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Autophagy and Its Potential Role in Stress and Feed Efficiency Using Avian Lines

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Autophagy and Its Potential Role in Stress and Feed Efficiency Using Avian Lines

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ABSTRACT

Autophagy is a highly conserved cellular mechanism that is responsible for the degradation and recycling of damaged organelles. Recently, autophagy has been involved in critical roles during overall development of the organism and degradation of damaged cellular components. This pathway has witnessed dramatic growth in the last few years and has been extensively studied in yeast and mammals, however, there is a paucity of information in avian (non-mammalian) species. First, we characterized genes involved in the autophagy pathway in male and female Jungle Fowl to determine gender and tissue specific differences. Secondly, tissue and genotype differences in Japanese quail selected for resistance (R) or susceptibility (S) to restraint stress was determined. Stress, whether external (temperature stress, disease, crowding) or internal (endogenous oxidative stress) reduces animal production efficiency. A major source of oxidative stress in cells is mitochondrial reactive oxygen species (ROS). Mitochondria are responsible for 90% of cellular energy (ATP) production and also a major site of ROS production. Oxidative damage of membranes induces lipid peroxidation with increased production of aldehydes and peroxides including 4-hydroxy-2-nonenal (4-HNE). Cells must expend energy to repair oxidative damage caused by 4-HNE protein adduct formation, possibly through utilization of the autophagy pathway. Lastly, as a result of the genetic selection of broiler (meat-type breeder) chickens for enhanced growth rate and lower feed conversion ratio, it has become necessary to restrict feed intake. When broilers are fed *ad libitum*, they would become obese and suffer from several health-related problems of which, autophagy may be a key regulator. In relation to this, feed efficiency (FE) is a very important genetic trait in poultry and livestock that can be negatively impacted by stress. Preliminary data from our laboratory indicates that autophagy expression of several genes is upregulated in muscle of broilers

exhibiting a high FE phenotype compared to the low FE phenotype. This suggests that part of the cellular basis of FE may hinge on the ability of the cell to maintain optimal functionality by a more active endogenous repair system offered by the autophagy pathway.

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PUBLISHED PAPERS

All of Chapter 4 comes from the published paper:

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1. INTRODUCTION

Autophagy is a highly conserved cellular mechanism that is responsible for the degradation and recycling of damaged organelles. In recent years, autophagy has been shown to play critical roles during overall development of the organism as well as degradation. Christian de Duve is credited to be the founding father of this pathway, making important observations leading its discovery and name (deDuve and Wattiaux, 1966). The term autophagy was used to distinguish the 'eating' (phagy) of part of the cell's self (auto) from the breakdown of extracellular material (heterophagy) (Klionsky, 2007). The name was coined from the observation of electron microscopy studies that showed novel single or double-membrane vesicles containing organelles in various stages of degradation (Clark, 1957; deDuve and Wattiaux, 1966) and, therefore, distinguishes it from the Ub-proteosome pathway that is specific for the degradation of short-lived or damaged proteins. Although the autophagic pathway was considered to be primarily non-specific, de Duve suggested it was also possible that autophagy may have selective types that allowed targeted degradation of abnormal cellular constituents; an idea in autophagy that has gained much prominence (Klionsky, 2007).

Autophagy was first observed in mammalian cells with the molecular mechanisms having been delineated in yeast (Nakatogawa et al., 2009; Klionsky, 2007). A number of protein complexes, and signaling pathways that influence the regulation of autophagy, have been identified in yeast and many have mammalian orthologs. Identification of autophagic genes in higher eukaryotes made it possible to analyze mammalian cells that express autophagy proteins that were tagged with fluorescent markers (Klionsky, 2007). Through images from time-lapse studies, data suggested that autophagosome formation proceeds in a step-wise manner marked by expansion of the sequestering membrane (Mizushima et al., 2001). A breakthrough for studying

the molecular basis of this pathway was through identifying the Atg (autophagy-related) gene (Yorimitsu and Klionsky, 2005). There are currently 27, of the more than 30, genes that are purely ATG genes which have been identified in yeast as well as functionally characterized orthologs of the ATG gene products in higher eukaryotes including: mammals, insects, worms, and plants (Reggiori and Klionsky, 2002; Levine and Klionsky, 2004). Studies in yeast have also advanced our understanding of molecular mechanisms required for not only the autophagy pathway, but also pexophagy (degradation of peroxisomes) and the Cvt (cytoplasm to vacuole targeting) pathway which is a highly selective process that involves the sequestration of at least two specific enzyme cargos Ape1 (aminopeptidase I) and Ams1 (α-mannosidase) (Scott et al., 1997; Hutchins and Klionsky, 2001). The discovery of the autophagy pathway has led to many new discoveries involved in the development of treatments ranging from cancer to Alzheimer's disease. While most studies have been aimed at human health, it is becoming clearer that autophagy could be very important in disease and/or stress-related illnesses in animal agriculture.

Stress, whether external (e.g. temperature stress, disease, crowding) or internal (e.g. endogenous oxidative stress) reduces animal production efficiency. A major source of oxidative stress in cells is mitochondrial reactive oxygen species (ROS). Mitochondria are responsible for 90% of cellular energy (ATP) production and also a major site of ROS production. Low levels of ROS modulate translation and transcription processes and high levels can oxidize proteins, lipids, and DNA. Heat stress has been shown to increase mitochondrial ROS production in broilers and layers (Mujahid et al., 2007a; Mujahid et al., 2007b; Azad et al., 2010) which could lead to mitochondrial autophagy (mitophagy) if radical (atom, molecule, or ion that has unpaired valence electrons) generation becomes excessive (Levine and Kroemer, 2008).

Due to autophagy being a critical pathway for homeostasis, the process must be tightly controlled to avoid extensive self-digestion. Preliminary data (as stated in following chapters) obtained from our laboratory indicated that; 1) heat stress alters expression of autophagy genes in quail muscle (QM7) cells *in vitro*, and 2) differential expression of autophagy genes occurs in breast muscle of broilers associated with the phenotypic expression of FE *in vivo*. By maintaining optimal functionality of proteins and organelles, as well as providing an alternative to cell death by necrosis or apoptosis, autophagy likely has important roles at the organismal level ranging from generalized stress, feed efficiency, and nutrient limitation, to disease response and toxin insult.

An animal that produces either greater body mass with the same feed intake or the same body mass with less feed intake would be considered more efficient than its contemporaries. That having been said, birds that are considered to have better feed efficiency typically have a lower proportion of feed intake to body mass. Using this definition, one can determine the relative efficiency of several species of agriculturally important animals including poultry. This is important worldwide due to substantial challenges animal agriculture is facing, including a steep projected increase in demand and the need to adapt to changing environmental conditions. Due to a predicted increase in world population to 9 and 10 billion, United Nations FAO estimates that by 2050 there will be a 73% increase in meat and egg consumption and a 58% increase in dairy consumption over 2011 levels (Alexandratos, 2006). With heat wave frequency and intensity projected to rise during the next century. Reducing the impact of climate change and cost of animal protein production is essential to achieve a sustainable, affordable, and secure animal protein supply. To do so, mechanistic understanding (at molecular and cellular levels) of heat stress and feed efficiency response are necessary and of uppermost interest.

