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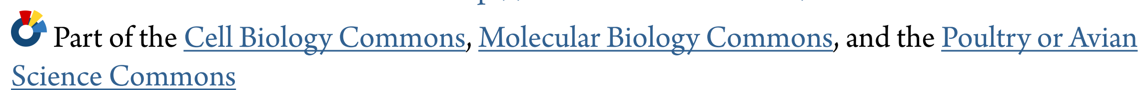
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Leptin Regulates the Expression of Autophagy-related Genes in Chickens

A Thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science in Poultry Science

By

Peter Olawale Ishola
Mississippi State University
Bachelor of Science in Poultry Sciences, 2013

December 2015
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ABSTRACT

Autophagy or cellular self-digestion, a lysosomal degradation pathway that is conserved from yeast to human, plays a key role in recycling cellular constituents, including damaged organelles. It also plays a pivotal role in the adaptation of cells to a plethora of distinct stressors including starvation. Autophagy has been extensively studied in mammals and yeast, but little is known in avian species. Thus, the major objective of the present study was to determine the effects of leptin on autophagy-related genes in chicken hypothalamus, muscle and liver. Leptin is an adipocytokine that is mostly produced by white adipose cells in mammals (as fat storage increases), mediating sensing mechanism for fat deposition, signaling the brain via leptin receptor-mediated signal transduction to inhibit feed intake and increase energy expenditure. In the present study, recombinant chicken leptin (625 pmol, 10 μL) diluted in artificial cerebrospinal fluid was injected intracerebroventricularly (ICV) in one week-old Hubbard x Cobb 500 chicks (n=10) and feed intake was recorded at 30, 60 and 180 min after injection. At the end of the experiment, hypothalamii, muscle, and liver were collected for gene expression and protein level analysis. Leptin significantly reduce feed intake after 30 min compared to the control group. ICV administration of both chicken and ovine leptin significantly down-regulated genes (mRNA and protein levels) in hypothalamic and muscle tissues. In the muscle, leptin upregulates the expression of AMPKβ1 and AMPKϒ1, and downregulates the expression of mTOR, upstream regulator of autophagy pathway. Expectedly, there were upregulations of leptin receptors, ObR ($P<0.05$). Our results support a novel link between metabolic control and autophagy that warrant further investigations.

Key Words: Leptin, autophagy, hypothalamus, muscle, liver, gene expression.

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INTRODUCTION

Subsequent to the challenge of global food security due to fast growing world population, the fast growing birds (broilers) was genetically selected to meet up with increasing demand for high quality animal protein. Although genetic selection has made spectacular progress (high growth rate, muscle development, low feed conversion ratio[weight gain/feed intake]), there have been a number of undesirable changes including metabolic disorders. For instance, broilers do not adequately regulate voluntarily feed intake to achieve energy balance. When given ad libitum access to feed, broilers exhibit hyperphagia leading to excessive accumulation of fat, making these birds prone to obesity [1]. Several other health related problems occur including reproductive failure of overweight male and female broiler breeders [2], white striping [3], congestive heart conditions, ascites and lameness [4]. Since feed is a major component of the total cost (up to 70%) of producing live birds, and because of metabolic disorders related to hyperphagia, poultry producers adopt feeding programs including feed restriction in order to cope with these problems.

Autophagy has been described as the **self-eating** process (occurring during nutrient depletion and stressful conditions) that is highly conserved from yeast to humans during which cells degrade and recycle their own components (cytosol and organelles) within the lysosomes [5, 6]. The word autophagy takes its origin from Greek Word "**auto**" which means **self**, and "**phagein**", meaning **to eat.** Autophagy, which is a unique morphological feature or process in a dying cell was often erroneously presumed to be a preceding pathway to cell death, but in contrast, it has now been evidently clarified that, one of its major function is to fight or avert cell death and consequently keep it alive even when undergoing stressful and life-threatening conditions. In recent years, autophagy has appeared to play critical roles in several cellular functions and

physiological processes including reproduction, development [9, 10, 11], immunity [12], inflammation [7], neurodegenerative diseases [5, 9, 13], cardiovascular diseases [14, 15], metabolic syndrome [5, 9, 11], and energy homeostasis [16].

There are three major types of autophagy; micro-, macro-autophagy, and chaperone-mediated autophagy [10, 17, 18, 19]. Micro- and macro-authophagy can selectively engulf large structures such as mitochondria and endoplasmic reticulum (referred to as mitophagy or reticulophagy, respectively [17, 19, 20] or by non-selective mechanisms (e.g. bulk cytoplasm), whereas chaperone-mediated autophagy degrades only soluble proteins [17, 18]. Although, the autophagy pathway is not completely defined in birds, it has been largely studied in yeast and mammals, and has been suggested that it is regulated by genetic, environmental, nutritional, and hormonal factors.

Among several key metabolic hormones that attract scientific attention that may have an effect over autophagy is the leptin hormone. Leptin, also called the obese hormone, is the central mediator in a negative feedback loop regulation of energy homeostasis. Mammalian adipocytes produce and secrete more leptin in the bloodstream as feed intake and fat storage increases [5, 21] signaling the brain via leptin receptors [16] and in turn, inhibits feed intake in mammals and in avian species [21, 23].

In addition to its role in the regulation of feed intake, leptin has been reported to play several key physiological roles including the regulation of energy expenditure, lipid metabolism, reproduction, bone metabolism [18]. Recently, leptin has been shown to regulate autophagy in mammals. It is not known if leptin has similar effect on autophagy in avian species. Therefore, the objective of the present study is to determine the effect of a single intracerebroventricular

administration of recombinant chicken and recombinant ovine leptin on the expression of autophagy-related genes in broiler chickens.

CHAPTER 2: LITERATURE REVIEW

2.1 Autophagy System

Autophagy is a highly conserved cellular mechanism that is responsible for the degradation and recycling of damaged organelles. The induction of autophagy during nutrient depletion or starvation triggers the response of more than 30 autophagy-related genes (Atg) [5]. However, how Atg proteins are regulated is not well understood, but it's clear that all signals reporting on availability of carbon and nitrogen sources converge on the mechanistic target of rampamycin (mTOR) signaling pathway, and that, Atg proteins are downstream effectors of mTOR pathway [23]

The formation of autphagosome involves three steps, and the first step is initiation, during which phagophore (outer mitochondrial membrane, plasma membrane, endoplasmic reticulum membrane etc) undergo nucleation [8]. The second step undergoes elongation, cycling, expansion and closure, forming an autophagosome [8]. The third and final step is referred to as maturation, which involves the advancement of autophagosome into amphiosome, which is an acidic and hydrolytic vacuole. It is this hydrolytic vacuole that is ripe for degradation and recycling of nutrients [18].

Under fed condition (normal nutrient-energy adequate), the nutrient sensor, mTOR is activated, which in turn phosphorylates UNC-51 like kinase 1(ULK1) and thereby sequestering the ULK1-Atg13-FIP200 (focal adhesion kinase family interacting protein of 200 kDa) complex in an inactive state at the mTOR complex [23, 20]. In contrast when nutrients are limited (e.g. during stress or starvation), the energy sensor, adenosine mono-phospate protein kinase (AMPK) is activated. AMPK activation inhibits mTOR activity leading to a reduced ULK1 phosphorylation and consequently releases the ULK1-Atg13-FIP200 complex from mTOR to the site of

autophagosome formation and induction of autophagy. In the second step of autophagy, Beclin1 forms a lipid kinase complex with Atg4, Vacoular protein sorting 15 (Vps15), and Vps34 that phosphorylates phosphatidylinositol (PI) to form inositol-3-phosphate (PI3P) and is essential for induction of autophagy [23]. Accumulation of PI3P in specific sub-domains of the endoplasmic reticulum (ER) increases membrane curvature at the site of autophagosome formation. The elongation step involves two ubiquitin-like reactions of the pre-autophagosomal structures. First, the ubiquitin-like protein Atg12 is conjugated to Atg5 by the action of Atg7 and Atg10 after which Atg16 multimerizes to form the Atg12-Atg5-Atg16 complex. Next, Atg4 cleaves soluble microtubule-associated protein light chain 3-I (LC3-I) to form the membrane-bound LC3-II [35]. Both of these two ubiquitin-like systems are required for elongation and closure of the phagophore. During maturation and fusion, autophagosomes will first fuse with endosomes then with lysosomes. Any mutation or loss of proteins important for formation of multivesicular bodies (MVBs) can lead to inhibition of maturation of autophagosomes [10]. Some genes involved in this step include Ultraviolet Radiation Resistance-associated Gene (UVRAG), a Beclin 1 interacting protein that recruits the fusion machinery on the autophagosomes. Another Beclin 1 interacting protein, Rubicon, also functions in the maturation of autophagosomes where it is thought to be a part of a distinct Beclin 1 complex containing hVps34, hVps15, and UVRAG that suppresses autophagosome maturation [18]. Working together, these steps complete the formation of the autolysosome and its lysis, that releases proteins and amino acids that can be used as an energy source during times of low energy availability or increased energy demand (stress) for the organism (Figure 1).

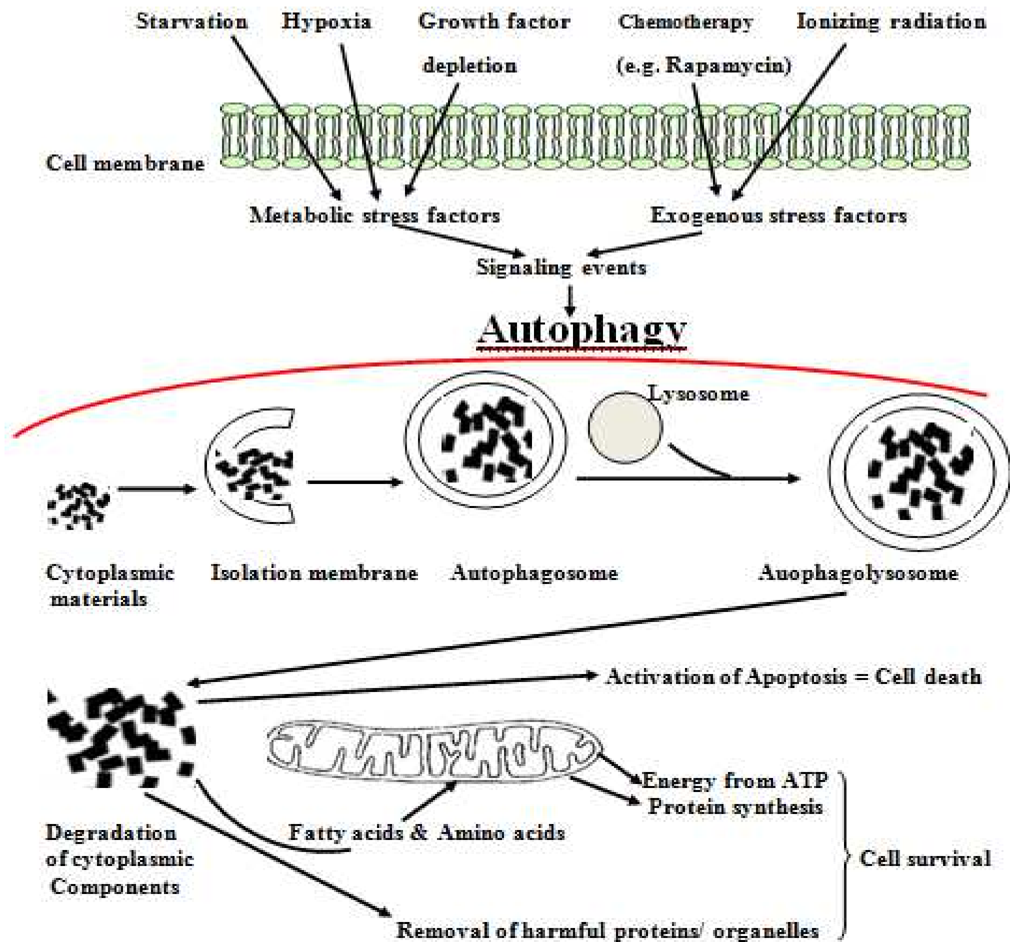

Figure 1: The Autophagy pathways and its physiological role.
Both the metabolic and exogenous stress factors are among the crucial factors that do activate the induction of autophagy, the process which involves the sequestration of cytoplasmic content. During the process of autophagy, both fatty acid and amino acids components of cytoplasm are re-synthesized into energy in form of ATP and proteins respectively, to enhance cell survival. Also, the removal of harmful proteins and organelles to promote the survival of essential long lived cells is orchestrated through the process of autophagy. Apoptosis of cancerous cell is sometimes warranted or enhanced by the process of autophagy to inhibit tumorigenesis. This figure was adapted from; [43].

