, line×feed condition, sex×feed condition. All values are reported as means ± SE. Significant differences imply $P \leq .05$.

**RESULTS**

*Polymorphism*

Leghorn ghrelin contained 8 extra base pairs in the 5’-untranslated region compared to broiler ghrelin cDNA. Based on the broiler and Leghorn ghrelin cDNA sequences, primers were designed to amplify the HWS and LWS chicken proventriculus cDNA (Figure 8-1). It was found that in the same 5’-untranslated region, HWS and LWS lines of chickens had 6 extra base pairs, which is different from that of broilers and Leghorns (Figure 8-2).

*Ghrelin Gene Expression of HWS and LWS, Male and Female Chickens, 84 and 363 Day-of-age.*

*Ghrelin CT/ Beta Actin CT*

Line and age, line by sex, and age by sex interactions were found for the CT ratio (Table 8-1). The LWS male chickens had a higher CT ratio than HWS male and LWS female birds. The 84 day-old male chickens had a higher CT ratio than 84 day-old females, or 363 day-old male chickens (Table 8-1).

*Inflection Point Cycle Number*

The ghrelin inflection point cycle number was different between sex and age (Table 8-2). The female and 363 day-old of chicken ghrelin inflection point cycle numbers were lower than in the male and 84 day-old chickens.
**Ghrelin Gene Expression in 84 Day-old HWS and LWS, Male and Female, Ad Libitum, and Fasting Chickens.**

**Ghrelin CT/ beta actin CT**

Line and feeding conditions were different in the CT ratio. The HWS CT ratio was lower than in the LWS chickens. A food condition by sex interaction was significant, since the *ad libitum* fed female chicken CT ratio was significantly lower than *ad libitum* male, and fasting female chickens (Table 8-3).

**Inflection point cycle number**

There was a feed condition by sex interaction for the inflection point cycle number. In the *ad libitum* fed group, the male inflection point cycle number was significantly higher than in female chickens. In both male and female chickens, the *ad libitum* group was greater than the fasting group (Table 8-4).

**DISCUSSION**

**Polymorphism**

Ukkola et al. (2001) studied mutations in the ghrelin gene in obese humans. They demonstrated that 6.3% of patients had a Arg51Gln mutation in exon 2 of preproghrelin. This mutation disrupts the proteolytic cleavage site and causes a defective or inactive protein. Sequence variations in the ghrelin gene may play a role in the etiology of obesity. Hinney et al. (2002) detected a frameshift mutation with a 2 bp deletion at codon 34, which lead to insertion of 36 aberrant amino acids and to a stop codon at position 71. This frameshift mutation affects the coding region of mature ghrelin. An A to T transversion leads to a non-conservative Gln90Leu mutation. Ghrelin has somatotropic, orexignic and adipogenic affects (Ukkola and Poykko, 2002). In human studies, these
mutations are associated with obesity and diabetes. Ukkola and Kesaniemi (2003) reported Leu72Met polymorphism was associated with diabetic human subjects.

Broiler chicken ghrelin is composed of 26 amino acids, and has 54% sequence identity to human ghrelin (Kaiya et al., 2002). The extra base pairs in the 5’-untranslated region of Leghorn, HWS, and LWS chickens may cause a frameshift that changes the amino acid sequence of the chicken ghrelin gene, and may alter the promoter region of the sequences further affecting the regulation of ghrelin.

Gene Expression

The effect of ghrelin on appetite and food intake has been investigated in humans (Wren et al., 2001). Subjects receiving ghrelin had increased food intake. However, ghrelin has no effect on gastric emptying (Wren et al., 2002). Nutritional state is a determinant of plasma ghrelin concentration in humans. Ghrelin concentration was significantly lower in obese subjects compared to subjects with normal body weight or anorexia nervosa patients (Wren et al., 2002). Shiiya et al. (2002) suggested ghrelin concentration is up-regulated under conditions of negative energy balance and down-regulated during positive energy balance.

Ghrelin responses differ between avian and mammalian. Recently, the feed intake response to ICV injection of chicken ghrelin (Saito et al., 2002), rat ghrelin (Furuse et al., 2001), bullfrog ghrelin (Saito et al., 2002), and growth hormone releasing peptide-2 (Saito et al., 2002) to neonatal chicks was investigated. All varieties of ghrelin and growth hormone-releasing peptide-2 decreased feed intake. It is possible that in birds, the control of GH secretion differs from that in mammals, and that chicken GHRH
is not an effective secretagogue for GH in chickens (Harvey, 1999). Chicken GH release is more likely induced by thyrotropin-releasing hormone (Ahmed and Harvey, 2002).

In avians, low expression of ghrelin decreases ghrelin concentration which is caused by negative energy balance in the body, which induced food intake. The LWS male chickens had a highest ghrelin CT ratio, which corresponds to less ghrelin expression, and ghrelin concentration. Some LWS chickens are anorexic (Dunnington and Siegel, 1997); the decreased ghrelin concentration may be the initiator of feed intake. The anorexic conditions in chicken and human are similar (Shiiya et al., 2002; Horvath et al., 2003). Male chickens at 84 day-of-age had the lowest ghrelin expression (based on the result from the CT ratio) compared to other chickens. Hence, ghrelin may further stimulate the chicken orexigenic system to increase the food intake, and provide energy for growth.