Willems and colleagues have stated that generally accepted feed costs represent about 70% of the cost of poultry production making a bird's ability to use feed efficiently very important (Willems et al., 2013). Changing methods in lighting, temperature, and nutrition with birds have all been ways the industry has used to improve feed efficiency (FE) in meat producing poultry. Perhaps the most recent area, as well as the one most likely to produce a lasting effect on the industry, is genetic selection for feed efficiency. Originally bred for body weight gain, significantly larger birds were produced but, as feed costs began to increase, it became clear that, in order to be profitable, selection needed to include other traits (Willems et al., 2013). While there are a multitude of ways for measuring feed efficiency, the two most often used are feed conversion ratio (FCR) and residual feed intake (RFI). FCR can be defined as the amount of feed consumed per unit of weight gain, and is a composite trait of starting and ending body weight and feed intake (Skinner-Noble and Teeter, 2003). RFI, on the other hand, is defined as the difference between actual and predicted feed intake based on the regression of requirements for production and body weight maintenance (Van der Werf, 2004). The main problem is that feed utilization efficiency has not kept up with the growth rate of broilers (Aggrey et al., 2010). Due to biofuel policies and a growing global demand for animal protein, feed, fuel, and fertilizer costs have been on a steady rise leading to intensified focus on the development of selection strategies for the improvement of FE in poultry and livestock production (Steinfeld et al., 2006). These increases in feed cost have driven an increase in live production costs which, in turn, decreases profitability for the industry. To alleviate this problem and make the industry more profitable, FCR has been implemented to make these improvements possible. As stated earlier, feed intake is a heritable trait and, as Pym and colleagues demonstrated over a decade ago, genetic studies for FCR show that it could be improved by selection on growth (Pym, 1990).

Many researchers are searching for new and improved ways of developing feed efficient animals by studying genes and pathways that may be of importance to this trait. The autophagy pathway is one such pathway that our laboratory has been studying in poultry. Feed efficiency in poultry is very important to the industry therefore, using poultry as a model, we studied common genes involved in autophagy and compared how this pathway may be involved in birds that are selected for either high feed efficiency (HFE) or low feed efficiency (LFE). We believe this study is the first of its kind in poultry, with feeding-type studies having been performed in cattle and *Caenorhabditis elegans.* Looking for candidate genes influencing feed efficiency traits, Rolf and colleagues found that autophagy was present in Angus cattle when measuring for residual feed intake (RFI) (Rolf et al., 2012). In another model, Morck and colleague indicated that the long-term starvation seen in *C. elegans* mutants that are "feeding-defective," activates autophagy, and leads to depletion of fat deposits, small cell size, and small body size. These experiments as well as others dealing with starvation, show that the autophagy pathway is involved in how these organisms cope with a certain feeding regime. The question to which our feed efficiency experiment hopes to address is: to what degree does autophagy affect the high or low feed efficiency trait, and could this be a potential aid in helping to solve the feed efficiency problem in the poultry industry?

Autophagy, although a relatively new area of study, may be a pathway that leads to many new discoveries as well as help to uncover previously unknown mechanisms underlying diseases. The aforementioned research suggests that this pathway is an important part of many topics covering a wide range of research; interest in it is only becoming more prevalent year to year. With advances in animal agriculture becoming ever present, as well as a need to produce affordable, high quality animal protein world-wide, this pathway may be an important source for

many solutions to these problems. The discoveries in this research have the potential to better understand heat stress and feed efficiency in animal agriculture, specifically commercial poultry; furthermore, it may lead to methods that will alleviate heat stress and improve feed efficiency in field.

1.2 OBJECTIVES

The objectives of my research in this dissertation were to characterize the autophagy pathway in avian species, to determine its regulation, by using challenging stressors, and effect on autophagy in how heat and oxidative stress are handle, and finally to determine autophagy expression levels in broilers and quail specifically phenotyped and carefully selected for high or low feed efficiency.

Specific objectives for this dissertation are as follows:

1. To characterize the autophagy pathway in a) male and female jungle fowl, and b) in Japanese Quail selected for resistance and susceptibility to restraint stress.
2. To determine the effect of oxidative stress and heat stress on the autophagy pathway in avian muscle cells *in vitro* and Japanese Quail *in vivo*.
3. To understand the role that the autophagy pathway may play in the phenotypic expression of feed efficiency in broilers and quail

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2. REVIEW OF THE LITERATURE

2.1 INTRODUCTION

Autophagy is a highly conserved cellular mechanism that is responsible for the degradation and recycling of damaged organelles, proteins, and other cytosolic components. In recent years, autophagy has been shown to play a critical role during overall development of the organism as well as degradation. Christian de Duve is credited to be the founding father of autophagy (deDuve and Wattiaux, 1966). The term autophagy was used to distinguish the 'eating' (phagy) of part of the cell's self (auto) from the breakdown of extracellular material (heterophagy) (Klionsky, 2007). The name was coined from the observation of electron microscopy studies that showed novel single or double-membrane vesicles containing organelles in various stages of degradation (Clark, 1957; deDuve and Wattiaux, 1966) and, therefore, distinguishes it from the Ub-proteosome pathway that is specific for the degradation of short-lived or damaged proteins. Although the autophagic pathway was considered to be primarily non-specific, de Duve suggested it was also possible that autophagy may have selective types that allowed targeted degradation of abnormal cellular constituents; an idea in autophagy that has gained much prominence (Klionsky, 2007).

Autophagy was first observed in mammalian cells with the molecular mechanisms having been delineated in yeast. A number of protein complexes, and signaling pathways that influence the regulation of autophagy, have been identified in yeast and many have mammalian orthologs. An overview of different components of autophagy, particularly the three major steps of induction, elongation, and vacuole formation, is shown in Figure 1. These steps will be discussed in greater detail below.

Identification of autophagic genes in higher eukaryotes made it possible to analyze mammalian cells that express autophagy proteins that were tagged with fluorescent markers (Klionsky, 2007). Through images from time-lapse studies, data suggested that autophagosome formation proceeds in a step-wise manner marked by expansion of the sequestering membrane (Mizushima et al., 2001). A breakthrough for studying the molecular basis of this pathway was through identifying the Atg (autophagy-related) (Yorimitsu and Klionsky, 2005). There are currently 27 of the more than 30 genes that are purely ATG genes which have been identified in yeast as well as functionally characterized orthologs of the ATG gene products in higher eukaryotes including: mammals, insects, worms, and plants (Reggiori and Klionsky, 2002; Levine and Klionsky, 2004). Studies in yeast have also advanced our understanding of molecular mechanisms required for not only the autophagy pathway, but also pexophagy (degradation of peroxisomes) and the Cvt (cytoplasm to vacuole targeting) pathway which is a highly selective process that involves the sequestration of at least two specific enzyme cargos Ape1 (aminopeptidase I) and Ams1 (α-mannosidase) (Scott et al., 1997; Hutchins and Klionsky, 2001). The discovery of the autophagy pathway has led to many new discoveries involved in the development of treatments ranging from cancer to Alzheimer's Disease. While most studies have been aimed at human health, autophagy could be very important in disease and/or stress-related illnesses, agricultural animal health is also a research topic of importance.