2.2.0 Leptin system

2.2.1 Leptin genes and proteins

Friedman and co-workers previously cloned and characterized ob gene (leptin) in rodent and humans [25]. The leptin gene consists of three exons with the two coding regions that are

separated by two introns. It was assigned to mouse chromosome 6 [26] and human chromosome 7q31.3 [26]. The name leptin (coined from the Greek word "leptos" meaning **lean**) contains 146 amino acids (AA) and a 21 AA signal peptide cleaved during translocation into the microsome. The 16-kDa mature leptin circulates in serum both as a free and as a protein-bound entity. The main site of ob gene expression and leptin secretion is mammalian white adipocytes. Its expression and secretion occur exclusively within the differentiated adipocytes [11, 26, 27]. However, in other organism, leptin is also produced in several cell types including; osteoblasts [28], and pituitary [27], brain [11], gastric cells in the walls of the stomach [13], follicular papilla cells of hair follicles [11, 26], placenta [27, 29], and skeletal muscle [11]. Additionally, leptin expression has been demonstrated in the ovary (granulose and theca cells, corpora lutea, and interstitial gland) [29] and in the mammary gland [27]. There is high homology of leptin in mammalian species. Furthermore, chicken leptin cDNA has been characterized by this groups, but not by others, but still a matter of controversies since 1998. The chicken leptin, which has 145 amino acids is expressed not only in adipose tissue, but also in liver [30]. It contains, in contrast to mammalian leptin, an unpaired cystein at position 3 after the signal peptide [30].

2.2.2 *Leptin Receptor*

The leptin receptor was first identified in mouse choroids plexus through expression cloning techniques and then in human using infant total brain library [31]. It is a single transmembrane-spanning receptor (Figure 2) and a member of the cytokine receptor superfamily that includes the gp130 signal-transducing component of the receptors for interleukin 6 (IL-6), granulocyte colony stimulating factor (G-CSF), and leukemia-inhibitory factor (LIF) [25]. The Ob-R extracellular domain consists of 816 AA and is followed by a 23-AA transmembrane domain and intracellular domain which varies in length from 30 to 303 AA, depending on alternate splicing. The alternate

splicing of the Ob-R gene generates multiple variants of leptin receptor mRNA that encode at least six Ob-R (Ob-Ra,b,c,d,e & f) isoforms [25,32] . All these isoforms differ in the length of their intracellular domains, but they share the same or common extracellular and (excluding ob-Re) trans-membrane domains [32].

Ob-R is primarily expressed in the hypothalamus. It is particularly prominent in areas important in regulation of energy balance such as arcuate (ARC) and paraventricular nuclei (PVN) [25, 32]. Expression of Ob-R was also detected at lower levels in a large number of peripheral tissues including skeletal muscle, heart, adrenals, kidney, adipose tissue, liver, pancreatic β-cells and immune cells [27]. The short isoforms are expressed at higher levels in a variety of tissues and were elegantly reviewed by Friedman and Halaas [23]. The ubiquitous expression of leptin and its related receptors indicates that leptin may have several physiological roles [23].

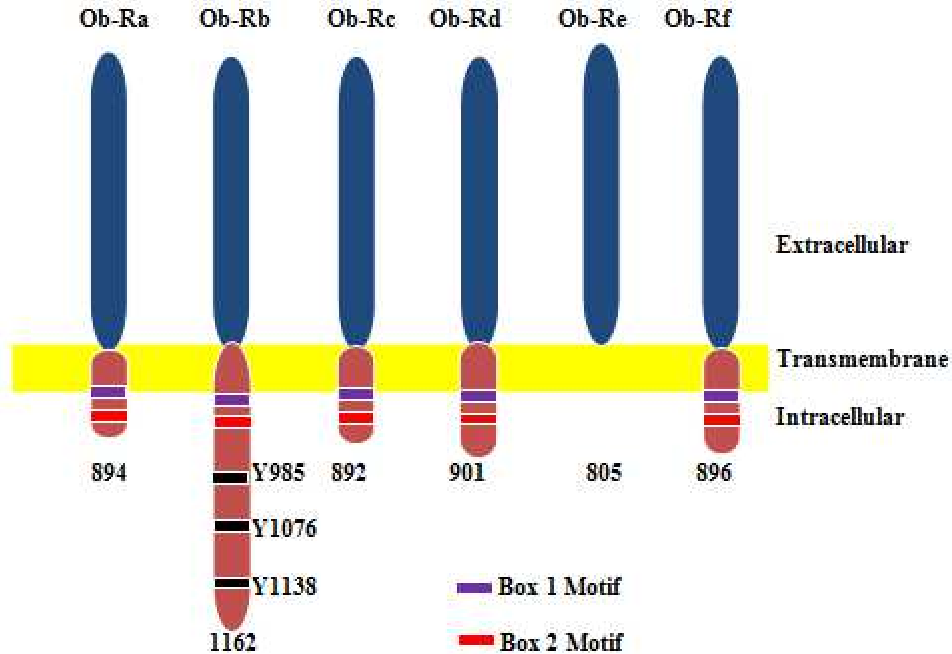

Figure 2: ***Figure 2 Leptin receptor isoforms***. There are at least six different isoforms of the leptin receptor, Ob-Ra, b, c, d, e, and f, they all share identical extracellular domains but they differ at their intracellular domain. They have transmembrane domains, with the exception of the ObRe. The Box 1 and Box 2 motifs that are encoded only by ObRb enable it to activate JAK-STAT pathway of signal transduction. Also, the C-terminus of Ob-Rb has three conserved tyrosine, which include Y985, Y1077, and Y1138. Y1138 functions as a docking site for STAT3. The ob-Re lacks intra-membrane domains, and it is the soluble circulating leptin-binding protein. This figure was adapted from; [44].

2.2.3 ***Physiological roles of leptin***

It is well established that leptin has potent food intake and body weight reducing effects in mammals [30] and this effect is mediated via the activation of Proopiomelanocortin and cocaine- and amphetamine-regulated transcript (POMC/CART) and inhibition of Neuropeptide Y and Agouti related peptide (NPY/AgRP) neurons [25]. The molecular basis for stimulation of POMC gene expression likely involves Janus kinase and signal transducer and activator of transcription (JAK-STAT) activation [30, 42], while the phosphoinositol 3-kinase (PI3K) pathway may play a specific role in the repression of NPY and AgRP gene expression by leptin [25, 31]. Leptin has been reported to interact also with other hypothalamic peptides including orexin, Melanocortin

receptor (MCR), Corticotropin releasing hormone (CRH), Glucagon-like peptide-1(GLP-1), ghrelin, Cholecytokinin (CCK), bombesin to regulate feeding behavior [5]. Leptin also increases energy expenditure [15], induces lipolysis, reduces lipogenesis [23, 32,], regulates reproduction, immunity, and bone mass [23, 32, 33].

2.4 *Leptin signaling pathways*

The biological effect of leptin is primarily mediated through its receptors [16, 32]. The longest intracellular domain of ob-Rb upon its ligand binding, activates the protein tyrosine kinase that belongs to the Janus kinase (JAK) family [32, 33]. When activated, JAKs phosphorylate signal transducers and activators of transcription (STAT), and the phosphorylated STAT proteins translocate to the nucleus where they regulate the expression of target genes [32], (figure 3). Also, the Ob-Rb receptor is able to undergo signal transduction through mitogen activated protein kinase (MAPK) pathway [32]. The short isoforms of leptin receptor (ob-Ra, ob-Rc, ob-Rd, and ob-Rf) are unable to activate JAK-STAT pathway, they may cause signal transduction through other mechanism such as MAPK pathway and phosphatidylinositol 3-kinase (PI3K) pathways [32, 33]. These short isoforms of leptin receptors are expressed in many peripheral tissues. pathways [32, 33]. These short isoforms of leptin receptors are expressed in many peripheral tissues. The ob-Re lacks intra-membrane domains, and is the soluble circulating leptin-binding protein.

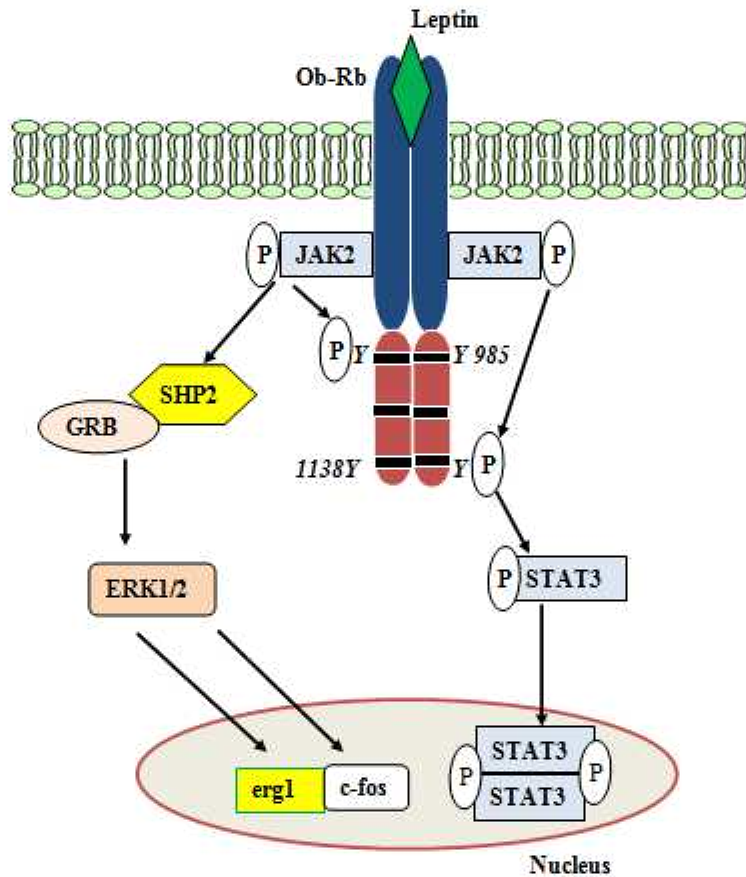

Figure 3: The Leptin, JAK-STAT, and ERK Pathways
Here, it is clear that all the pathways are induced and dependent on JAK2 signaling pathway. When activated, JAKs phosphorylate STAT, then the phosphorylated STAT proteins translocate to the nucleus where they regulate the expression of target genes. It is also shown that the activation of ObRb enhances the phosphorylation of Tyr 985 by Jak2 forming the units that can further phosphorylate SHP-2. The phosphorylated SHP-2 can then modulate GRB-2 which eventually activates the ERK. The activated ERK then facilitates the transcription of egr-1 and c-fos This figure was adapted from; [42].