Parhar et al. (2003) reported that in the female tilapia, ghrelin expression was higher than in males. The differences in somatic and gonadal growth in tilapia could be a consequence of age- and sex-related synthesis of gastric ghrelin. However, in rats, there was an age, but no sex difference in the ghrelin expression from the stomach, and it was concluded the sex hormones do not influence ghrelin expression (Gualillo et al., 2001). However, in the present study, it was found that LWS and 84 day-old females had a lower CT ratio, and ghrelin inflection point cycle numbers were less than in male chickens reflecting greater ghrelin expression. Our results demonstrate a difference in the ghrelin expression between sexes.

In mammals, ghrelin concentration and expression in the stomach and hypothalamus are increased during 48 hours of fasting (Toshinai et al., 2001). Fasting is
similar to anorexia, which increases concentration of ghrelin restores normal energy balance. Fasting also up-regulates ghrelin receptor expression, which increases the ghrelin action in a negative-energy balance state (Kim et al., 2003). The fasted female chicken CT ratio is higher, thus expressing less ghrelin in the proventriculus than other birds. However, the fasted female inflection point cycle number was less, which is contrary to the CT ratio comparison. These results show that the definitions of CT ratio analysis and inflection point cycle number analysis are different for the explanation of the gene expression condition.

Real time PCR was used to quantify ghrelin gene expression. Gene expression levels that are calculated from Real time PCR data are based on the assumption that fewer thermo-cycles are required for samples to reach cycle threshold. It is also assumed that the slope and shape of each reaction profiles are similar. However, in the present study we found slope and shape were unique for each sample. Thus, we used a secondary method to determine gene expression. We determined each reaction’s inflection point cycle number and used this for analysis. This point indicated the cycle number at which the reaction was at a maximal velocity. This may be more precise and repeatable model of analysis. Conclusions revealing significant effects were dependent upon the specific parameter analyzed from Real Time PCR data. The analysis conducted here compared the CT ratio, based upon the signal to noise ratio, and cycle number at the inflection point that indicates the cycle at maximal reaction energy. It was found that CT and inflection point cycle numbers were not correlated among samples. Our data demonstrate that samples can have similar inflection point with dissimilar CT. The factors contributing to these differences are unknown. Problems associated with interpreting Real Time PCR
data have been reviewed by Bustin (2002). Muller et al. (2002) indicated the major problem with real time PCR is not the technique itself, but rather the statistical analysis of the data. Several statistical methods have been developed to fit this new technology (Muller et al., 2002; Pfaffl et al., 2002). Since the accepted statistical analysis for this new equipment has not been agreed upon, the two aforementioned modes of analysis were explored here.


Broiler ghrelin complete cDNA (AB075215)- 836bp. Primers (1S-CCTTGGACAGAAACTGCTC and 1AS-CACCAATTTCAGGAACG) were chosen for real time PCR to detect chicken ghrelin gene expression level. Primers (2S-CAGCAGTTTGGTGGAAGCAAC and 2AS-TCAGGCCATCTAGTGCTGTG) were chosen for amplify cDNA and polymorphism determination.
Figure 8-2. Ghrelin proventriculus cDNA comparison between Leghorn, broiler, HWS and LWS chickens

CTTTTGCCAGTTTCC

Leghorn

HWS & LWS

Broilers

TCTGTAATTCTCTCTGCTAACCTGTCTGGTCCAGTC

TCTGTAATTTCTCT--GCTAACCTGTCTGGTCCAGTC

TCTGTAATTTCTCT------------CTGTCTGGTCCAGTC

CAGTTATAGAAGAAAACACATTTTGAGACTGCTAAAGAAGACATGTGTTTCAGAGTTATTCTGCTAGGAATTCTCTCTGCTAGCATTCC

TTCAGCATCCTTTGGACAGAAACTGCTCTGGCTCTAGTCTCTGAGGCTTAAAGCCCTACATATATAAAAAACATACAGCAACAAGAACAG

ATACAAAGAAAACCAACAGCAAGATTCATCGCCGAGGCACAGAAAGCTTTTGGGATACAGATGAAACAGAGGGAGAAGATGACAATAACAGTGTTGATATCAAGTTTAACGTTCCTTTTGAAATTGGTGTCAAGATAACAGATGAAACAGAGGGAGAAGATGACAATAACAGTGTTGATATCAAGTTTAACGTTCCTTTTGAAATTGGTGTCAAGATAACAGAAAGAGTATCAAGAGTATGGACA

ACCACAGCTATGCTAGCTACAGGACATTTTGGCAGAGAATGCTGAGAAACTCAGACAAAAAGCTGAAGCAAGCTGAAGGACACCACAGCACTAGATGGCCTGATATAAGTTTTACTTTAAAACAAAACTATGACTGAATTTAGATCTCTAAGAAGAAAGAAGCATACTTGAGTAGTTTGCTCTAAGAAAAAGACAAGAATCTACATGCAGAACTTCAAAGGGCAATGTGAATCACAGGCTGAC