2.2 TYPES OF AUTOPHAGY

Autophagy is a general term for the process in which organelles are enclosed in lysosomal structures for degradation and recycling of materials. It has been found that there are three main types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy, with macroautophagy the most extensively studied using *Saccharomyces cerevisiae*

(baker's yeast) (Tomotake et al., 2011) (See Table 1). Macroautophagy is the term used in reference to the major lysosomal pathway for the degradation and recycling of cytoplasmic components.

Microautophagy refers to the process of invagination of the lysosomal membrane which then delivers a small part of the cytoplasm into the lysosomal lumen for degradation. Chaperone-mediated autophagy is the translocation of cytosolic proteins across the lysosomal membrane which requires protein unfolding mediated by chaperone proteins (Mizushima and Yoshimori, 2010). Along with these main types of autophagy are more specific, selective types of autophagy such as pexophagy, for example, which itself has two types: macropexophagy and micropexophagy. Pexophagy, discovered using methylotrophic yeast, involves a degradation pathway for peroxisomes which are single membrane organelles involved in metabolic processes such as fatty acid oxidation and detoxification of hydrogen and lipid peroxides (Mizushima and Yoshimori, 2010). Another selective autophagy is piecemeal microautophagy of the nucleus, a specific microautophagic process that was discovered in *S. cerevisiae*. During this type of microautophagy, blebs of nuclear membrane, as well as part of the nucleoplasm, are pinched off into the vacuole and degraded (Roberts et al., 2003). Mitophagy, reticulophagy, ribophagy, and lipophagy are yet more selective types of microautophagy which can be used to remove damaged mitochondria, endoplasmic reticulum, and ribosomes respectively.

2.3 SIGNALING PATHWAYS THAT REGULATE AUTOPHAGY

The main signaling pathway as well as a master regulator of autophagy is mTOR, (mechanistic target of rapamycin) a highly conserved serine/threonine protein kinase that acts as a sensor of growth factors, nutrient signals, and energy status (Yang and Klionsky, 2010). It exists in two complexes: mTORC1 and mTORC2 which are conserved from yeast to mammals.

In yeast, when mTORC1 is inhibited, (e.g. via rapamycin treatment or nitrogen depletion) autophagy is activated. This complex inhibits autophagy through activation of the class I PtdIns3K (phosphatidylinositol 3-kinase) protein kinase B (PKB) pathway (Figure 1). PKB also activates mTORC1 by inhibiting a downstream protein complex, tuberous sclerosis complex 1/2 (TSC1/TSC2). Phosphorylation of this TSC2 by PKB or extracellular-signal-regulated kinases (ERK1/2) leads to the disruption of its association with TSC1 and activates mTOR (Yang and Klionsky, 2010). The TORC2 complex also regulates autophagy. For PKB to be fully activated, mTORC2 is required. Inhibition of PKB, caused by mTORC2 depletion, activates forkhead box O (FoxO3). FoxO3 is a transcription factor that stimulates autophagy in muscle cells independent of the activity of mTORC1 (Yang and Klionsky, 2010).

AMPK (AMP-activated protein kinase) is involved in another autophagy signaling pathway and is an important sensor of cellular bioenergetics, especially in response to energy stress (e.g. starvation or high energy demand). When energy or nutrients are depleted, AMPK is activated by an increase in the AMP/ATP ratio through the upstream liver kinase B1 (LKB1) which also regulates cell polarity and functions as a tumor/growth suppressor. Once AMPK is activated, it leads to the phosphorylation of TSC1/TSC2 and thus the inhibition of mTORC1 complex (Yang and Klionsky, 2010). Activation of AMPK can also occur in response to an increase in the cytosolic free Ca^{2+} concentration and cytokines such as TNF-related apoptosis-inducing ligand (TRAIL) via activation of Ca^{2+}/ calmodulin-dependent kinase kinase-β (CaMKKβ) and transforming growth factor-β-activating kinase 1 (TAK1) (Høyer-Hansen et al., 2007; Herrero-Martín et al., 2009). These pathways are required for Ca^{2+}-induced or TRAIL-induced autophagy (Høyer-Hansen et al., 2007; Herrero-Martín et al., 2009).

The MAPK (mitogen activated protein kinase) and Erk1/2 pathway have a mostly inhibitory role on autophagy. The Erk1/2 pathway activates mTOR, thereby inactivating autophagy and increasing cell synthesis and energy usage. However, it has recently been suggested that the Erk1/2 pathway may regulate the induction of the autophagy pathway through glucose which was formally thought to inhibit autophagy (Moruno-Manchón et al., 2013).

The tumor suppressor p53 and its pathway exerts both positive and negative effects on autophagy. Genotoxic stress or oncogenic activation of p53 induces autophagy by phosphorylation of AMPK which activates TSC1/TSC2 complex and leads to the inhibition of the mTORC1 pathway (Feng et al., 2005). This tumor suppressor has also been known to induce autophagy through upregulation of the damage-regulated autophagy modulator (DRAM) (Crighton et al., 2006). As a negative autophagy regulator, the inhibition of p53 or its proteasomal degradation, favors autophagy induction.

Another important signaling pathway comes through the B-cell lymphoma 2 (Bcl-2) protein family which also plays a dual role in autophagy regulation. The antiapoptotic proteins: Bcl-2, B-cell lymphoma-extra-large (Bcl-$_X$L), Bcl-2-like protein 2 (Bcl-L2L2), and induced myeloid leukemia cell differentiation protein (Mcl-1) all can inhibit autophagy, whereas proapoptotic BH3-only proteins, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), Bcl-2-associated death promoter (BAD), BCL2-interacting killer (BIK), Noxa, p53 up-regulated modulator of apoptosis (PUMA), and BimEL, can induce autophagy (Levine and Kroemer, 2008). When Bcl-2 binds to Beclin1, Beclin1 association with Vps34 is disrupted and this decreases the Beclin1 associated hVps34 PtdIns3K activity, inhibiting autophagy.