2.2.5 Interaction of leptin and autophagy in the regulation of energy homeostasis

In vitro treatment with recombinant leptin inhibited autophagy in human CD4(+)CD25(-) conventional (T conv) T cells and this effect was mediated via mTOR activation [14,21]. However, leptin knockdown attenuated hypoxic-preconditioning induced autophagy in bone marrow derived mesenchymal stem cells [14, 34], indicating that the effect of leptin on autophagy might be tissue and cell-specific. Enteral leptin administration has also been shown to

inhibit intestinal autophagy in piglets [14, 35]. In heart, however, leptin promoted autophagosome formation as evidenced by increased LC3-II, beclin 1 and Atg5 expression [13, 14]. Malik and co-workers reported in 2011 [36], that peripheral administration of recombinant leptin induced autophagy in peripheral tissues including skeletal muscle, liver and heart. Moreover, leptin stimulated autophagy in cultured human and mouse cell lines and this effect was likely mediated through the activation of AMPK and inhibition of mTOR [11, 14, 16]. More so, in 2012, three recent studies have implicated Central nervous system (CNS) autophagy in the regulation of energy homeostasis. Conditional specific depletion of Atg7 in POMC neurons resulted in higher body weight, hyperphagia, impaired glucose tolerance, increased adiposity and leptin resistance [37]. Moreover, deficient Atg7 in hypothalamic POMC neurons impaired leptin induced signal transducer and activator of transcription 3, STAT3 activation. In line with these data, Malhotra and coworkers [38], recently showed that upon high-fat diet consumption, mice lacking Atg12 in POMC positive neurons exhibit accelerated weight gain, adiposity and glucose intolerance which is associated with increased food intake and decreased leptin sensitivity. Mice lacking Atg5 in POMC neurons do not exhibit these phenotypes observed in Atg7 and Atg12 deficient mice [35]. These results indicated that autophagy-related genes might exert different physiological function based on tissue or cell type. Furthermore, it has been proposed that autophagosome-mediated form of secretion in POMC neurons controls energy homeostasis by regulating alpha-melanocortin stimulating hormone (α-MSH) production [12]. Kaushik and his group [39] also demonstrated a role for autophagy in hypothalamic agouti-related peptide (AgRP) neurons in the regulation of food intake and energy balance. Activation of hypothalamic mTOR has been shown to regulate feeding behavior and energy homeostasis [14, 27, 36] and mTOR pathway has been shown to downregulate leptin and upregulate autophagy [14, 27, 40].

Leptin, mTOR and autophagy have all been reported to be regulated by starvation and nutritional state [40].

2.3 OBJECTIVES

Since leptin is anorexigenic in both mammalian and avian species, we hypothesized that leptin may regulate autophagy in a tissue-specific manner in broiler. Therefore, the aim of the present study is to determine the effect of a single central administration of recombinant chicken and recombinant ovine leptin on the expression of autophagy-related gene in three metabolic important tissues; (i) brain (main site for regulation of feed intake), (ii) muscle (main site for thermogenesis), and (iii) liver (main site for lipogenesis).

Our lab has recently characterized autophagy-related genes in chickens and quail and showed a genotype and gender dependent expression [41]. Additionally, autophagy pathway exhibited differential expression between a stress-sensitive and stress resistant quail lines suggesting its potential role in stress response in avian species [41]. The molecular mechanism-based understanding of leptin-autophagy interaction will provide new insights in chicken response to stress and will likely contribute to a framework whereby new effective nutritional and/ or genetic strategies could be developed.

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CHAPTER 3

3.1 MATERIALS AND METHODS

In Vivo Study

The studies were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol #13039).

3.1.1 Animal, diet, Leptin treatment and experimental design

This work was performed in collaboration with Dr. Mark Cline (Virginia Tech) and Dr. Ohkubo (Ibaraki University, Japan). One week-old Hubbard x Cobb 500 chicks were assigned into three body weight matched groups and subjected to two treatments. One group was intracerebroventricularly (ICV) injected with recombinant Chicken Leptin and another group with recombinant Ovine Leptin (625 pmol, 10 µL) diluted in artificial cerebrospinal fluid (CSF)(n=10). The control group (n=10) received ICV injection of CSF. Feed intake was recorded at 30, 60, and 180 min after injection. At the end of the experiment, the birds were euthanized, the samples (hypothalamus, muscle and liver) were collected, snap frozen in liquid nitrogen and kept at -80^{C} until use.

3.1.2 In vitro Study

The quail myoblast (Qm7), Chicken embryo liver cells (Celi), Human brain cells (SHSY5Y), and Chinese Hamster Ovary cells (CHO) were cultured in appropriate media. At 80-90% confluence, the complete medium was removed in each case, and replaced with serum deprived media overnight (fasting). Thereafter, QM7 cells and Celi were treated separately with recombinant chicken leptin and ovine leptin for 24 hr, each treatment having 2 replicates. The untreated cells were used as control. However, The cultured human brain cells (SHSY5Y) and CHO cells were

also treated with recombinant chicken leptin and ovine leptin, but on time dependent manner, at time variation of: 0, 0.25, 0.5, 1, 4, and 24hrs, respectively.

3.1.3 RNA Extraction

Total RNA was isolated from broilers tissues (hypothalamus and muscle) and avian cell lines using Trizol (Life technologies, Catshed, CA) according to manufacturer's recommendation. Briefly, 1 mL of Trizol was added to 1.5ml tube that already contained the beads (Next Advance) and tissue samples , then chloroform was added, and then centrifuged (12000 rpm , 15min, 4^0 C) , and the upper phase was carefully removed, then isopropanol was added and then centrifuged (12000 rpm, 10min, 4^0 C), and then DEPC-EtOH was added to supernatant collected, and then centrifuged (9500 rpm, 5 min, 4^0 C) to get the RNA pellet. The RNA was suspended in appropriate volume of DEPC-H_2O. The RNA quantity and integrity were determined at 260/280 using Synergy HT multi-mode microplate reader (BioTek) and by electrophoresis.

3.1.4 Reverse Transcription and real time Quantitative PCR (qPCR)

1 ul of otal RNA, 15 ul of ultra pure water (Eup H_2O) and 4ul of cDNA SuperMix (5X) [Quanta] (Biosciences) were added into 0.5 tubes in order to make cDNA. The cDNA were then subjected to qPCR using Sybergreen and Chicken Atg specific primers (see Table1) [41]. The qPCR was run using Applied Biosystems 7500 machine (Life Technologies). The relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method [1].

Gene	Accession number[a]	Primer sequence (5' ⟶ 3')	Orientation
Beclin1	NM_001006332	TGCATGCCCTTGCTAACAAA	Forward
		CCATACGGTAGAAGACGGTATCTTT	Reverse
Atg3	NM_001278070	GAACGTCATCAACACGGTGAA	Forward
		TGAGGACGGGAGTGAGGTACTC	Reverse
Atg5	NM_001006409	TCACCCCTGAAGATGGAGAGA	Forward
		TTTCCAGCATTGGCTCAATTC	Reverse
Atg9A	NM_001034821	AGTATGCCTCCACTGAGATGAGTCT	Forward
		GGCATGCTGCTTGTGCAA	Reverse
Atg10	XM_424902	CATCTCACCAGATCTCAAGAAGGA	Forward
		CGACATGCGTAAGCAACGTT	Reverse
Atg12	XM_003643073	GCACCCGCACCATCCA	Forward
		GAGGCCATCAGCTTCAGGAA	Reverse
Atg14	XM_426476	GCGCTGCGAGGGTGTTAAT	Forward
		TTCTGTTACAAAAGCGTTCCTTGA	Reverse
Atg13	XM_003641387	GGTCCCCCGAGCCAAATA	Forward
		ATGAGGTGCGGAGCTGTAG	Reverse
Atg7	NM_001030592	ACTGGCAATGCGTGTTCAG	Forward
		CGATGAACCCAAAGGTCAGA	Reverse
Atg4B	NM_213573	CCCCGATGAAAGCTTCCA	Forward
		GCTCAGCGATGCTCATTCTG	Reverse
Atg4A	NM_001271986	CACAGCAGTGCACATTTGCA	Forward
		CAGAGTCCTGCTGCGTTCCT	Reverse
Atg16L1	XM_003641751	TGCATCCAGCCAAACCTTTC	Forward
		CGACGCTGGTGGCTTGTC	Reverse
UVRAG	NM_001030839	GGGCTCATGGTCAGATGTGA	Forward
		CTTTGGAACGGGAATTGCA	Reverse
Ambra1	XM_001233288	GGGATGTTGTGCCTTTGCA	Forward
		CCTGGTGTGGGAAGAGAGAAGA	Reverse

AMPKY1	NM_001034827	CAAGCCGITGGTCTGCATCT	Forward
		GGGAGGAGACGGCATCAA	Reverse
AMPK-β1	NM_001039912	TTGGCAGCAGGATCTGGAA	Forward
		AAGACTGTTGGTCGAGCTTGAGT	Reverse
AMPK-α1	NM_001039603	CCACCCCTGTACCGGAAATA	Forward
		GGAAGCGACTGCCAGAGTTC	Reverse
ObR	NM_204323	GCAAGACCCTCTCCCTTATCTCT	Forward
		TCTGTGAAAGCATCATCCTGATCT	Reverse
mTOR	NM_020009	CATGTCAGGCACTGTGTCTTCTC	Forward
		CTTTCGCCCTTGTTTCTTCT	Reverse
Rab7	XM_003641978	GTGCCAAGGAGGCCATTAC	Forward
		AAGTGCATTTCGTGCAATC	Reverse
Lamp2	NM_001001749	TCAATAGCTGAAGAATGCGC	Forward
		TGCCAACTGCGACTGGAAT	Reverse

Table1: The Real-Time quantitative PCR Primers' sequences**.** This figure is adapted from; [2**]**

3.1.5 ***Western Blotting***.

Tissues (hypothalamus and muscle) were homogenized in lysis buffer [10 mM Tris base (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40], phosphatase inhibitors [5ul NaF, 5ul $NavO_3$, 5ul PMSF(200Mm)] and Protease Inhibitors [1 ul Leupeptin, 1 ul Aprotinin, 1ul Pepstain] (Roche)]. Protein concentrations were determined using a Bradford assay kit (Bio-Rad) with BSA as a standard. Proteins (35-100 μg) were run on Bis-Tris gels (Invitrogen) and then transferred to Immun-Blot PVDF membranes (Bio-Rad). The transferred membranes were thereafter blocked for 1 h at room temperature and incubated at 4 °C overnight with antibodies against Atg3, 5, 7, 12, Phospho STAT3, T-STAT3, Phospho and Total STAT6, Beclin1, Phospho and Total mTOR, Raptor, p-AMPKalpha1, LAMP2, Rab7, ObR (all from Cell Signaling Technology; 1:1,000). Protein loading was assessed using an anti-B-

Actin (1:1,000; Cell Signaling) or anti-GAPDH antibody (1:1,000; Santa Cruz). The secondary antibodies were used (Anti-rabbit [Cell Signaling] and Anti-Goat [Santa Cruz] 1:5,000) for 1 h at room temperature. Finally, the signal was visualized by enhanced chemiluminescence (ECL plus) and captured by FluorChem Multi Fluor System (Protein Simple). Image Acquisition and Analysis were performed by Alpha View software (Protein Simple).

3.1.6 Immunofluorescence

Immunofluorescence was performed as previously described (Dridi et al, 2012 [3]. Briefly, QM7 and CHO cells were shown to about 70% confluence in chamber slides (LabTek). After treatment, as mentioned above, cells were fixed in methanol for 10 min at −20 °C. Cells were blocked with Protein Block Serum-Free (Dako) and incubated with LC3B (for QM7), Total-STAT3 and ObR (CHO cells) (1:200; Cell Signaling Technology) overnight at 4 °C and visualized with Alexa Fluor 488-conjugated secondary antibodies. After DAPI counterstaining slides were coverslipped in Vectashield (Vector Laboratories). Images were obtained using the Leica SP-5 or Zeiss Axio Observer Z1 microscopes.

3.1.7 ***Statistical analysis***

Data were analyzed by Student's t-test, using Graph Pad Prism software. Significance was set at $P<0.05$. Data are expressed as mean ± SEM.