TCTGTAATTTCTCT------------CTGTCTGGTCCAGTC

CTTTTGCCAGTTTCC
Table 8-1. Mean ± SE of ghrelin/ beta actin CT ratio of proventriculus tissue where line × sex and age × sex interactions were significant ($P \leq .05$)

<table>
<thead>
<tr>
<th>Line</th>
<th>Age (days)</th>
<th>Line</th>
<th>Age (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HWS</td>
<td>LWS</td>
<td>84</td>
</tr>
<tr>
<td>Male</td>
<td>1.36 ± 0.02</td>
<td>*</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Female</td>
<td>1.39 ± 0.02</td>
<td>NS</td>
<td>1.39 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.42 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.36 ± 0.03</td>
</tr>
</tbody>
</table>

1Value represents Mean ± SE. Where line by sex, sex by age interaction were significant.

* = Means when line, sex, and age was significant.

NS = Means when line, sex, age was not significant.
Table 8-2. Mean ± SE of ghrelin inflection point of proventriculus tissue by line, sex, and age (P ≤ .05)

<table>
<thead>
<tr>
<th></th>
<th>Line</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin inflection point</td>
<td>HWS</td>
<td>LWS</td>
<td></td>
</tr>
<tr>
<td>27.07 ± 0.76</td>
<td>NS</td>
<td>26.24 ± 0.80</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>27.75 ± 0.82</td>
<td>*</td>
<td>25.62 ± 0.69</td>
<td></td>
</tr>
<tr>
<td>Age (days)</td>
<td>84</td>
<td>363</td>
<td></td>
</tr>
<tr>
<td>28.66 ± 0.67</td>
<td>*</td>
<td>23.77 ± 0.39</td>
<td></td>
</tr>
</tbody>
</table>

1 Value represents Mean ± SE.
* = Means when line, sex, and age were significant.
NS = Means where line, sex, and age was not significant.
Table 8-3. Mean ± SE of ghrelin/ beta actin CT ratio of proventriculus tissue where sex × feed condition interactions was significant (P ≤ .05)\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Food Control</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ad libitum</td>
<td></td>
<td>Fasting</td>
</tr>
<tr>
<td>Male</td>
<td>1.49 ± 0.02</td>
<td>NS</td>
<td>1.49 ± 0.02 NS</td>
</tr>
<tr>
<td>Female</td>
<td>1.42 ± 0.02</td>
<td>*</td>
<td>1.52 ± 0.02</td>
</tr>
</tbody>
</table>

\(^1\)Value represents Mean ± SE. Where sex by feed condition interaction was significant.

*= Means when sex, and feed condition was significant.

NS = Means when sex and feed condition was not significant.
Table 8-4. Mean ± SE of ghrelin inflection point of proventriculus tissue where sex × feed condition interactions was significant (P ≤ .05).

<table>
<thead>
<tr>
<th></th>
<th>Food Control</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ad libitum</td>
<td>Fasting</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30.22 ± 0.84</td>
<td>*</td>
<td>27.57 ± 1.01</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>29.98 ± 1.29</td>
<td>*</td>
<td>26.85 ± 0.92</td>
</tr>
</tbody>
</table>

\(^1\text{Value represents Mean ± SE. Where sex by feed condition interaction was significant.}
\ \text{* = Means when sex, and feed condition was significant.}
\ \text{NS = Means when sex and feed condition was not significant.}
CHAPTER IX

EPilogue

Obesity is not controlled by a single factor. As reviewed in Chapter II, studies have focused on understanding regulation of food intake though the autonomic nervous system (ANS) by neuropeptides, and the possibility of controlling these pathways to regulate body weight.

Obesity is a serious health problem. The understanding of food intake regulation has significant implications for obesity. In the past few years, there has been considerable research focusing on genetic regulation of food intake, body fat stores and fat metabolism. As each new gene is identified, a new question arises about how expression of the protein changes in response to peripheral signals that maintain energy homeostasis. While researchers have identified hormones and neuropeptides involved in energy homeostasis, the real deficit in the understanding of these complex feedback mechanisms is a lack of knowledge of the transcription factor and intermediate signaling molecules that are involved in these processes.

Previous studies had shown that the high body weight selected (HWS) line of chickens exhibits lower sympathetic tone with regards to cardiovascular activity while the low body weight selected (LWS) line has greater sympathetic nervous system activity. In the present research, intraperitoneal injection reserpine caused a greater effect on neurotransmitter levels in the LWS line, but had less of an effect on body weight and food intake in this line than in the HWS line. The results indicated the LWS line has greater sympathetic tone, which may explain why reserpine caused less of an effect on body weight and food intake.
Most neuropeptides affecting food intake are secreted in the brain where they stimulate or inhibit the sympathetic nervous system. However, leptin and ghrelin are secreted in the peripheral system, pass through the blood brain barrier, and then activate receptors in the hypothalamus. This provides an interaction between the central and peripheral nervous system in the regulation of the food intake. The intracerebroventricular injection of leptin caused a linear decrease in food intake in the LWS, but not in the HWS line of chickens suggesting that the activity of the leptin receptor between these lines might be different. Plasma leptin concentrations were similar for these two lines of chickens at 35 days-of-age, and different at 363 days-of-age (HWS<LWS). The concentration of insulin like growth factor (IGF)-1 and 2 are also different between these two lines (HWS<LWS). The peripheral leptin regulation and related hormones maybe different between the two lines at different ages.