2.3.1 GENES ASSOCIATED WITH AUTOPHAGY

Characterization of genes in the autophagy pathway was carried out using mutagenesis-based screens in *S. cerevisiae* (Marino and Lopez-Otin, 2004) (An overview of the genes/proteins involved in the autophagy pathway is shown in Figure 1, p. 42). A high degree of homology between yeast and mammalian genes indicates the pathway is conserved in mammals as well. Interestingly, the autophagic pathway in yeast seems to overlap with the cytoplasm-to-vacuole targeting (Cvt) pathway, the only known biosynthetic pathway to utilize the machinery of autophagy for operation, as well as pexophagy. These different pathways often differ in genetic screens (a type of phenotypic screen that can provide information on gene function as well as molecular events that underlie biological pathways) which has led to autophagic genes having different names because of their involvement in overlapping processes (Marino and Lopez-Otin, 2004). Most of these genes are involved in signaling complexes and pathways that are directly involved in the development of an autophagic response. Among these are: mTOR (mechanistic target of rapamycin), ATG1 complex, and Vps34/class III PI3K (phosphatidylinositol 3-kinase) complex. mTOR is a serine/threonine kinase that is involved in control of cellular processes in response to nutrient changes which are mostly monitored by AMPK (AMP-activated protein kinase). Treatment with rapamycin, an immunosuppressant drug that inhibits TOR activity, will block cell cycle progression thus triggering autophagy (Raught et al., 2001). ATG1 is a protein kinase that forms part of a protein complex that is involved in triggering Cvt and autophagy pathways (Kamada et al., 2000). Triggered by nutrient deprivation or treatment with rapamycin, another gene, ATG13, becomes partially dephosphorylated, leading to ATG1-ATG13 interaction which triggers autophagy and the generation of autophagosomes instead of Cvt vesicles (Marino and Lopez-Otin, 2004). In mammals, the ATG1 ortholog is

ULK1 (unc-51-like kinase) and may be functionally related to the ATG1 protein complex through interaction between two mammalian orthologs of ATG8 (a protein essential for autophagosome formation) (Marino and Lopez-Otin, 2004). Other Atg genes include, a.) Atg14 determines the localization of the autophagy-specific PI3-kinase complex, b.) Atg16L1 is part of a large protein complex that is necessary for autophagy also involving Atg12 and Atg5, c.) E1-like activating enzyme is similar to Atg3 which is an E2-like conjugating enzyme with both having importance in the formation and sequestration of the autophagosome, d.) Atg 4 is important in the processing of LC3 (mammalian homolog of yeast Atg8) into LC3-II that is important in final sequestration of the autophagosome and used as a marker for the occurrence of autophagy (Mizushima and Yoshimori, 2010). LC3-II is a protein located in the inner and outer membrane of autophagosomes and when ubiquitinated proteins at lysine 63 recruit the LC3-interacting protein p62, p62 is then recognized by LC3-II. Protein substrate tagged with ubiquitin-63, p62 and LC3-II forms a complex which is then engulfed by the autophagosome, and subsequently degraded by the lysosomes (Ichimura and Komatsu, 2010). Vps34/class III PI3K complex is an enzyme family involved in processes such as intracellular trafficking, proliferation, and assembly of cytoskeletal elements (De Camilli et al., 1996). This family of protein kinases was implicated in the autophagy pathway through the discovery that 3-methyladenine (3-ME), a PI3K inhibitor, had an inhibitory effect on autophagy (Seglen and Gordon, 1982).

2.4 AUTOPHAGY IN YEAST AND MAMMALS

Yeast, specifically *S. cerevisiae*, were the first organisms in which the molecular mechanism of the autophagy pathway was discovered, and although autophagy has been highly studied in mammalian cells, scientists have only been able to develop this molecular

understanding through the application of yeast genetics (Wang and Klionsky, 2003). In 1992, the laboratory of Yoshinori Ohsumi demonstrated that the autophagy morphology in yeast was similar to that in mammals, which proved to be a crucial step for further studies in this organism (Takeshige et al., 1992). While this pathway occurs in all eukaryotic cells, in yeast it is maintained at basal levels under normal conditions and is induced by starvation (Wang and Klionsky, 2003). Under starvation conditions, the activity of the autophagy pathway allows for excess proteins to be degraded and the now free amino acids to be reused for the synthesis of proteins essential for survival or used for energy production (Yorimitsu and Klionsky, 2005). Similar trends have been seen in nutrient starved cultured cells and tissues as well as nutrient depletion seen in response to birth and severing of the trans-placental food supply (Kuma et al., 2004; Mizushima et al., 2004). However, multicellular organisms, mammals in particular, have more diverse roles for autophagy than in lower eukaryotes (Marino and Lopez-Otin, 2004). For example, although this pathway has an original function during starvation, it is also involved in programmed cell death, as stated earlier, as well as tissue specific functions. It has been noted that research in mammalian autophagy has had major hurdles to overcome. Two hurdles in particular are capturing a "dynamic process" with "static measurements" as well as separating "form" from "function," and avoiding a common pitfall of assigning particular functions to this process given a certain setting (Mizushima and Yoshimori, 2010). These challenges, along with others, have been partially overcome by applying advances in the molecular mechanisms of autophagy to the development and understanding of new methods in research of this area of study (Mizushima and Yoshimori, 2010).

2.4.1 AUTOPHAGY IN NON-MAMMALIAN MODELS

The autophagy pathway is best characterized in mammalian and yeast models, from which genes were identified and most mechanistic work has been conducted. However, there are many other models in which the autophagy pathway has been observed that indicate that the action of most of these genes may be highly conserved among species. For example, upon nutrient deprivation or overcrowding, *Dictyostelium discoideum*, a soil amoeba, will undergo a developmental cycle which ends in the production of a multicellular organism (Kessin, 2001). This process is thought to protect the developing *Dictyostelium discoideum* spores from the noxious environment of the soil, but insertional mutagenesis of orthologs of yeast Atg5, Atg6, Atg7, and Atg8 genes, results in loss of cellular viability and aberrant multicellular development during starvation (Otto et al., 2003; Otto et al., 2004). *Caenorhabditis elegans*, with limited nutrients or increased temperature, will reversibly arrest into an alternative third larval stage that is suited to survive in an unfavorable environment (Riddle, 1997). It has been shown that autophagy is enhanced during dauer development (an alternative long living larvae stage where the nematode is dormant) in seam cells (during postembryonic development, they can act as stem cells to produce neurons and support cells) important in formation of dauer cuticle and radial constriction of the nematode body (Meléndez and Levine, 2009). Furthermore, inactivation of Atg genes (orthologs of Atg1, Atg6, Atg7, Atg8, and Atg18) in *C. elegans* blocks morphogenetic and physiological features of dauer development, inhibits seam autophagy, and prevents dauer survival (Meléndez and Levine, 2009). Plant processes similar to autophagy in mammals have been described in a number of biochemical and morphological studies (Matile, 1975; Moriyasu and Hillmer, 2000). In plants, mutations in autophagy genes AtAPG7 and AtAPG9 does not disrupt completion of the life cycle, but does cause phenotypic alterations that may result from a defective ability to mobilize nutrients (Doelling et al., 2002; Hanaoka et al., 2002). Although a

wealth of information exists on the autophagy pathway in a variety of mammalian and non-mammalian species, very little information is available in avian species. Therefore, the present dissertation aimed to characterize the autophagy pathway in male and female jungle fowl (Chapter 2), and to study its regulation by heat and oxidative stress (Chapter 3), as well as genetic selection through use of stress resistant (R) or susceptible (S) Japanese quail lines (Chapter 4).