3.2 RESULTS

3.2.1 Intracerebroventricular administration of recombinant leptin inhibits feed intake in young broiler chicks

As shown in Figure 4A, ICV administration of recombinant chicken leptin significantly inhibits feed intake in young broiler chicks 30 minutes after the injection. Feed intake remained numerically lower in leptin treated group compared to placebo-treated groups after 1 and 3h, but the difference was not statistically significant. The same effect was observed with recombinant ovine leptin (Fig 4B)

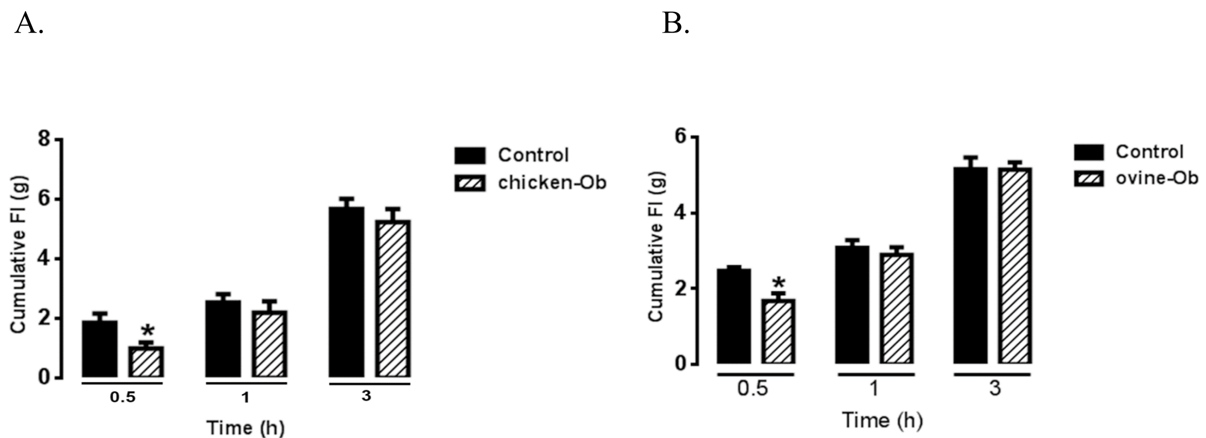


Figure 4: Recombinant leptin inhibits feed intake in young broiler chicks. (A) Chicken leptin, (B) Ovine leptin. Data are mean ± SEM (n=10).*p<0.05

3.2.2 ICV leptin administration upregulates the hypothalamic leptin receptors gene expression in broiler chicks

As shown in Figure 5 A and B, ICV administration of recombinant chicken or ovine leptin significantly upregulates the expression of hypothalamic leptin receptor in young broiler chicks ($p<0.05$).

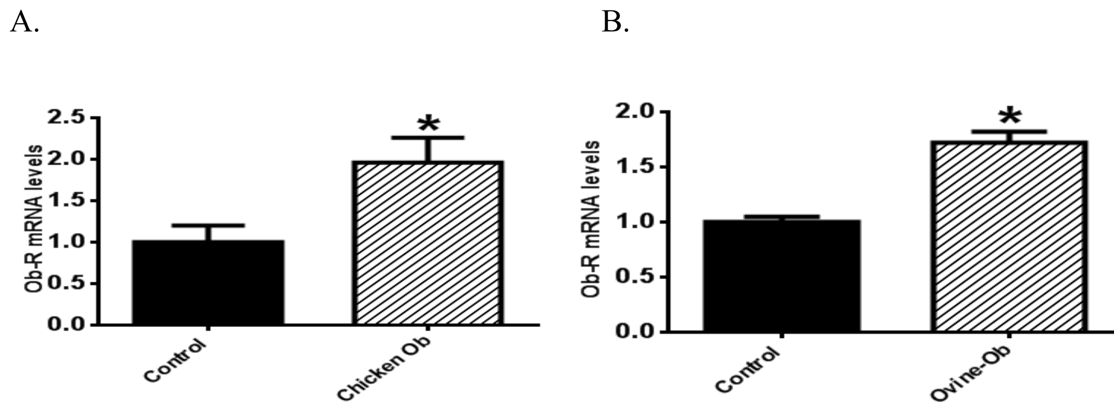


Figure 5: Intracerebroventricular injection of recombinant Chicken leptin (A) or Ovine leptin (B) upregulates leptin receptor (Ob-R) genes expression in the hypothalamus of young broilers chicks. Data are mean ± SEM (n=10).*p<0.05.

3.2.3 ICV administration of recombinant leptin downregulates autophagy related genes in broiler chicks' hypothalamus

The result shown in Fig 6 A shows that recobinant chicken leptin downregulates most of the autophagy related genes (Atg), including Atg3, 2b, 12, 13, Lamb2, and Rab7 ($p<0.05$). Ovine leptin had the same effect on broiler chick hypothalamus (Figure 6B).

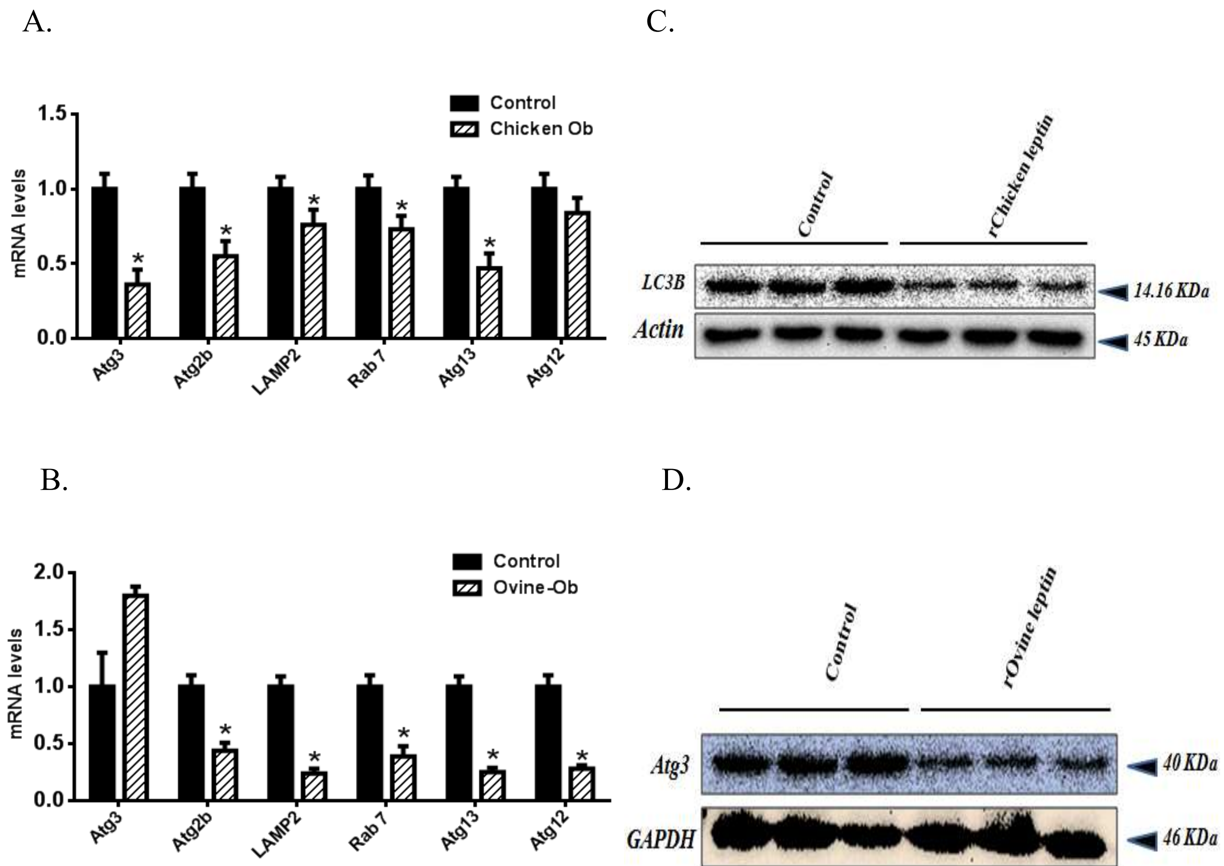


Figure .6: Recombinant leptin downregulates autophagy related genes and proteins in broiler chicks's *hypothalamus tissues*. Chicken leptin (A & C). Data are mean ± SEM (n=10).*p<0.05. Ovine leptin (B & D).

Also, the results for protein analysis show that chicken leptin and ovine leptin administrations down-regulated LC3B and ATG3 proteins respectively in chicken hypothalamus tissues (Fig 6 C & D)

3.2.4 ICV administration of recombinant leptin alters autophagy related genes in broiler chicks' skeletal muscle tissue

As shown in Figure 7, the ICV administration of recombinant Ovine leptin downregulated Atg3, 4a, 7, 9, 10, 12, 13,14 and beclin1(p<0.05). Interestingly, there was upregulation of leptin

receptor (ObR).

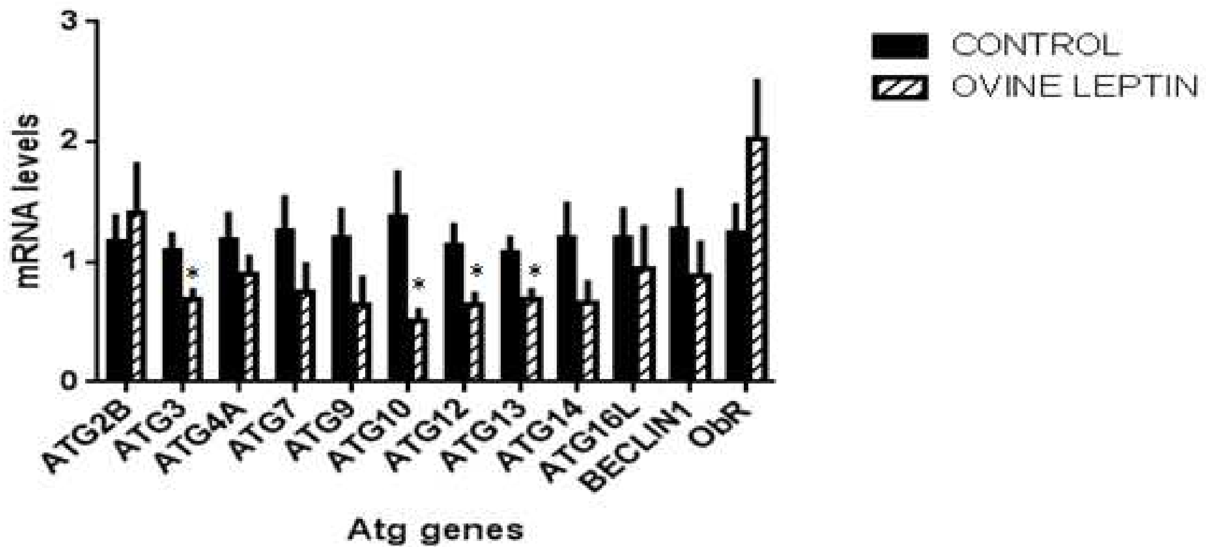


Figure 7: Recombinant Ovine leptin down regulates autophagy related genes in chicken muscle**.** Data are mean ± SEM (n=10).*p<0.05

3.2.5 Leptin treatment regulates Autophagy related genes in quail myoblast (Qm7 cells)

The results as shown in figure 8A and B reveal that the recombinant chicken and ovine leptin downregulated the expression of autophagy related genes in QM7 compared to untreated cells. In addition, leptin treatment alters the expression of AMPK (energy sensor) and mTOR (nutrient sensor).

Interestingly, STAT3 was down-regulated in both treatments compared to control, the regulation of which was statistically significant ($p<0.05$) in ovine leptin treatment. Similarly, there was downregulation of STAT6 in both treatments, but this was statistically ($p<0.05$) significant in chicken leptin treatment($p<0.05$).