Polymorphisms between the HWS and LWS lines were found in both the intron and exon regions of the leptin receptor gene, and the leptin receptor expression was also different between lines, ages, and sexes in brain, liver and adipose tissue. These observations indicate that leptin receptor regulation is different between the central and peripheral system of these two lines of chickens. The higher leptin receptor expression may be correlated to the higher leptin concentration in the body, which further caused reduced body weight. The polymorphism and different gene expression level also raises the possibility that leptin receptor proteins are different between these two lines of chickens, which results in different food intake response after intracerebroventricular injection in them (LWS>HWS).
Ghrelin gene expression was affected by line, age, and different feeding condition, which is similar to the results for leptin receptor gene expression. The ghrelin expression could be the initiator, which regulates food intake in HWS and LWS chickens. Leptin receptors affect food intake and body weight, which is further related to energy balance, and impacts growth and sexual maturity. Ghrelin is an indicator of energy balance in the body. Leptin concentration, IGF-1 and IGF-2, leptin receptor and ghrelin were expressed differently between age and sex in these two lines of chickens. The regulation of leptin, leptin receptor, and ghrelin are important for growth development, and sexual maturity, and their changes are correlated with energy status in the body. Growth rate (HWS>LWS) and age at sexual maturity (LWS>HWS) are different between the two lines of birds. It is believed that the changes of leptin, leptin receptor, and ghrelin in these two lines of birds are strongly involved with energy balance and developmental status (Figure 9-1).

From current information, it is clear that the concentration of leptin is not the only factor that could regulate the food intake. The leptin receptor, and its cellular signal pathways, appears to be more important in performing the action of leptin rather than leptin itself. Knockout animals have been designed to study the dysfunction of leptin or the leptin receptor that cause hyperphagia or obesity; however the results are not always applicable to humans. Mammalian species increase food intake when ghrelin concentration is increased; however avians have the opposite response. The interaction and networking between these neuropeptides, and where the receptors are located, needs further studied.
Numerous studies are related to understanding of the cause of obesity, but not anorexia. Serotonin receptor and alpha melanocortin dysfunction are known to cause anorexia. It is known the dysfunction of serotonin could cause both obesity and anorexia. It is important to further study serotonin regulation in both obesity and anorexia.

Numerous studies also focused on the neuropeptide Y (NPY)/agouti related peptide neuron pathway activated by leptin. The NPY/gamma-aminobutyric acid (GABA) neuron is one of the interneurons between the NPY/agouti related peptide (AGRP) and pro-opiomelanocortin (POMC) neurons. The GABA neurons could inhibit the POMC neuron action to regulate the food intake. The GABA neuron located in the ventromedial and ventrolateral part of the arcuate nucleus contain leptin receptors, indicating that GABA is directly involved with the leptin activity in the central nervous system. Immunofluorescence and in situ hybridization histochemistry confirmed the existence of the leptin receptor on the GABA neuron. However, the transcriptional gene for the GABA-synthesizing enzyme glutamic acid decarboxylase and vesicular GABA transporter were not observed. Mechanisms other than transcriptional regulation for leptin to influence arcuate GABA neurons may exist. To understand the regulation of GABA in HWS and LWS lines of chickens may provide more information on the difference between these two lines of birds.

Regulation of neurotransmitters is the primary action of the ANS. Various neuropeptides have either direct or indirect interaction on the sympathetic nervous system, and most of them are involved with beta receptor activity. The effect of intracerebroventricular administration of norepinephrine and epinephrine to HWS and LWS chickens should be investigated. The gene sequence, expression, protein
concentration, and activity of norepinephrine, epinephrine, as well as alpha and beta receptors should also be evaluated. This information could elucidate differences in the basic sympathetic nervous system activity or regulation between these two lines of chickens.

Real time PCR was used to determine gene expression in our study. This new technology is able to detected small changes in the expression of genes in a short period of time, and is more sensitive than the traditional Northern blotting technique. Microarray is another technology that can detect genes that exist or are expressed in a particular sample. It has been reported that real time PCR and microarray are better technologies to evaluate gene expression level. However, both methods have their pros and cons. First, because the sensitivity of these two methods is greater than Northern blotting, higher observation numbers are not required. Second, both real time PCR and microarray are more expensive to use than the Northern blotting technique. Because of the expense, it is possible that experiments are designed with too few observations to adequately test a hypothesis. Third, there were 13 statistical analysis methods being used to analyze real time PCR data; however, no one particular method has been recognized as the primary method. Most of the statistical methods have focused on the mathematics, while not including factors involved in numerous of the biological studies. Furthermore, the methods reply on transformations to recalibrate the data, which may lose the true meaning of the data. Development of appropriate statistical methods for analysis of real time PCR and microarray data are needed.