2.5 AUTOPHAGY AS A CELL DEATH AND DEVELOPMENTAL PATHWAY

After the discovery of autophagy, existing cell death pathways were restructured. Apoptosis, the best known type of programmed cell death (PCD), is now referred to as type I PCD and autophagy is characterized as type II PCD (Wang and Klionsky, 2003). The reasoning behind this is due to the morphology of the two pathways. Type I PCD occurs when chromatin is fragmented and the cytoplasm condenses, whereas, type II PCD involves the appearance of autophagosomes that are important markers of the final sequestration stages of autophagy. Autophagy is mainly a cytoprotective pathway, functioning during normal cellular development and growth. However, if cellular damage is extreme, superfluous autophagy can be used by the cell to initiate type II PCD. For example, autophagy can act as a cellular defense mechanism to prevent infection by pathogenic bacteria and viruses but, on the other hand, it is also involved in type II PCD, contributing to certain disease pathologies (Cuervo, 2004; Kirkegaard et al., 2004; Shintani and Klionsky, 2004; Levine and Yuan, 2005). Although both type I and II PCD pathways can involve the other for successful completion of each process, autophagic dysfunction is associated with various diseases (Klionsky and Emr, 2000). Autophagy is active under basal conditions and helps to regulate the balance between protein synthesis and degradation.

A defect in autophagic genes such as Beclin1, an important gene in autophagy initiation and a tumor suppressor, can lead to carcinogenesis. Links have also been found between defective autophagy and a number of neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's diseases (Klionsky and Emr, 2000). In fact, the question of whether or not autophagy is beneficial or destructive, came from a study investigating neuronal cell death (Ravikumar and Rubinsztein, 2004). The study noted that axotomy, (severing of an axon) may cause neuronal cell death in neonatal or embryonic animals as this is the time when neurons are dependent on their targets for survival (Dixon, 1967; Holtzman et al., 1967). In mature animals, on the other hand, survival factors are derived locally or through autocrine loops. Axotomy, cutting or severing of an axon, of motorneurons can lead to a regenerative response without neuronal death (Matthews and Raisman, 1972; Matthews, 1973; Holtzman, 1989). In both of these situations, autophagy increased which has led to two opposing theories: 1) autophagy is a mechanism that clears the way for neuronal regeneration, or 2) autophagy is a mechanism for cell destruction (Ravikumar and Rubinsztein, 2004). Most researchers believe there is no reason why this pathway cannot perform both roles. For example, caspases which are normally found in apoptosis pathways, may be important in cell survival under certain conditions where death by another means, i.e. autophagy, may prevail (Yu et al., 2004). A strong association between autophagy and neurodegeneration is best seen in the *Lurcher* mouse model. In this model, heterozygotes with an activating mutation in a glutamate receptor develop cerebellar ataxia within the first four weeks of birth due to degeneration of the cerebellar cortex (Ravikumar and Rubinsztein, 2004). The response in this model is usually death of the Purkinje cell, which plays a fundamental role in controlling motor movement, through activated apoptotic responses, but many studies show missing mechanisms. Yue and colleagues set about

identifying these mechanisms and reported that Beclin1 was a component of the complex that binds to the glutamate receptor (Yue et al., 2002). Two possibilities arose; either 1) the autophagy pathway was the missing pathway that contributed to Purkinje cell death, or 2) that the coupling of autophagy to neurotransmitter receptors plays an important role in regulating neuronal function (Yue et al., 2002). They discovered that there is a biochemical link between the neurotransmitter for the glutamate receptor and autophagy. The model that emerged was one where both apoptosis and autophagy contributed to excitotoxic cell death with Beclin1 being a key molecule in both pathways (Ravikumar and Rubinsztein, 2004).

2.6 PHYSIOLOGICAL ROLE OF AUTOPHAGY

There has been an increase in autophagy research in the past decade which has led to an increase in our knowledge regarding the connections between autophagy and animals (patho)physiological and cellular systems. These studies show a dual role for autophagy: positive or negative effects of this pathway depending on the specific disease and its level of progression (Huang and Klionsky, 2007). A study by Ravikumar and colleagues showed that inhibition of mTOR, which induces autophagy, reduced the toxicity of certain aggregation-prone proteins like those found in cases of Huntington's disease (Ravikumar et al., 2002). Similar studies in murine models found that knockouts of Atg5 and Atg7 prevented symptoms of neurodegeneration in healthy organisms (Hara et al., 2006; Komatsu et al., 2006). While an increase in the number of autophagosomes are seen in different physiological and pathological states in the nervous system, there is still confusion over why this is observed. Is it simply the result of increased autophagic activity (induction of autophagosome formation), or is it a decrease in autophagosome-lysosome fusion (that decreases autophagosome removal and increases apoptosis)? In neurological diseases, increased numbers of

autophagosomes/autophagic vacuoles (AVs) have become a feature of a number of neurological diseases, certain neuronal cell death pathways, and after neuronal injury (Ravikumar and Rubinsztein, 2004). For scientists to understand whether this is a protective or destructive pathway, these phenomena have to be studied more in depth. While these are more general examples, there are many ways autophagy plays a role in physiology.

Bacterial pathogens in humans can cause a wide variety of diseases through infection. Autophagy is a defense mechanism that can be activated by the immune system to target intracellular bacteria that have escaped the phagosome of the immune system, and target them for sequestration in the autophagosome that then fuses with a lysosome, thus eliminating the pathogen (Huang and Klionsky, 2007). This elimination route by autophagy is specific to bacteria, whether it is in the cytosol, within immature phagosomes, or in damaged phagosome-like vacuoles (Birmingham et al., 2006). Some bacteria, however, have developed mechanisms to avoid the autophagy pathway and instead propagate in the cytosol or subvert the autophagy pathway and utilize it to replicate (Huang and Klionsky, 2007).

Viral pathogens also trigger the autophagic pathway through the immune system, but do so with a different initial mechanism. Viruses, like bacteria, have evolved mechanisms to block or utilize autophagy to their advantage. When a virus infects their host, host cells may secrete interferons (IFN), which are cytokines that can trigger antiviral mechanisms to restrict replication of the virus. IFN has recently been shown to upregulate autophagy by activation of protein kinase R (PKR) which inhibits protein synthesis and restricts viral replication (Huang and Klionsky, 2007). Using overexpression of Beclin1 in neurons it was shown that autophagy was inhibited during Sindbis virus replication, the cause of fatal encephalitis (Liang et al., 1998). Some positive-strand RNA viruses are able to induce autophagosome formation and use them as

replication sites thereby increasing the intracellular yields of the virus through autophagy (Jackson et al., 2005; Espert et al., 2007).