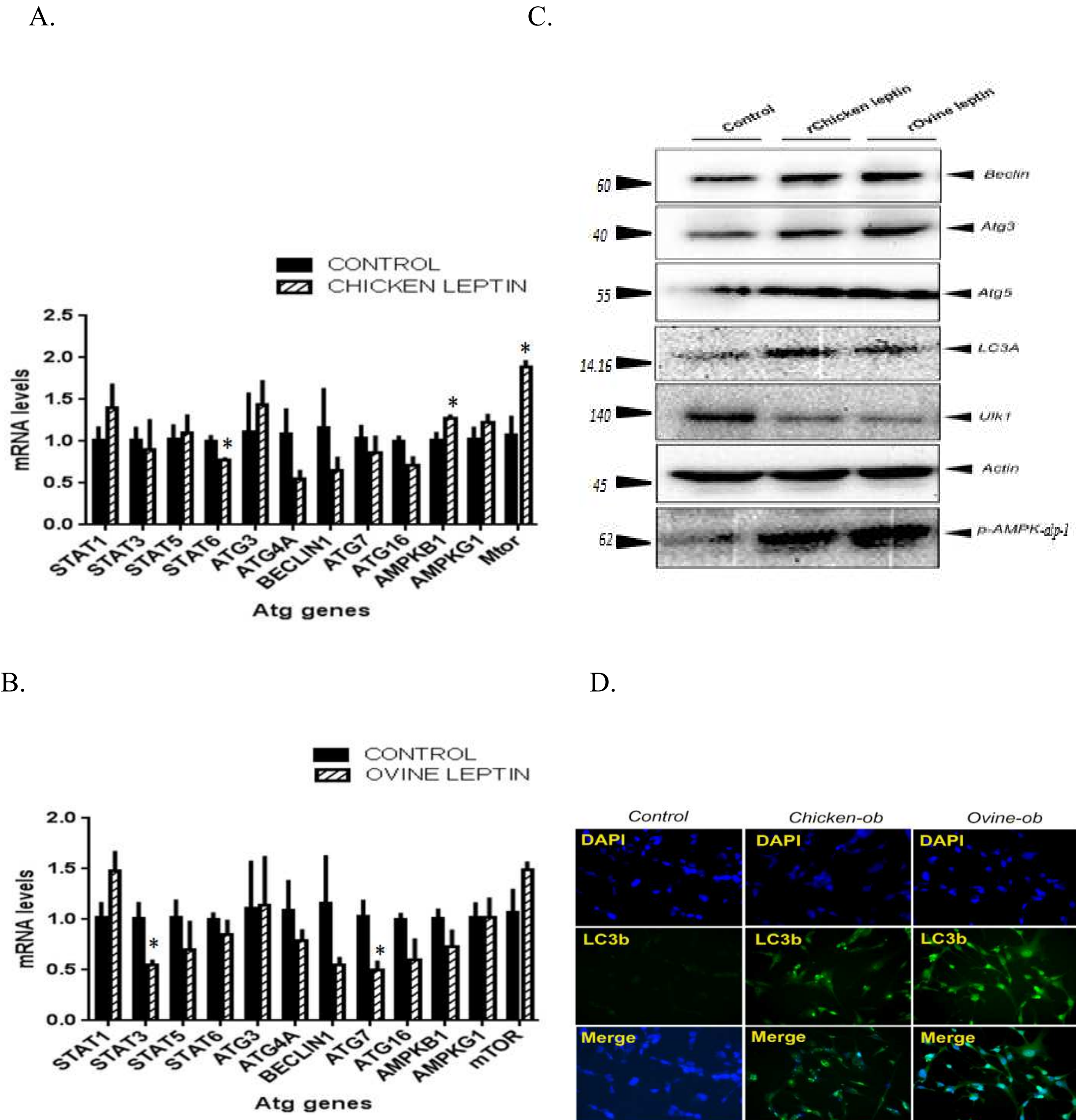


Figure 8: Recombinant Chicken leptin and Ovine leptin regulated autophagy related genes and proteins in quail myoblast (Qm7cells) respectively. Chicken leptin (A), Ovine leptin (B). Data are mean ± SEM (n=4).*p<0.05. Western blot (C) and, Immunofluorescence (D)

The results of protein analysis as shown in figure 2.8C shows that Atg3, 5, Beclin, LC3A and Phospho-AMPKalpha1 proteins(energy sensor) were more induced in both treatment group

compared to control. However, there was reduction in ULK1 protein level in both treatment groups compared to control.

3.2.6 ***Autophagy related gene expression in fasted Qm7 cells.***

In other to verify and validate beyond any doubt on the effect of both chicken leptin and ovine leptin on autophagy in chicken, the real time qPCR was carried out to examine the expression of few autophagy related genes, aiming to see if fasting (treated with medium that lacks bovine serum) alone will induce autophagy, particularly in the same manner as leptin treatment). The results (Figure 9) obtained showed that there was no significant effect (p>0.05)

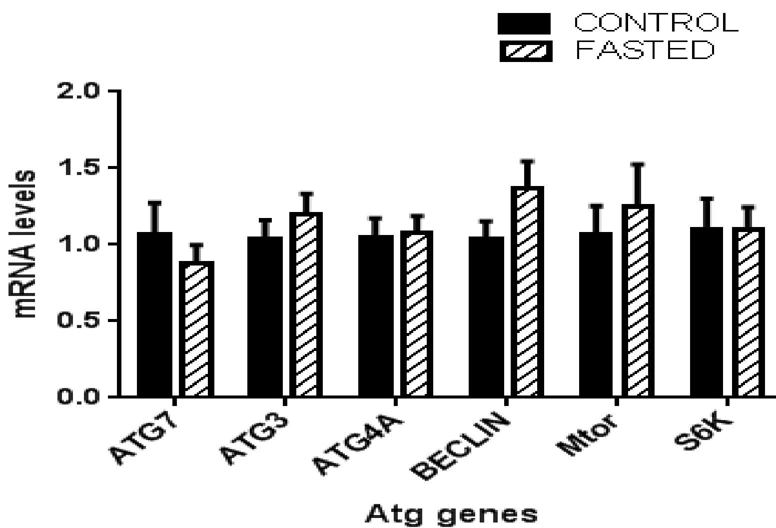


Figure 9: Gene expression of Autophagy related genes in fasted Qm7 cells. Data are mean ± SEM (n=4).*p<0.05

3.2.7 Recombinant leptin administration regulates Autophagy related gene expressions in CELi cells

As shown in figure 10A and C, chicken leptin administration downregulated the expression of Atg3, 7, 9, 10, 12, 13, 14, 16L, Beclin1, AMPK-gama1, mTOR, LC3A and STAT6.. However, Atg2B, AMPK-beta1, LC3B, STAT1, STAT3, and STAT5 were upregulated by chicken leptin

administration. Also, ovine leptin administration downregulated Atg2B, Atg3, Atg7, 9, 10, 12, 13, 14, 16L, Beclin1, AMPK-beta1, AMPKgama1, and mTOR. But interestingly, LC3A, LC3A, STAT1, STAT3, STAT5, and STAT6 were upregulated by ovine leptin administration. Also, the results shown in Figure 11 (B&D) show that regulation of Atg12, 13, 14, AMPKgama1, LC3B and STAT3 by Ovine leptin treatment were statistically significant($p<0.05$).

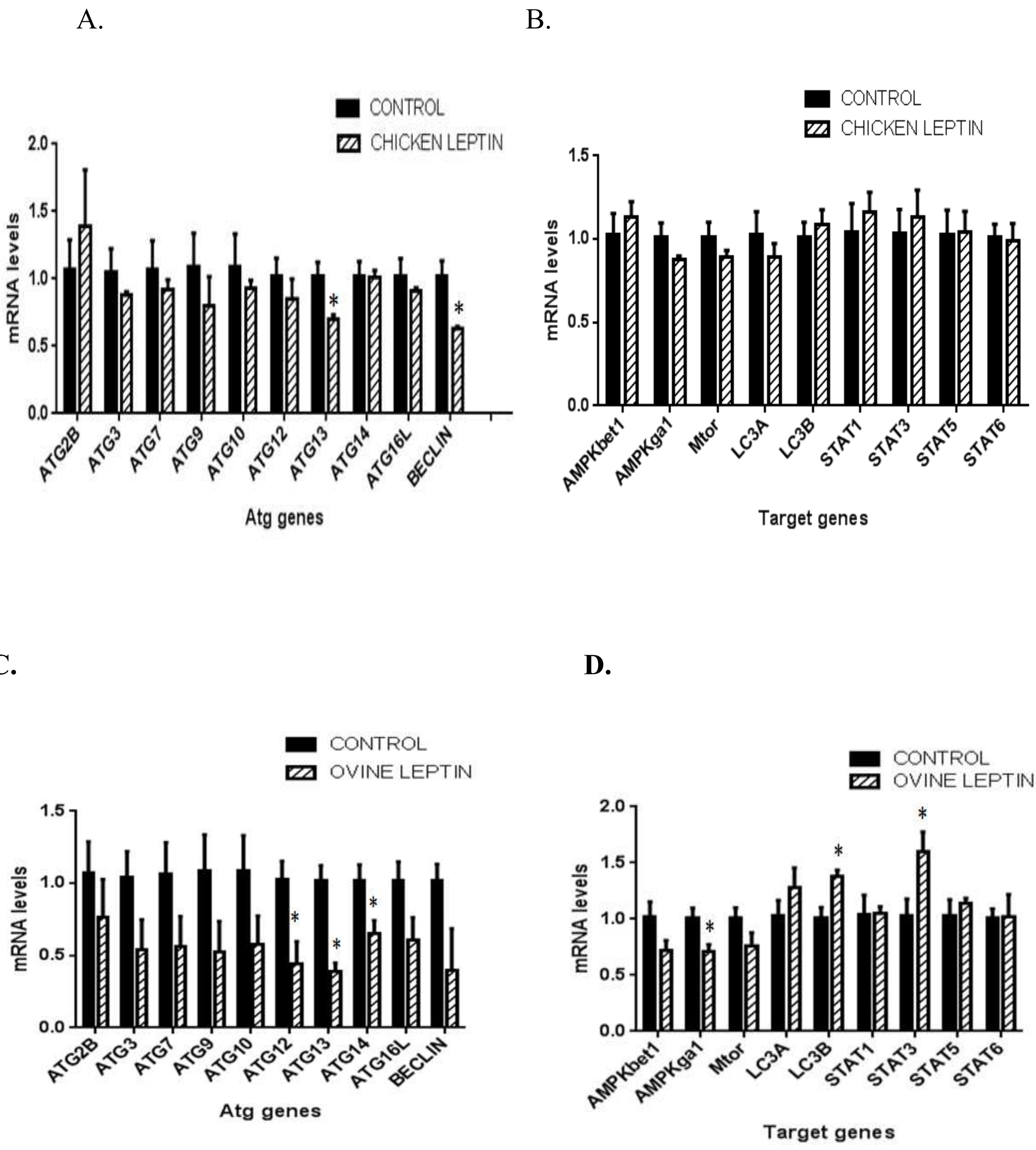
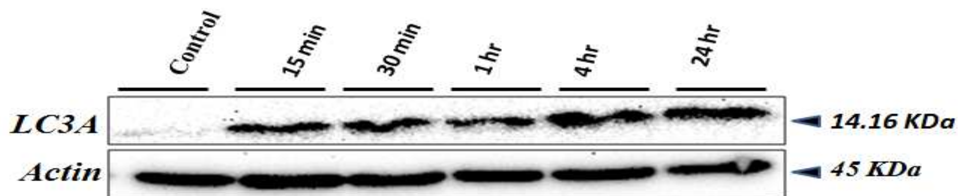


Figure 10: Leptin administration regulates Autophagy related genes expressions in CELi cells. Data are mean ± SEM (n=4).*$p<0.05$.

3.2.8 Leptin treatments alter Atg protein in SHSY5Y

Figure 11 (A&B) shows that the regulation of LC3A by Ovine leptin treatment on SHSY5Y cells seems to be time dependent, whereas, the regulation of Beclin1 was not dependent on time course.

A.



B.