We used chickens that have undergone long-term selection for a quantitative trail. The difference between knockout animals and the experimental chickens used in
these studies were selected for multiple genes, which may be more similar to humans populations. Although, chickens are not traditionally used in human research modeling compared to mice or rats, but they are more closely related to human as compared to other laboratory animals. Many biological studies are based on a human medical hypothesis and use rodents as research model. The regulation, gene sequence, gene expression, and protein of the neuropeptides in humans, rats, and chickens should be compared, to provide a better understanding of these compounds, and how they function.
Figure 9-1. Combined results

NPY/AGRP Neuron

ICV Leptin
Linear Effect on
Feed intake
LWS>HWS

Leptin Receptor
CT Analysis
Sex x Age

28D
F>M

Inflection Point
Age

Leptin
Line x Sex
HWS F ↓
LWS>HWS

Leptin Receptor
CT Analysis
Line x Age

2D 363D
LWS>HWS HWS>LWS

Inflection Point
Line
HWS>LWS

Leptin Receptor
CT Analysis
Line x Age

Food Condition-Ghrelin
CT Analysis
Food x Sex

Inflection Point
Food x Sex
Ad Lib F ▲
Ad Lib M ↓

Ghrelin

Inflection Point
Sex x Age

F>M 363>84
APPENDIX A

RESERPINE EFFECT ON FOOD INTAKE AND BODY WEIGHT DATA
LANGUAGE

options nodate ls=90 ps=53 pageno=1;

DATA WK1;
Input Pen $ TRT $ LINE $ REP BIRDWT No FEEDIN FEEDOUT AVNO
AVWTSTEX AVWTSTPD;
PREVFD = 0;
week=1;
CARDS;
;
DATA WK# (List for every weeks);
INPUT PEN $ TRT $ LINE $ REP BIRDWT NO FEEDOUT FEEDIN AVNO;
week=2;
CARDS;
;
proc sort data=wk1; by pen;
proc sort data=wk#; by pen;
data wk1mer; set wk1;
avwtstpd=birdwt/no;
prevfd=(FEEDIN - FEEDOUT)/AVNO + prevfd;
keep pen avwtstpd avwtstex prevfd;
data wk#a; merge wk# wk1mer; by pen;
data wk#amer; set wk#a;
avwtstpd=birdwt/no;
prevfd=(FEEDIN - FEEDOUT)/AVNO + prevfd;
keep pen avwtstpd avwtstex prevfd;
data all2; set WK1 WK2a WK3a WK4a WK5a WK6a WK7a;
linetr=trim(line)||left(trt);
AVWT  = BIRDWT / NO;
GAINPD  = AVWT - AVWTSTPD;
GAINCUM  = AVWT - AVWTSTEX;
FEEDCONS  = FEEDIN - FEEDOUT;
AVFDCNS  = FEEDCONS/ AVNO;
CMFDCNS  = AVFDCNS + PREVFD;
AVFDEFF  = GAINPD / AVFDCNS;
CMFDEFF  = GAINCUM/ CMFDCNS;
proc print data=all2;
title "all the data";

proc sort data=all2; by linetrt;

proc mixed data=all2;
class line trt week pen;
model AVWT=week line trt line*trt week line*week line*trt*week line*week line*trt*week line*trt*week line*trt*week;
repeated week / type=un sub=pen(line*trt) r rcorr;
lsmeans line*trt line trt week line*week trt*week line*trt*week line*trt*week line*trt*week line*trt*week line*trt*week line*trt*week;
title " AVWT lsmeans";

proc mixed data=all2;
class linetrt week pen;
model AVWT=week linetrt linetrt*week;
repeated week / type=un sub=pen(linetrt) r rcorr;
contrast 'high vs. low' linetrt 1 1 1 -1 -1 -1;
contrast 'linear funct' linetrt -1 0 1 -1 0 1;
contrast 'quad funct' linetrt -1 2 -1 -1 2 -1;
contrast 'line by linear' linetrt -1 2 -1 1 -2 1;
contrast 'line by quad' linetrt -1 2 -1 1 -2 1;
estimate 'high vs. low' linetrt 1 1 1 -1 -1 -1/divisor=3;
estimate 'linear funct' linetrt -1 0 1 -1 0 1;
estimate 'quad funct' linetrt -1 2 -1 -1 2 -1;
estimate 'line by linear' linetrt -1 0 1 1 0 -1;
estimate 'line by quad' linetrt -1 2 -1 1 -2 1;
title " AVWT contrast";
run;
APPENDIX B

NEUROTRANSMITTER DATA ANALYSIS LANGUAGE

options nodate pageno=1 ps=53 ls=80;
data BRAIN;
input line $ trt$ sex$ NuM NE Epi DOPAC DA HIAA HT;
DAO=DOPAC/DA;
HTAA=HIAA/HT;
datalines;
;
run;

proc print data=BRAIN;
title 'BRAIN DATA';
run;

proc sort; by line;
proc means n mean std min max stderr cv; by line;
var NE Epi DOPAC DA HIAA HT DAO HTAA;
title 'DIFFERENCE BY LINE ';
run;

proc sort; by sex;
proc means n mean std min max stderr cv; by sex;
var NE Epi DOPAC DA HIAA HT DAO HTAA;
title 'DIFFERENCE BY sex ';
run;