The autophagic pathway has also been implicated in protein aggregation diseases. As an adaptive response, degradation systems such as the ubiquitin-proteasome system and autophagy may be activated to eliminate the resulting abnormal inclusion bodies (Huang and Klionsky, 2007). Both degradative pathways are triggered by protein aggregates but their effectiveness lies in the degradation of different forms of the substrates (Teckman et al., 2000; Perlmutter, 2006). There are many examples of protein aggregate diseases such as endoplasmic reticulum (ER) storage diseases like alpha-1-antitrypsin (AT) deficiency or hypofibrinogenemia. In hypofibrinogenemia, degradation of mutant fibrinogen, Augadilla γD, aggregates in the hepatic ER and is dependent on autophagy, whereas soluble mutant proteins are subjected to ER-associated degradation (ERAD) by the proteasome (Perlmutter, 2006). A recent study showed that increased activation of the ubiquitin-mediated autophagy–lysosomal degradation pathway induced by either corticosterone or Aβ (amyloid beta) treatment, may contribute to the pathological changes in pre-synaptic proteins and its functions (Wuwongse et al., 2013). This group showed that corticosterone-induced toxicity is associated with upregulation of the autophagy pathway which they believed was used as a potential mediator of protein degradation when protein aggregates formed as a result of treatment (Wuwongse et al., 2013). It has also been reported that autophagy is responsible for the degradation of a mutant form of dysferlin, found in muscular dystrophy and Miyoshi myopathy, in which the mutant aggregates in the ER induce autophagy-mediated degradation (Fujita et al., 2007).

As mentioned earlier, autophagy is heavily involved in certain neurodegenerative diseases. In regard to cytosolic aggregate-prone proteins, the role of autophagy is most clearly

seen in some forms of Parkinson's disease, Huntington's disease (HD), and Alzheimer's disease (AD) (Rubinsztein et al., 2005; Nixon, 2006). Autophagosome accumulation has been observed in studying these diseases in brains of patients, mouse models, and cell lines. Although autophagy protects against the toxicity of aggregate-prone proteins, its activity must be controlled because excessive autophagy leads to cell death (Huang and Klionsky, 2007).

Cardiomyopathies and lysosomal storage disorders are also associated with autophagy. If the function of a lysosome is altered, excessive amounts of undigested material will accumulate with the lysosome and eventually become toxic to the cell. Interestingly, autophagy often correlates with these "autophagic vacuolar myopathies" (Huang and Klionsky, 2007). Due to reliance on primarily morphological observations, it is not yet clear how these autophagic vacuoles are formed or whether the autophagic response is cytoprotective or contributive to the disease (Terman and Brunk, 2005).

Muscular disorders have been associated with deregulated autophagy, that is mainly observed in non-proliferative cells (muscle and neuronal) where accumulation of damaged materials is more severe (Shintani and Klionsky, 2004). Analyses of unrelated patients with Danon's disease (a disease in which weakening of the muscles that leads to myopathy) identified mutations in the gene for lysosomal-associated membrane protein 2 (Lamp2) (Nishino et al., 2000). In addition, a study performed in mice with a homozygous deletion of the Lamp2 gene, resulted in a phenotype typical of Danon's disease which includes massive accumulation of autophagic vesicles in many tissues (Eskelinen et al., 2004). Even with this evidence, it is still unclear whether the accumulation of autophagic vesicles in vacuolar myopathy results from the promotion of autophagosome formation or the decrease in fusion of the autophagosome with lysosomes (Shintani and Klionsky, 2004).

Perhaps the oldest known link between human disease and autophagy was seen about twenty years ago in cancer, although the elucidation of the possible molecular mechanisms was only recently achieved (Botti et al., 2006; Kondo and Kondo, 2006). Autophagy has had a two pronged role in cancer with it being implicated in tumorigenesis in both a positive and negative role and now the balance has tilted more towards its role as a tumor suppressor. As tumors grow, cancer cells may need autophagy to survive nutrient-limiting as well as reduced oxygen availability conditions because of the poorly vascularized internal region of the tumor (Cuervo, 2004). It may also protect cancer cells against some forms of ionizing radiation by removing damaged mitochondria (mitophagy) which could protect against apoptosis and allow the continued survival of these transformed cells (Paglin et al., 2001; Alva et al., 2004). Some evidence of this includes: a.) Reduced autophagic activity in tumor cells, b.) Absence of Beclin1, MAP1LC3, and Atg7 in different cancers, c.) Autophagy is induced in many anticancer therapies, and d.) Tumor suppressor genes such as PTEN (phosphatase and tensin homolog), p53, and the DAPK (death-associated protein kinase) protein family are involved in signaling autophagy induction (Crighton et al., 2006; Gozuacik and Kimchi, 2006; Kondo and Kondo, 2006).

Autophagy is thought to play an important role as an anti-aging mechanism because of its role in cellular and tissue remolding during morphogenesis in developing organisms. Although function of autophagy in many tissues of an adult organism is minimal, protein and organelle turnover by autophagy is essential in homeostatic or housekeeping functions through removal of unwanted or damaged organelles and proteins (Levine and Klionsky, 2004). Many believe that autophagy is related to anti-aging through these functions by removing or reducing reactive oxygen species and other toxic substances that could contribute to genotoxic stress (Levine and

Klionsky, 2004). Studies using biochemical and genetic analyses, as well as protein caloric restriction on life span extension in diverse species (Bergamini et al., 2003), show a more direct role of autophagy in anti-aging pathways. Perhaps the best-characterized pathway, is the insulin/insulin-like growth factor 1 (IGF-1) pathway which is highly conserved from yeast to human (Longo and Finch, 2003). For example, the aging process related to autophagy has been analyzed in *C. elegans*, *Drosophlia*, and mice. From studies done with *C. elegans*, it is now known that several components of the insulin-like signaling that affect the adult life-span in this species are known to regulate autophagy (Guarente and Kenyon, 2000; Bergamini et al., 2003).