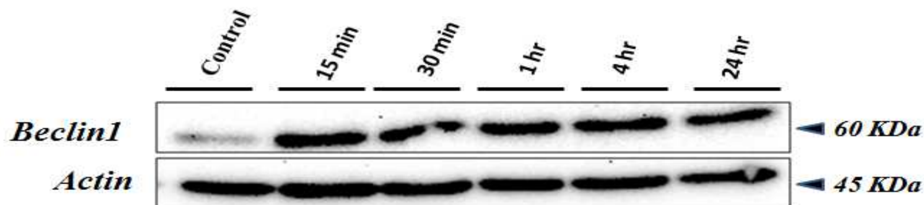
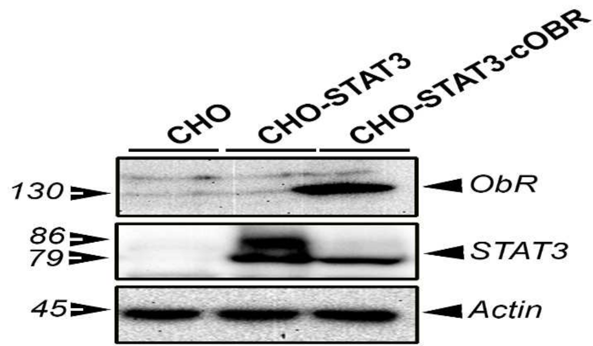


Figure 11: Leptin treatments alter Atg protein in SHSY5Y. (A&B Ovine leptin)

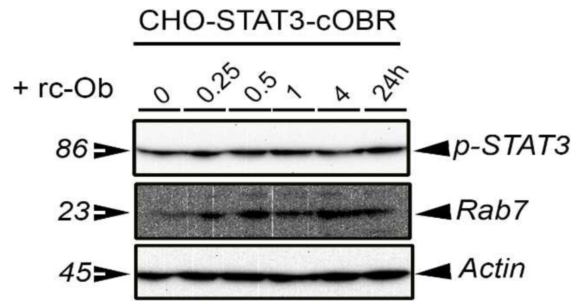
3.2.9 Leptin administration alters Autophagy related proteins in transfected CHO Cells

Figure 12A and B show the successful transfection of CHO cells with STAT3 and chicken leptin receptor (cObR). The results shown in Figure 12C and D clearly show that autophagy was induced in transfected CHO cells. The induction of LC3A and B as shown in Figure 12D were time dependent.

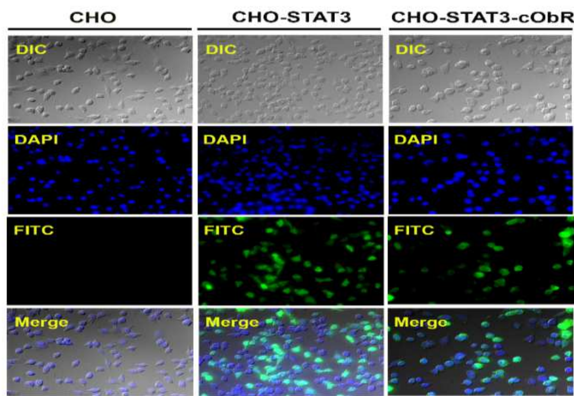
A.



C.



B.



D.

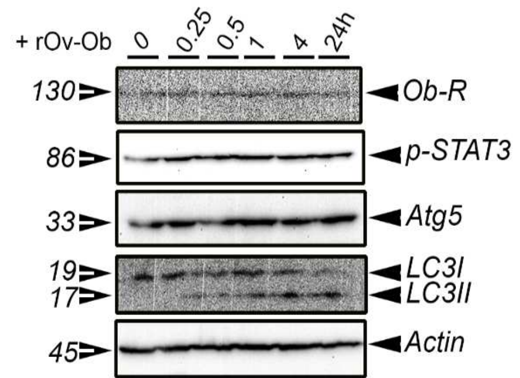


Figure 12: Recombinant leptin administration induced autophagy related proteins intransfected CHO cells

3.3 DISCUSSION

The goal of this study was to determine the effect of recombinant chicken and ovine leptin on autophagy pathway in chicken. The aim of the study was to examine the potency or physiological activity of chicken leptin and its receptor and the mechanism by which leptin transduces its signals in broiler chicks. Treatment with chicken leptin and ovine leptin (both having the same potency) [4] will enable us to carry out such investigation of the effects of recombinant chicken leptin on autophagy in chicken. In the chicken leptin treated Qm7 cells, the downregulation of most Atg genes, and upregulation of AMPKbeta1&2 (energy sensors) were similar to the report by Russell in et al [5]. From this data, it appears that leptin treatment had reduced nutrient utilization, but such reduction in nutrient utilization seems to have negative effect on autophagy induction in Qm7 cell lines. The downregulation of STAT6 and STAT3 in chicken and ovine leptin group were indicative of potency of leptin to downregulate autophagy in chicken which is similar to previous report by Beltowski [6], and also, this result is in support of experiment by Villanueva and Myers [7], who published that Leptin produces various biological responses via activation of JAK/STAT pathway.

It is noteworthy that mTOR genes were downregulated in Qm7 cells that were treated with both chicken leptin and ovine leptin, as well in the fasted Qm7 cells. This result reflected here that leptin induced autophagy in Qm7 cells through the downregulation of mTOR.

Contrastingly to the result from leptin treated Qm7 cells, the result from those Qm7 cells that were fasted alone, (but no further treatment with leptin), indicated that there was no significant difference. The upregulation of AMPK-β1 gene as well as induction of phospo-AMPK-α1 protein are clear evidences that nutrient utilization was reduced by chicken leptin treatment, still supporting the fact that chicken leptin potentially reduces nutrient utilization in Qm7 cells.

However, ULK1, one of the autophagy initiator, was downregulated in both treatments groups compared to control. These findings provide a strong proof about the potency of leptin to regulate autophagy as previously reported by Malik SA et al in 2011, which claimed that recombinant human leptin induced autophagy in HeLa cells [8]. This result also is very similar to the report from Malik et al 2011 who stated that LC3 is one of the molecular marker for monitoring the autophagic flux.

Interestingly, from the in vivo experiment with one week old broiler chicks that were injected with chicken and ovine leptin, there were remarkable effects of both chicken and ovine leptin on feed intake of the chicks. There was reduction in feed intake , (which is similar to previous report by Cassy S. et al, 2004) [9], after 0.5,1 and 3 hours of intra-cerebroventricular injection of leptin, howbeit, the reduction in feed intake was statistically significant after 30 minutes of treatment (injection), these may suggest that leptin reduces feed intake in time specific manner . More so, the result obtained from the real time to determine the mRNA level of lepin receptors on the hypothalamus of those broilers injected with chicken leptin and those with ovine leptin, these results showed that both chicken leptin and ovine leptin receptors were upregulated, this is a clear proof of bio-potency of chicken leptin and its receptor .

However, having confirmed the physiologic effect (reduced feed intake) of leptin on broiler chicks, we further proceeded to examine the physiological changes at molecular level on the chicken hypothalamus (tissues), and the results from real time PCR to examine leptin role on autophagy in chicken hypothalamus showed that most autophagy related genes (Atg) were downregulated in the hypothalamus. Also, this result also indicated that both chicken leptin and ovine leptin have the same affinity for chicken leptin receptor (cObR), and this is similar to previous report by Dridi S et al, 2000 [4]. The result from western blot performed on

hypothalamus tissues from chicken leptin treatments indicated that LC3B (which is a molecular marker for autophagy) was downregulated compared to control group. In the same trend, Atg3 protein (one of the principal Atg genes/proteins) was downregulated in ovine leptin treated group compared to control. These both consolidate those results from real time quantitative PCR from *in vitro* and *in vivo* studies, and the results obviously is different from the previous report from Malik SA et al 2011, who reported that intraperitoneal leptin injections in mice upregulated autophagy in several peripheral tissues (muscle, heart and liver) [8]. The differences in this results compared to those reported by Malik SA , 2011, could be due to evolutionary differences between mammals and avian species.

Furthermore, examining the effect of Ovine leptin on autophagy in chicken muscle (Figure 7) reveals that most of the Atg genes were down-regulated in the same trend as in *In vitro* studies and chicken hypothalamus. The time specific downstream of LC3A/I and upstream of LC3B/II (i.e, as LC3I was decreasing, LC3II was increasing) as seen in transfected CHO cells (fig 12 D) which clearly reveals the mechanism through which recombinant chicken leptin probably regulate autophagy in avians species, is obviously similar to the report of Tamotsu Yoshimorim, et al, 2010 who reported that conversion of LC3 1 to LC3 2 is one of the principal method to monitor autophagic flux [10].

And more excitingly, the energy sensors AMPKβ1 & ϒ1 genes were upregulated. The upregulation of these isoforms of AMPK were indicative of the nutrient depletion. ObR was also upregulated in chicken skeletal muscle, this also is an evidence of the biopotency of chicken leptin whose administration activates synthesis of its Receptor.

In the same manner, the downregulation of Atg13, Beclin1, and AMPKbeta1 as well as upregulation of LC3B, and STAT3 in CELI cells that are treated with chicken further

strengthened evidences that validate the potency of chicken leptin in autophagy induction in chicken. Also, the time dependent regulation of LC3A protein was observed in the result of analysis of Autophagy related protein in SHSY5Y cells. Emphatically, the results obtained from this study clearly reveal the equipotency of both chicken and ovine leptin in signal transduction, and also, it is clearly shown that leptin receptor equally recognizes both.

In summary, recombinant chicken leptin reduced nutrient utilization in Qm7, CHO cells, SHSY5Y, and CELi cells, and reduced feed intake in the *in vivo* experiment performed on broilers chicks, and this was evidenced by the activation of some isoforms of AMPK genes and also induction of phospho-AMPKα1 protein. The downregulation of LC3A genes in CELi and in CHO cells treated with recombinant Chicken and Ovine leptin respectively and that of LC3B protein in the broiler chicks hypothalamus were sufficient evidences that chicken leptin downregulates autophagy in avian species. The upregulation of STAT1 and STAT5 in chicken muscle, and STAT3 in CELI leptin treatment further shed light on the suggestive pathway whereby leptin transduces its signal to regulate autophagy in chicken [11]. However, the pathway by which leptin precisely induced autophagy in chicken warrant further investigation.

In conclusion, since chicken leptin downregulated most of the Atg genes and proteins in *In vivo* and *In vitro* studies, it is probably the fact that exogenous administration of recombinant Chicken leptin is not adequately efficient in upregulating autophagy in avian species, the reason which may be partly due to evolutionary differences of avian species compared to mammalian, or other animal species, or due to the phylogenetic changes that resulted from the genetic selection of the current breeds of domestic birds. However, since few Autophagy related genes and proteins were shown to be upregulated, the recombinant chicken leptin may be used as the molecular marker for regulation of autophagy in chicken.

CHAPTER 3 LITERATURE CITED

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4. CONCLUSION

The results from both *In vitro* and *In vivo* studies demonstrated that Recombinant chicken leptin administration obviously reduced nutrient utilization and feed intake respectively (as evidenced by upregulation of the energy sensors AMPKβ1 & ϒ1 genes) , and subsequently downregulated autophagy related genes and proteins in avian species. The valid proof to these findings were demonstrated by the downregulations of LC3A genes in CELi and in CHO cells treated with recombinant Chicken and Ovine leptin respectively, as well as the downregulation of LC3B protein in the broiler chicks' hypothalamus. The difference observed in the result for this study on avian species when compared to those previously reported in the mammalians, regarding leptin's role on autophagy could be due to phylogenetic and evolutionary variations which were thought to be attributed to genetic selection of the current breeds of domestic birds. However, since some few autophagy related genes were also upregulated, the use of Recombinant chicken leptin as molecular marker for induction of autophagy in chickens could prove significant for further study that may contribute to a framework whereby new effective nutritional and/ or genetic strategies could be developed for poultry industries.