proc sort; by trt;
proc means n mean std min max stderr cv; by TRT;
var NE Epi DOPAC DA HIAA HT DAO HTAA;
title 'DIFFERENCE CHANGE BY TRT ';
run;

proc sort; by line trt;
proc means n mean std min max stderr cv; by line trt;
var NE Epi DOPAC DA HIAA HT DAO HTAA;
title 'CATECHOLAMINE LEVEL IN BRAIN BY LINE TRT';
run;

proc glm data=BRAIN;
title GLM for all nontransformed variables;
class line trt sex;
model DAO HTAA NE Epi DOPAC DA HIAA HT DAO HTAA=line sex trt line*trt sex*trt/ss3;
lsmeans line trt line*trt /pdiff adjust=tukey stderr;

data brain2; set brain;
linetrt=line||trt;

proc glm data=brain2;
class linetrt sex;
model NE Epi DOPAC DA HIAA HT DAO HTAA =linetrt sex/ss3;
lsmeans sex linetrt;
contrast 'linear trt' linetrt -1 0 1 -1 0 1;
contrast 'quad trt' linetrt -1 2 -1 -1 2 -1;
contrast 'line diff' linetrt -1 -1 -1 1 1 1;
contrast 'linear trt*line' linetrt 1 0 -1 -1 0 1;
contrast 'quad trt*line' linetrt 1 -2 1 -1 2 -1;
estimate 'linear trt' linetrt -1 0 1 -1 0 1;
estimate 'quad trt' linetrt -1 2 -1 -1 2 -1/ divisor=2;
estimate 'line diff' linetrt -1 -1 -1 1 1 1/ divisor=3;
estimate 'linear trt*line' linetrt 1 0 -1 -1 0 1;
estimate 'quad trt*line' linetrt 1 -2 1 -1 2 -1/divisor=2;
run;
quit;
APPENDIX C

INTRACEREBROVENTRICULAR INJECTION DATA ANALYSIS LANGUAGE

data a;
input IDN LIN TRT TIM FEI;
datalines;
;
proc print data=a;

proc sort; by lin tim;
proc means n mean min max std stderr cv; by lin tim;
var FEI;

proc glm; by lin tim;
class TRT IDN;
model FEI= idn trt/ss3;
means TRT/duncan etype=2;
run;

proc sort; by lin tim;
proc mixed; by lin tim;
class TRT IDN ;
model FEI = idn TRT / ddfm=satterth;
lsmeans trt ;
contrast 'lin trt' trt -7 -3 1 9;
contrast 'quad trt' trt 7 -4 -8 5;
run;
APPENDIX D

INFLECTION POINT DATA CALCULATION LANGUAGE

options nodate ls=80 ps=53 pageno=1;
libname akdata 'c:\test sas\Alice\akdata';

*rtpcr-####;

data id; set akdata.####id;

data id2; set id;
if organ="####";

data ####; set akdata.####newanplate;
if read>56 then delete;
cycle=read;
drop read;

proc sort data=####; by plate cycle;

data ####2; set ####; by plate cycle;
array obs(96) a1-a12 b1-b12 c1-c12 d1-d12
e1-e12 f1-f12 g1-g12 h1-h12;
do i=1 to 96;
wellno=i;
wellob=obs(i);
output;
end;
keep plate cycle wellno wellob;

data ####3; set ####2;
length well $ 8;
if wellno/12<=1 then well='A'||left(wellno);
else if wellno/12<=2 then well='B'||left(wellno-12);
else if wellno/12<=3 then well='C'||left(wellno-24);
else if wellno/12<=4 then well='D'||left(wellno-36);
else if wellno/12<=5 then well='E'||left(wellno-48);
else if wellno/12<=6 then well='F'||left(wellno-60);
else if wellno/12<=7 then well='G'||left(wellno-72);
else if wellno/12<=8 then well='H'||left(wellno-84);
drop wellno;

proc sort data=####3; by plate well cycle;

data ####4; set ####3;
retain lind;
ind=lind;
lwell=lag(well);
if well=lwell and wellob=0 then ind=0;
else if wellob=0 then ind=1;
if ind=1;
lind=ind;
lcyc=log10(cycle);

proc means noprint; by plate well;
var wellob cycle;
output out=maxflor max=mflor mcyc;

data ###5; merge ###4 maxflor; by plate well;
if mflor=wellob then delete;
if wellob=0 then delete;
ratio=wellob/mflor;
lograt=log(ratio/(1-ratio));

data dum1; set ###5; by plate well;
if first.plate or first.well;
lcyc=0; lograt=.;
keep plate well lcyc lograt;

data dum2; set ###5; by plate well;
if first.plate or first.well;
lcyc=1; lograt=.;
keep plate well lcyc lograt;

data ###6; set dum1 dum2 ###5;
proc sort data=###6; by plate well;

proc reg noprint; by plate well;
model lograt=lcyc/r;
output out=###7 p=yhat;

data int one; set ###7;
if lcyc=0 then output int;
if lcyc=1 then output one;

proc sort data=one; by plate well;

data one2; set one; by plate well;
if first.plate or first.well;
data int2; set int;
int=yhat;
keep int plate well;