2.6.1 STRESS AND AUTOPHAGY

Autophagy is triggered by stressors such as: nutrient limitation, heat stress, oxidative stress, and/or the accumulation of damaged or excess organelles and abnormal cellular components (Huang and Klionsky, 2007). With stress, autophagy is induced as a degradative pathway where it is involved in the elimination of potentially toxic components coupled with the recycling of nutrients that then aids in cell survival (Levine and Klionsky, 2004). It is believed that from an evolutionary standpoint, autophagy may have developed as a mechanism to protect unicellular organisms against starvation and other forms of environmental stress (Levine and Klionsky, 2004). This is due to the suggestion that stimuli to degrade organelles may have created a cellular medium that favored the acquisition of other advantages such as differentiation and development (Levine and Klionsky, 2004). These processes both require cells to undergo changes and, therefore, must have some way to breakdown and recycle the components, making autophagy not just a coincidence, but rather that differentiation is triggered by environmental stressors that stimulate autophagic activity (Levine and Klionsky, 2004). An example of a pathway involved in both stress and aging is the insulin/insulin-like growth factor 1 (IGF-1)

signaling cascade that involves a tyrosine kinase receptor, PtdIns 3-kinase, and Akt/PKB. In *C. elegans,* inactivation of this cascade can extend life-span up to 300% as well as increase heat and oxidative stress resistance, possibly leading to life-span extension (Shintani and Klionsky, 2004). Also, due to Akt/PKB pathway controlling the activity of mTOR (the autophagy inhibitor), down-regulation of this pathway may also induce autophagy, leading to life-span extension (Shintani and Klionsky, 2004). Due to the rate of autophagy decreasing with age, as many pathways often do, some groups have looked at caloric restriction and induction of autophagy as a means to increase longevity. This life-span extension is thought to be brought on by an increase in protection against oxidative damage, through mitophagy, and an increase in antioxidants, as well as by mechanisms involved in repair and replacement of damaged or defective DNA, lipids, and proteins (Shintani and Klionsky, 2004; Terman et al., 2004).

2.7 ANIMAL AGRICULTURE, HEAT STRESS, AND AUTOPHAGY

Animal agriculture is facing substantial challenges, including a steep projected increase in demand and the need to adapt to changing environmental conditions. Due to a predicted increase in world population to 9-10 billion, United Nations FAO estimates that by 2050 there will be a 73% increase in meat and egg consumption and a 58% increase in dairy consumption over 2011 levels (Alexandratos, 2006); with heat wave frequency and intensity projected to rise during the next century. Reducing the impact of climate change and cost of animal protein production is essential to achieve a sustainable, affordable, and secure animal protein supply. To do so, mechanistic understanding (at molecular and cellular levels) of heat stress response is necessary.

Modern poultry are particularly sensitive to heat stress due to highly metabolically active tissues. As the Earth's climate changes with global warming, this has become a very important

matter for the poultry industry. At temperatures exceeding 38°C, there is marked mortality in flocks (Squibb and Wogan, 1960). Problems including reduced growth rates, reduced egg production with thin shells, smaller sizes, and reduction in hatching are all results of heat stress. Zhou and colleagues studied heat stress in tomato plants and found that heat stress activate autophagy genes with accumulation of autophagosomes (Zhou et al., 2014). We believe that similar mechanisms will be induced by heat stress in birds. Reports of "thermal conditioning" indicate that exposure of embryos (pre-hatch) or neonates (during the first 4 d post hatch) results in greater resistance to heat stress and reduced body temperatures (DeBasilio et al., 2003). Part of this protection is likely due to increased expression of protective proteins, such as the family of heat shock proteins (HSPs). Some argue that early exposure could affect weight gain and growth, and studies have shown that short-term exposure to heat stress during the first week of life did result in slow growth, however, this was immediately followed by compensatory growth with higher feed intake that counteracted any decrease in weight gain (Yahav and Hurwitz, 1996; Yahav et al., 1997; Yahav and Plavnik, 1999). These and many other studies have proven that thermal conditioning of chicks results in improvements in performance by the time they reach market age, clearly showing that heat stress and ways to combat this stress, is of major importance to the poultry industry worldwide. Since the effect of heat stress on the autophagy pathway in poultry has not been documented, our laboratory set out to discover if this pathway had any influence on causing or protecting against heat stress. Current and future studies, unlocking the potential of this pathway, may prove invaluable to the understanding of heat stress in livestock animals and how we can further prevent this costly issue.

2.7.1 AUTOPHAGY IN FEEDING, STARVATION, AND FEED EFFICIENCY

As the incidence of obesity or metabolic syndrome continues to rise, there is a clear demand to identify new and efficient therapeutic strategies. Therefore, insights into the molecular mechanisms of this devastating disease using different experimental models are of uppermost interest. We decided to monitor if this pathway plays a role in efficiency and, if it does, how we can apply this to further improving feed efficiency in agricultural animals. Feed efficiency has always been a topic of interest for many livestock industries and further research in this field, especially involving autophagy, may be applicable to help alleviate this issue or, at the very least, used as a tool to aid in further understanding of the mechanisms underlying this trait.

Rodents are very useful models for the study of obesity, but it could be suggested that another equally good model for this study would be chickens (*Gallus gallus*). Whereas lipogenesis occurs in both adipose tissue and liver in rodents, (Goodridge and Ball, 1967; Leveille et al., 1968; Leveille et al., 1975) chickens are similar to humans in that lipogenesis occurs exclusively in the liver and is exported *via* the circulatory system to adipose tissue (Trayhurn and Wusteman, 1990). In addition, chickens are characteristically hyperglycemic compared with mammals, with their blood glucose levels averaging three times that found in humans (300 *vs.* 100 mg/dl) (Krzysik-Walker et al., 2008). Genetic selection for production efficiency (rapid growth rate and feed efficiency) necessitates feed restriction in commercial meat-type chicken (broiler) breeders that are hyperphagic, heavy, and prone to obesity. Broilers voraciously consume approximately 4.1 kg of feed to achieve a 40-fold increase in body weight after hatch that is concomitant with tremendous increase in muscle development as well as abdominal fat during a period of 42 days (Scheuermann et al., 2003). Relationships of effects of starvation on the autophagy pathway may be very pertinent to this established practice of skip-a-

day feeding programs in broiler breeders used to prevent obesity and decreased reproductive performance.

Starvation studies in yeast were some of the earliest to document the induction of the autophagy pathway. It is thought that because autophagy defective yeast mutants are not able to survive during nitrogen starvation, this pathway may be important for the cellular response to starvation as well as normal cytoplasmic constituent turnover (Tsukada and Ohsumi, 1993). On the other hand, mammals encounter the first and most severe period of starvation during the early post-natal period. It is well known that carbohydrate and lipid reserves are used during this period to overcome any life-threatening situations (Medina et al., 1992). However, in addition to these reserves, it is thought that autophagy must also be activated to maintain an adequate amino acid pool until nutrient supply from milk reaches a steady state (Kuma et al., 2004). The amino acids produced through autophagy can be used as an energy source, synthesis of proteins required for the appropriate starvation response, or converted into glucose in the liver (Kuma et al., 2004). For example, it has been noted that the level of autophagy in mice remains low during embryogenesis but is immediately upregulated in various tissues and maintained at high levels for hours after birth before returning to basal levels within a few days (Kuma et al., 2004). It was also shown that mice deficient for Atg5, (important in autophagosome formation) appear almost normal at birth and die within one day (exhibiting reduced amino acid concentrations in plasma and tissue) (Kuma et al., 2004). Results from these studies suggest that the production of amino acids by autophagic degradation of 'self' proteins is important for survival in the early hours after birth considered 'neonatal starvation' (Kuma et al., 2004). Evidence of autophagy and feed efficiency using this starvation model as a resource has been seen in many species and

may play a crucial role in studies of feed efficiency and restriction in avians (Mizushima et al., 2004; Chera et al., 2009).