4.1 APPENDIX 1

Invited review

Leptin and autophagy: when the two masters meet

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Abstract

Autophagy or cellular self-digestion, a lysosomal degradation pathway that is conserved from yeast to human, plays a key role in recycling cellular constituents, including damaged organelles. It also plays a pivotal role in the adaptation of cells to a plethora of distinct stressors including starvation. Leptin is an adipocytokine that is mostly produced by white adipose cells in mammals and functions as a hormonal sensing mechanism to inhibit feed intake and increase energy expenditure. In this review, we will describe the autophagy and leptin systems and summarized recent advances regarding their interactions in the regulation of energy homeostasis.

Key words: *Leptin, autophagy, food intake, energy homeostasis, molecular mechanisms.*

Introduction

The hormone Leptin, also called obese hormone, is the central mediator in a negative feedback loop regulation of energy homeostasis. Mammalian adipocytes produce and secrete more leptin in bloodstream as fat storage increases [1] signalling the brain via leptin receptors [2-5] and modulating the feeding-related (an)orexgenic hypothalamic neuropeptide system to suppress appetite and increase energy expenditure [3-4]. Leptin gene and its related receptors are expressed in a wide range of tissues indicating various potential physiological functions. Leptin has been reported to play a key role in reproduction [6], immunity [5], bone mass [7], blood pressure [4], hematopoiesis [4], and lipid metabolism [3, 4].

Hyperphagy, morbid obesity and diabetes were observed in rodents that were deficient in leptin (ob/ob mouse), or that lack certain isoform of leptin receptor (db/db mouse and fa/fa rat) [2, 4, 8, 9]. Interestingly a dysfunctional autophagic activity has been observed in these obese models, suggesting a potential interaction between leptin and autophagy.

Autophagy is a highly conserved cellular mechanism that is responsible for the degradation and recycling of damaged organelles. It is also considered as an alternative to apoptosis in programmed cell death. In recent years though autophagy has appeared to play critical roles in several cellular functions and physiological processes including reproduction, development [10] immunity [11], inflammation [11] neurodegenerative diseases [12], cardiovascular diseases [5], metabolic syndrome [13, 14], and energy homeostasis [15].

There are three major types of autophagy; micro-, macro-autophagy, and chaperone-mediated autophagy [16-18]. Micro- and macro-authophagy can selectively engulf large structures such as mitochondria and endoplasmic reticulum (referred to as mitophagy or reticulophagy, respectively [17, 18] or by non-selective mechanisms (e.g. bulk cytoplasm), whereas chaperone-mediated

autophagy degrades only soluble proteins [18]. Micro-autophagy refers to the sequestration of cytosolic components directly by lysosomes through invaginations in their limiting membrane. However, macro-autophagy that we will address in the present review refers to the sequestration of material within an autophagosome, a unique double membrane cytosolic vesicle. Autophagosomes fuse with late endosomes and lysosomes, promoting the delivery of organelles, aggregated proteins and cytoplasm to the luminal acidic degradative milieu that enables their breakdown into constituent molecular building blocks that can be recycled by the cell [19].

In recent years, interaction between leptin and autophagy has been a focus of research interest. After a brief description of leptin and autophagy systems, we will review here studies on the biological interaction between leptin and autophagy in the regulation of energy homeostasis.

Leptin system

The ob (leptin) gene has been previously cloned and characterized in rodent and human by Friedman and co-workers [20]. It consists of three exons with the two coding regions separated by two introns. It was assigned to mouse chromosome 6 [21] and human chromosome 7q31.3 [21]. The ob product, leptin (derived from the Greek word "leptos" meaning lean) contains 167 amino acids (AA) and a 21 AA signal peptide cleaved during translocation into the microsome. The 16-kDa mature leptin circulates in serum both as a free and as a protein-bound entity.

Mammalian white adipose tissue is the main site of ob gene expression and leptin secretion. Expression and secretion occur exclusively within the differentiated adipocytes [1, 22]. Leptin, however, is also produced in several cell types in other organs. In fact, it is produced by gastric cells in the walls of the stomach [23], in follicular papilla cells of hair follicles [1], in osteoblasts [7], in the placenta [6], in skeletal muscle [1], in the brain [1], and in the pituitary [22]. Additionally, leptin has been localized in the ovary (granulosa and theca cells, corpora lutea, and interstitial gland) [6] and in the mammary gland [22]. Intriguingly, leptin has been shown to particularly be expressed in the liver of several non-mammalian oviparous species such as chicken [24, 25], dunlin [26], thin-billed prions [24], fishes [26], amphibians [26] and reptiles [23].

Leptin exerts its function through its receptor Ob-R which is first identified in mouse choroids plexus by expression cloning techniques and then in human using infant total brain library [8]. It is a single transmembrane-spanning receptor and a member of the cytokine receptor superfamily that includes the gp130 signal-transducing component of the receptors for interleukin 6 (IL-6), granulocyte colony stimulating factor (G-CSF), and leukemia-inhibitory factor (LIF) [13]. The Ob-R extracellular domain consists of 816 AA and is followed by a 23-AA transmembrane

domain and intracellular domain which varies in length from 30 to 303 AA, depending on alternative splicing. The alternate splicing of the Ob-R gene generates multiple variants of leptin receptor mRNA that encode at least six Ob-R (Ob-Ra,b,c,d,e & f) isoforms [4, 5].

Ob-R is primarily expressed in the hypothalamus. It is particularly prominent in areas important in regulation of energy balance such as arcuate (ARC) and paraventricular (PVN) [4, 5]. Expression of Ob-R was also detected at lower levels in a large number of peripheral tissues including skeletal muscle, heart, adrenals, kidney, adipose tissue, liver, pancreatic β-cells and immune cells [22]. The short isoforms are expressed at higher levels in a variety of tissues and were elegantly reviewed by Friedman and Halaas [27]. The ubiquitous expression of leptin and its related receptors indicates that leptin may have several physiological roles.

It is well established that leptin has potent food intake and body weight reducing effects in mammals [1, 5] and this effect is mediated via the activation of POMC/CART and inhibition of NPY/AgRP neurons [5]. The molecular basis for stimulation of POMC gene expression likely involves Janus kinase and signal transducer and activator of transcription (JAK-STAT) activation [5, 8], while the phosphoinositol 3-kinase (PI3K) pathway may play a specific role in the repression of NPY and AgRP gene expression by leptin [5, 8]. Leptin has been reported to interact also with other hypothalamic peptides including orexin, melanocortin receptors (MCR), corticotropin releasing factor (CRF), glucagon-like peptide (GLP-1), ghrelin, cholecystokinin (CCK), and bombesin to regulate feeding behavior [1, 27]. Leptin also increases energy expenditure [2, 25, 27], induces lipolysis, reduces lipogenesis [27], regulates reproduction [6], immunity [22], and bone mass [7].

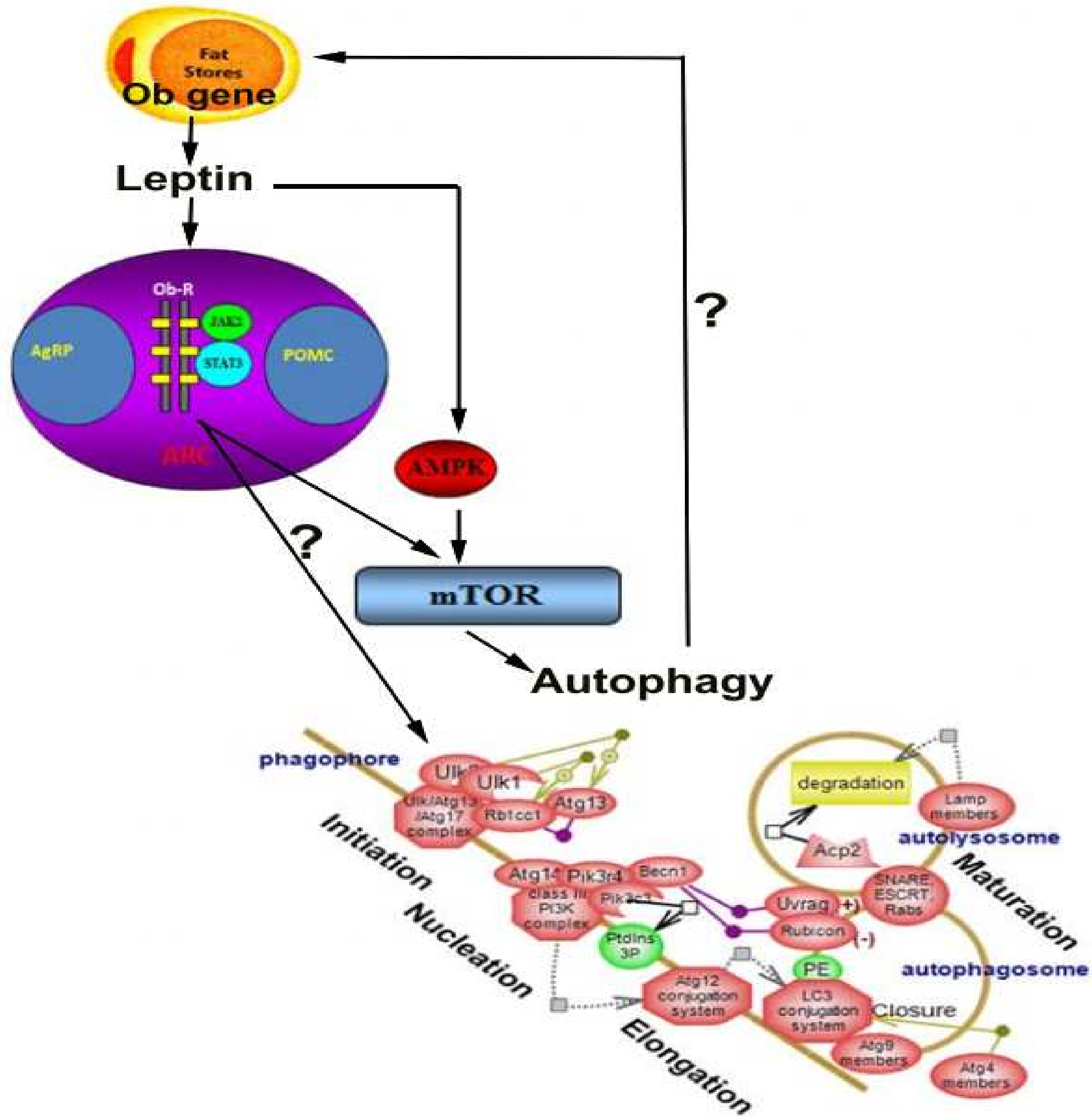

Figure 1: Potential model of leptin-autophagy interaction in the regulation of energy homeostasis.

Leptin is secreted from adipocytes, binds to the extracellular domain of its Ob-Rb receptor dimmer and activates the JAK2 tyrosine kinase and STAT3. In ARC neurons that coexpress Ob-Rb and POMC/CART, leptin increases POMC production via STAT3, which 17 generates an anorectic signal via α-MSH and MCR3/4. In ARC neurons that co-express Ob-Rb and NPY/AgRP, leptin inhibits AgRP production partly through STAT3 pathway, which disinhibits melanocortin signaling. Additionally, leptin can act through IRS-PI3K pathway. Leptin can alter autophagy directly via JAK-STAT, AMPK-mTOR or via other downstream signaling cascades that are not known yet. Whether autophagy alters the leptin expression in peripheral tissues directly or indirectly is unknown and warrant further investigations. AgRP, agouti-related peptide; AMPK, AMP-activated protein kinase; ARC, arcuate nucleus; JAK2, janus kinase 2;

mTOR, mechanistic target of rapamycin; Ob gene, obese gene; OB-R, leptin receptor; POMC, pro-opiomelanocortin

Autophagy system

Autophay has been described as a highly conserved self-eating process during which cells degrade and recycle their own components (cytosol and organelles) within the lysosomes [28]. The word autophagy was coined from Greek Word "**auto**" which means **self**, and "**phagein**", meaning **to eat.** Autophagy, which is a unique morphological feature or process in a dyeing cell was often erroneously presumed to be a preceding pathway to cell death, but on the contrast, it has now been evidently and clearly clarified that, one of its major function is to fight the cell death and consequently keep it alive even when undergoing stressful and life-threatening conditions [29]

Autophagy is induced upon nutrient depletion or starvation, thereby leading to the response of more than 30 autophagy-related genes (Atg) [30]. However, how Atg proteins are regulated is still under investigation, but it's clear that all signals reporting on availability of carbon and nitrogen sources converge on the mTOR signaling pathway, and that, Atg proteins are downstream effectors of mTOR pathway [30, 31]

There are three steps involved in formation of autphagosome, and the first is initiation, during which phagophore (outer mitochondrial membrane, plasma membrane, endoplasmic reticulum membrane, etc) undergo nucleation [19]. The second step undergoes elongation, cycling, expansion and closure, forming autophagosome [19]. The third and final step is referred to as maturation, which involves the advancement of autophagosome into amphiosome (fusion of autophagosome and endosome), which is acidic and hydrolytic vacuole. It is this hydrolytic vacoule that is ripe for degradation and recycling of nutrients [19].