data one3; set one2;
xone=yhat;

proc sort data=int2; by plate well;

data eqn; merge int2 one3; by plate well;
slope=xone-int;
lxint=-int/slope;
xint=10**(lxint);

data eqn2; set eqn;
keep plate well xint;

proc sort data=eqn2; by plate well;
proc sort data=id; by plate well;

data all1; merge eqn2 id; by plate well;
file 'c:/test sas/Alice/###xint1';
put plate well xint organ line sex age gene;

run;
APPENDIX E

EFFECT OF INTRAPERITONEAL INJECTIONS OF SALINE, 1.25MG/KG OF RESERPINE, AND 2.50 MG/KG RESERPINE ON BRAIN AND PLASMA NEUROTRANSMITTER CONCENTRATIONS OF HIGH AND LOW BODY WEIGHT SELECTED LINES OF CHICKENS
Figure 1. Effect of intraperitoneal injections of reserpine on brain norepinephrine concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 2. Effect of intraperitoneal injections of reserpine on brain epinephrine concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 3. Effect of intraperitoneal injections of reserpine on brain dopamine concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 4. Effect of intraperitoneal injections of reserpine on brain 3,4-dihydroxy-L-phenylalanine concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 5. Effect of intraperitoneal injections of reserpine on brain 5-hydroxyindole, 3-acetic acid concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 6. Effect of intraperitoneal injections of reserpine on brain serotonin concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 7. Effect of intraperitoneal injections of reserpine on brain dopamine turnover rate (3,4-dihydroxy-L-phenylalanine/dopamine) of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 8. Effect of intraperitoneal injections of reserpine on brain serotonin turn over rate (5-hydroxyindole, 3-acetic acid/serotonin) concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 9. Effect of intraperitoneal injections of reserpine on plasma norepinephrine concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 10. Effect of intraperitoneal injections of reserpine on plasma epinephrine concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 11. Effect of intraperitoneal injections of reserpine on plasma 3,4-dihydroxy-L-phenylalanine concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Figure 12. Effect of intraperitoneal injections of reserpine on plasma 5-hydroxyindole, 3-acetic acid concentrations of high and low body weight selected lines of chickens. HWS, high body weight selected line; LWS, low body weight selected line.
Curriculum Vitae

ALICE YI-WEN KUO

EDUCATION


2000  **M.S., Animal and Poultry Sciences:** Physiology - Virginia Polytechnic Institute and State University, Blacksburg. Area of specialization - Neuromodulation of cardiovascular response, body weight regulation, and feeding behavior via the autonomic nervous system.

1997  **B.A., Biology:** University of Hawaii at Hilo, Hawaii.

RESEARCH EXPERIENCES

Lead Investigator:

Ghrelin gene expression and sequence comparison in high (HWS) and low body weight selected (LWS) lines of chickens, 2003

Ghrelin mainly is secreted from the stomach and gastrointestinal tract and is a plausible mechanism for increasing feed intake in many species. This study was conducted to determine if and where differences exist in ghrelin gene and to monitor differences in gene expression by using real time PCR on samples from the HWS and LWS lines of chickens which had undergone 46 generations of selection.

Quantitative leptin receptor gene expression HWS and LWS lines of chickens, 2002-2003

Mutations in Zucker fatty rats and obese Koletsky rats have demonstrated that leptin receptor dysfunction causes hyperphagia and obesity. Leptin inhibit feed intake by activating the leptin receptor long form (Ob-Rl) isoform in the hypothalamus. The aim of this study was to evaluate the expression of the Ob-Rl in various organs in the HWS and LWS lines.

Comparison of leptin, IGF-1, and IGF-2 concentrations in chickens HWS and LWS lines of chicken, 2002-2003

Leptin is a feedback signal between adipocytes and the satiety center that is internally involved in body weight regulation and energy expenditure. Insulin-like growth factor-I (IGF-I) and IGF-II regulate perinatal growth, bone growth and metabolic regulation. It is
known that difference of body weight between the HWS and LWS lines of chicken are about 10- fold at selecting age. The aim of this study is to compare leptin, IGF-I and II concentration in the HWS and LWS chickens.

Effect on feed intake after intracerebroventricular injection of human recombinant leptin, in HWS and LWS lines of chickens, 2002-2003

Studies have shown that intracerebroventricular injection of leptin to mammals caused decreased feed intake. Human recombinant leptin also has been administrated to chickens through intracerebroventricular injection, that resulted in decreased feed intake, however this effect was not demonstrated when mouse leptin was administrated. The aim of this study was to compare the feed intake modulation in the HWS and LWS lines chickens by human recombinant leptin.

Determination of leptin receptor gene polymorphism in HWS and LWS chickens, 2001-2002

The db/db mouse has a mutated lepin receptor gene, however leptin secretion remains functional. Preliminary data (unpublished) showed that HWS and LWS chickens have the same leptin gene sequences. The aim of this study to compare leptin receptor sequences for polymorphism between the HWS and LWS lines.

The effect of reserpine on body weight gain, feed efficiency, catecholamine and indoleamine levels in the HWS and LWS lines of chickens, 2000-2001

Various neurotransmitters including norepinephrine, epinephrine, dopamine, and serotonin at the hypothalamus have an impact on the regulation of body weight and feed intake. Reserpine impairs the storgage of biogenic amines, in both the central and peripheral nervous systems, and disturbs the digestive system and decreases food intake. The aim of this study was to compare the response of HWS and LWS lines of chickens to reserpine.


Studies report decreased sympathetic nervous system function is associated with obesity. This study was conducted to compare the autonomic nervous regulation in cardiovascular system though changes in blood pressure and heart rates in HWS and LWS lines of chickens following the intravenous injection of various pharmacological agents.

Co-Investigator:

Identification of polymorphism in the corticotropin releasing hormone receptor 2 gene and quantitative corticotropine releasing hormone receptor 2 gene expression in the HWS and LWS lines of chickens, 2002-2003
Effect of feed intake and body weight regulation after intracerebroventricular injection of ovine corticotropin releasing hormone to chickens selected for body weight, 2002-2003

Effect of crop emptying after intracerebroventricular injection of ovine corticotropin releasing hormone to lines of chickens selected for body weight, 2002-2003

Research Assistant - Virginia Polytechnic Institute and State University

Served as a research assistant at swine nutrition laboratory at year 2001. Researches were funded by BASF to compare the phytase sources in broiler diets. My responsibility was including running various mineral assays, laboratory management, statistic analysis, and training undergraduate students.

Laboratory Technician - Virginia- Maryland Regional College of Veterinary Medicine

Recouped by Dr. Jortner and Ehrich at year 2000 to serve as laboratory technician in their neurotoxicity studies laboratory. Project was funded by United States Army to determine the factor that caused the Gulf War syndrome. The responsibility included brain dissection, running various immuno-histo-assays, neurotoxin administration, and processing sample for TEM

TEACHING EXPERIENCES

1) Guest Lecturer: Principle Biology (BIOL 101, Radford University), 4 credits, Fall 2002, 2003
2) Teaching Assistant: Animal Nutrition and Feeding (ALS 3204, Virginia Tech), 3 credits, Spring 2002
3) Guest Lecturer: Structure and Function (BIOL 311, Radford University), 4 credits, Spring 2002
4) Guest Lecturer: Advanced Physiology and Anatomy of Domestic Animals (ALS 5304, Virginia Tech), 5 credits. Fall 2002
5) Teaching Assistant: Anatomy and Physiology (Governor’s School for Agriculture, Virginia Tech), Summer 2002
6) Teaching Assistant: Animal and Poultry Seminar (APSC 3004, Virginia Tech), 1 credit, Spring 2001
7) Laboratory Instructor: Domestic Animal Anatomy and Physiology Laboratories (ALS 2304, Virginia Tech), 4 credits. Fall 2001
8) Teaching Assistant: Poultry Management (APSC 4404, Virginia Tech), 3 credits, Fall 1999
10) Graduate Mentor - Undergraduate Research Projects:
    a) Leptin modulation of feed intake (Virginia Tech), 2002
    b) Corticotropin releasing hormone modulation of feed intake (Virginia Tech), 2002
c) Corticotropin releasing hormone regulate crop emptying  
(Radford University), 2003

LABORATORY AND RESEARCH TECHNIQUES

Atomic absorption spectrometer  
Blood collection and processing  
Catheterization: Jugular vein, cannulation of brachial vessels and  
hepatic portal vein especially in poultry  
High performance liquid chromatograph with electrochemical detection  
Identification of internal parasites of domestic animals  
Immuno-histology - GFAP, and Flour-jade  
Polymerase chain reaction  
Poultry immunity: PHAP infection, and sheep red blood cells administration.  
Real time PCR  
Southern, Northern, and Western blotting  
Sterotaxic surgery  
Transmission electron microscope imaging

SKILLS

Computer: Microsoft Office suite, SAS for data analysis, MP 100 AcqKnowledge  
BIOPAC system for measuring various physiological functions, AMI-Pro, Lotus 123.  
Language: Speak and interpret Chinese, Taiwaness, Japanese, and English

PUBLICATIONS

Jortner, B. S., E. J. Lehning, S. K. Hancock, K. Lowe, A. Kuo, J. Hinckley, and  
R. LoPachin.  Electron microscopic evaluation of the cerebellar cortex in  
acrylamide exposed rats.  J. Toxicology Suppl. 66:17.

cardiovascular effects of pharmacological agents in chickens selected for high and  

BASF Technical Symposium, Mexico City, Mexico, September 28, 2001.

PRESENTATIONS

on body weight gain, feed efficiency, catecholamine and indoleamine levels in  
high and low body weight selected chickens. The Federation of American  
Societies for Experimental Biology 17(5.2):A865.
ABSTRACTS


AWARDS


Virginia Polytechnic Institute and State University Graduate Student Assembly Travel Funding (2002)

Virginia Polytechnic Institute and State University Graduate Student Assembly Research Scholarship (2002)

Virginia Polytechnic Institute and State University Higher Education Research/Teaching Assistantship (2001)

PROFESSIONAL AFFILIATIONS, ORGANIZATIONS and SOCIETIES

American Physiology Society
Poultry Science Association