Under fed (normal nutrient-energy) state, the nutrient sensor mechanistic target of rapamycin (mTOR) is activated and in turn phosphorylates ULK1 and thereby sequestering the ULK1-Atg13-FIP200 complex in an inactive state at the mTOR complex [32]. In contrast when nutrients are limited (e.g. during stress or starvation), the energy sensor AMPK is activated. AMPK activation inhibits mTOR activity leading to a reduced ULK1 phosphorylation and consequently releases the ULK1-Atg13-FIP200 complex from mTOR to the site of autophagosome formation and induction of autophagy. In the second step of autophagy, Beclin1 forms a lipid kinase complex with Vps15, Vps34 and Atg14 that phosphorylates phosphatidylinositol (PI) to form inositol-3-phosphate (PI3P) and is essential for induction of autophagy [33]. Accumulation of PI3P in specific sub-domains of the ER increases membrane curvature at the site of autophagosome formation. The elongation step involves two ubiquitin-like reactions of the pre-autophagosomal structures. First, the ubiquitin-like protein Atg12 is conjugated to Atg5 by the action of Atg7 and Atg10 after which Atg16 multimerizes to form the Atg12-Atg5-Atg16 complex. Next, Atg4 cleaves soluble microtubule-associated protein light chain 3-I (LC3-I) to form the membrane-bound LC3-II [34]. Both of these two ubiquitin-like systems are required for elongation and closure of the phagophore. During maturation and fusion, autophagosomes will first fuse with endosomes then with lysosomes. Any mutation or loss of proteins important for formation of multivesicular bodies (MVBs) can lead to inhibition of maturation of autophagosomes [28]. Some genes involved in this step include UVRAG, a Beclin 1 interacting protein that recruits the fusion machinery on the autophagosomes. Another Beclin 1 interacting protein, Rubicon, also functions in the maturation of autophagosomes where it is thought to be a part of a distinct Beclin 1 complex containing Vps34, Vps15, and UVRAG that suppresses autophagosome maturation [35]. Working together, these steps complete the

formation of the autolysosome and its lysis, that releases proteins and amino acids that can be used as an energy source during times of low energy availability or increased energy demand (stress) for the organism (Fig. 2).

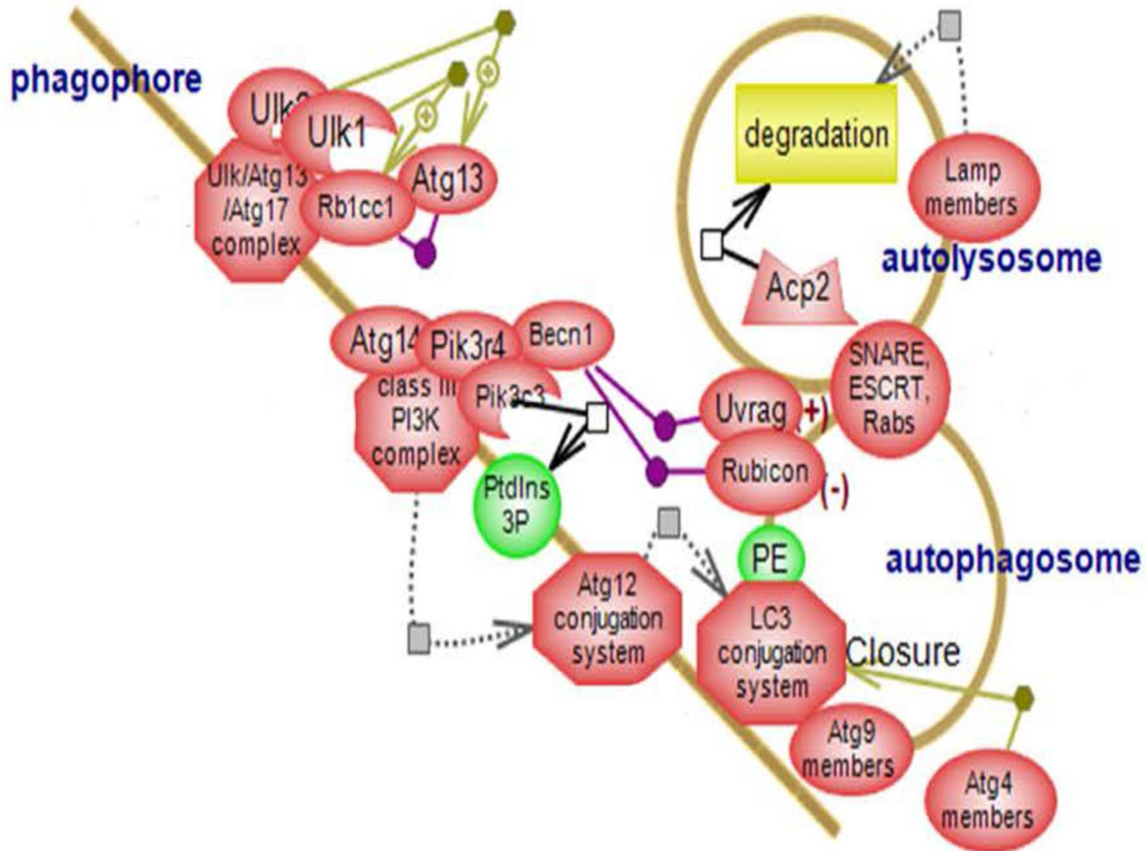

Figure 2: **Steps of autophagosome formation**

Autophagosome formation can be initiated via mTOR inhibition or AMPK activation during starvation or nutrient limitation. This results in the activation of ULK1 which in turn phosphorylates Atg13, Atg101 and FIP200. When autophagy is activated, Beclin 1 is liberated from Bcl-2 and is associated with Vps34, Vps15 and Atg14. ULK1 phosphorylates also AMBRA, a component of the PI3K CIII complex enabling it to relocate from the cytoskeleton to the isolation membrane. The activation of Vps34 generates PI3P which catalyzes the first of two types of ubiquitination-like reactions that regulates membrane elongation. Firstly, Atg5 and Atg12 are conjugated to each other in the presence of Atg7 and Atg10. Attachment of the Atg5-Atg12-Atg16L1 complex on the isolation membrane induces the second complex to covalently conjugate PE to LC3 which facilitates in turn the closure of the isolation membrane. The

complex Atg9-Atg2-atg18 cycles between endosomes, the Golgi and the phagophore possibly carrying lipid components for membrane expansion. LC3-II is formed by LC3 conjugation to its lipid target PE and Atg4 removes LC3-II from the outer surface of newly formed autophagosome, and LC3 on the inner surface is degraded when the autophagosome fuses with lysosomes. Atg, autophagy-related genes; LC3, microtubuleassociated protein light chain; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3 kinase; PIP3, phosphatidylinositol 3-phosphate; ULK1, UNC51-like kinase 1. **The figure was produced by the Pathway Studio software from Ariadne/Elsevier and is used by permission of the Rat Genome Database [47]**

Interaction between Leptin and autophagy in the regulation of energy homeostasis

Since both leptin and autophagy are dysfunctional in obese models and both are implicated in the regulation of lipid metabolism, increasing studies investigating the leptin-autophagy interaction have received considerable attention over the last few years. Activation of hypothalamic mTOR has been shown to regulate feeding behavior and energy homeostasis [2, 25] and mTOR pathway has been shown to be a downstream effector of leptin and upstream regulator of autophagy [36]. Leptin, mTOR and autophagy are all regulated by starvation and nutritional state [36]. In addition, appetite, energy expenditure and metabolism are tightly regulated by the central nervous system (CNS) particularly the POMC and AgRP neurons in the hypothalamic arcuate nucleus. These neurons act as major negative (anorexigenic) and positive (orexigenic) regulators of feed intake.

In 2012, three recent studies have implicated CNS autophagy in the regulation of energy homeostasis. Conditional specific depletion of Atg7 in POMC neurons resulted in higher body weight, hyperphagia, impaired glucose tolerance, increased adiposity and leptin resistance [37]. Moreover, deficient Atg7 in hypothalamic POMC neurons impaired leptin-induced signal transducer and activation of transcription 3 activation. In line with these data, Malhotra and co-workers [38], recently showed that upon high-fat diet consumption mice lacking Atg12 in POMC-positive neurons exhibit accelerated weight gain, adiposity and glucose intolerance

which is associated with increased food intake and decreased leptin sensitivity. Interestingly, mice lacking Atg5 in POMC neurons do not exhibit these phenotypes observed in Atg7 and Atg12 deficient mice [38]. These results indicated that autophagy-related genes might exert different physiological function depending on tissue or cell type. Kaushik et al. [39] proposed that autophagosome-mediated form of secretion in POMC neurons controls energy homeostasis by regulating α-MSH production. The same group demonstrated a role for autophagy in hypothalamic agouti-related peptide (AgRP) neurons in the regulation of food intake and energy balance [40]. They showed that starvation-induced hypothalamic autophagy mobilizes neuron-intrinsic lipids to generate endogenous free fatty acids which in turn regulate AgRP levels. Depletion of Atg7 in hypothalamic AgRP neurons promotes neuronal lipid accumulation, reduced AgRP levels, feed intake and adiposity [40].

Plasma leptin levels have been reported to be altered in *Zmpste24*-null mice, which show accelerated aging and exhibit an extensive basal activation of autophagy [41]. Mice with specific deletion of Atg7 in adipocytes exhibited markedly decreased plasma concentration of leptin [42]. In vitro treatment with recombinant leptin inhibited autophagy in human CD4(+)CD25(-) conventional (T conv) T cells and this effect was mediated via mTOR activation [43]. However, leptin knockdown attenuated hypoxic-preconditioning- induced autophagy in bone marrow-derived mesenchymal stem cells [44] indicating that the effect of leptin on autophagy might be tissue- and cell-specific. Enteral leptin administration has also been shown to inhibit intestinal autophagy in piglets [28]. In heart, however, leptin promoted autophagosome formation as evidenced by increased LC3-II, beclin 1 and Atg5 expression [45]. Malik and co-workers reported that peripheral administration of recombinant leptin induced autophagy in peripheral tissues including skeletal muscle, liver and heart [2]. Moreover, leptin stimulated autophagy in

cultured human and mouse cell lines and this effect was likely mediated through the activation of AMPK and inhibition of mTOR.

Together these elegant studies suggest that the interaction between the two masters; leptin-autophagy underscore a novel link that plays a crucial role in the regulation of energy balance and many other cellular processes (Fig. 3).

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4.2 APPENDIX 2

Office of Research Compliance

MEMORANDUM

TO: Sami Dridi
FROM: CraigN. Coon, Chairman
Institutional Animal Care
And Use Committee
DATE: May 8, 2013
SUBJECT: IACUC Protocol **APPROVAL**
Expiration date: **June 30, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13039- "Regulation of energy homeostasis and fat metabolism in avian species".** You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **06-30-2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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