While there are a multitude of ways for measuring feed efficiency, the two most often used are feed conversion ratio (FCR) and residual feed intake (RFI). FCR can be defined as the amount of feed consumed per unit of weight gain, and is a composite trait of starting and ending body weight and feed intake (Skinner-Noble and Teeter, 2003). RFI, on the other hand, is defined as the difference between actual and predicted feed intake based on the regression of requirements for production and body weight maintenance (Van der Werf, 2004). Although these two methods are by far the most widely used and understood, there are alternative methods for measuring feed efficiency. Some alternatives include: residual maintenance energy (RME_m) that, unlike RFI or FCR, aims to measure energetic efficiency without being perplexed by feed intake; residual gain (RG) which is defined as the residuals from the linear regression of average daily gain (ADG) on both feed intake and body weight; and residual intake and gain (RIG) which combines the beneficial characteristics of both RFI and RG such that RIG is independent of body weight, but when used for selection can increase weight gain as well as reduce feed intake simultaneously (Romero et al., 2009; Berry and Crowley, 2012). With many options, which method should one choose or which works best for a certain scenario?

Although each has its own advantages and disadvantages, usage of FCR, which can be used on a large scale, and RFI, which tends to be used on a smaller scale, are common, making these the top two choices for measuring feed efficiency that are widely accepted. FCR and RFI both require the measurement of individual feed intake, and use two different ways to obtain these feed intake values. The first requires individually caged birds, which need to have their feed recorded and refilled on a daily or weekly basis, as well as has been widely studied, is cheap

and simple, but requires a significant amount of barn space where there is very little to no social interaction. The second is an automated electronic feeding system that enables feed intake measurements on specific individuals to be taken in a group-house setting. This automated system measures a larger number of birds, as well as social interaction due to the group-housed environment (Howie et al., 2011; Tu et al., 2011). Selecting for weight gain has been shown to make indirect improvements in FCR and, as a related genetic response, feed intake is increased but at a slower rate, thereby improving FCR (Varkoohi et al., 2011). In contrast, RFI would not be affected by the indirect selection of weight gain since this is accounted for in the computation via regression on body weight (Kennedy et al., 1993; Van der Werf, 2004). FCR is used more often and selections based on this method have made significant improvements in feed efficiency. Experimental trials with RFI show promising theoretical results, although it is important to recognize Kennedy and colleagues (Kennedy et al., 1993), who showed that RFI provides no additional information to a breeding program over and above what is provided by its component traits (Willems et al., 2013).

Feed efficiency is important in the livestock industry, especially in poultry production. The cost of feed represents about 70% of the cost of production broilers, but feed utilization efficiency has not kept up with the growth rate of broilers (Aggrey et al., 2010). Due to biofuel policies and a growing global demand for animal protein, feed, fuel, and fertilizer costs have been on a steady rise leading to intensified focus on the development of selection strategies for the improvement of FE in poultry and livestock production (Steinfeld et al., 2006). These increases in feed cost have driven an increase in live production costs which, in turn, decreases profitability for the industry. To alleviate this problem and make the industry more profitable, FCR has been implemented to make these improvements possible.

In poultry, as a result of the genetic selection of broiler (meat-type) chickens for enhanced growth rate and lower feed conversion ratio, it has become necessary to restrict feed intake, creating a starvation-like atmosphere in breeding stock to prevent poor reproductive performance. When these animals are fed *ad libitum,* they become obese and suffer from several health-related problems (Richards et al., 2010). Broiler hens tested under these conditions displayed low egg production and a high proportion of defective eggs (which could be alleviated by feed restriction) as well as a delay in sexual maturity and low reproductive fitness (Heck et al., 2004). The modern commercial broiler under the same conditions displays, as an unintended consequence of this selective breeding, the loss of the ability for self-regulation of feed intake to closely match the requirements for maintenance, growth, and reproduction (Richards et al., 2010). Thus, this animal tends to overconsume feed which, similar to the broiler hens, can result in a range of metabolic disorders and health problems. To try to keep this situation under control before it becomes an issue, broiler breeder birds must be subjected to severe feed restriction early in life to ensure that appropriate body weight (BW) and composition are achieved at critical phases of the production cycle (Richards et al., 2010). Therefore, a vital adaptation to the restricted feed intake method that induces a starvation-like situation is autophagy. As stated earlier, autophagy was first discovered using a starvation model and was shown to help protect the cell by enabling to "self-eat' damaged organelles so as to utilize a new source of energy. This pro-survival mechanism initiates as a counter to apoptosis at first but, if continued with no positive results, the pathway can quickly become aggregated, leading to apoptosis. Autophagy is a promising pathway for finding a molecular signature to solve heat stress in poultry and, if useful, could be adapted for use in livestock as well. Therefore, the objectives of this dissertation research was as follows:

2.8 OBJECTIVES

From the literature cited above, it is apparent that autophagy is a very important pathway and linked to many different conditions, from starvation to stress, that could impact avian species development and function. Despite this importance, very little information is available on the autophagy pathway in birds or in how various types of stress or management conditions could affect the autophagy pathway. My hypothesis for the project is that the autophagy pathway will be prevalent in avians and that this pathway will exert an effect on both exogenous and endogenous stressors as well as the phenotypic trait of FE between species. Thus, the overall goal of research in this dissertation was to characterize the autophagy pathway in jungle fowl, to determine the effect of different types of stress on autophagy, and finally to determine if autophagy may contribute to the phenotypic expression of feed efficiency in broilers.

Specific objectives for this dissertation are as follows:

1. To characterize the autophagy pathway in a) male and female jungle fowl, and b) in Japanese Quail selected for resistance and susceptibility to restraint stress.
2. To determine the effect of oxidative stress and heat stress on the autophagy pathway in avian muscle cells *in vitro* and Japanese Quail *in vivo*.
3. To understand the role that the autophagy pathway may play in the phenotypic expression of feed efficiency in broilers and quail

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Table 1: Classification of different types of autophagy	**Definition**
Type of Autophagy	
1. Macroautophagy	Major lysosomal pathway for the degradation and recycling of cytoplasmic components.
2. Microautophagy	Invagination process of the lysosomal membrane that delivers a small part of the cytoplasm into the lysosomal lumen for degradation
3. Aggrephagy	Selective autophagic degradation of protein inclusions caused by aggregate-prone or misfolded proteins
• Pexophagy	Degradation pathway for damaged peroxisomes
• Mitophagy	Degradation pathway for damaged mitochondria
• Reticulophagy	Degradation pathway for endoplasmic reticulum
• Ribophagy	Degradation pathway for ribosomes
4. Chaperone-mediated Autophagy	Translocation of cytosolic proteins across the lysosomal membrane that requires protein unfolding mediated by chaperone proteins

Figure 1

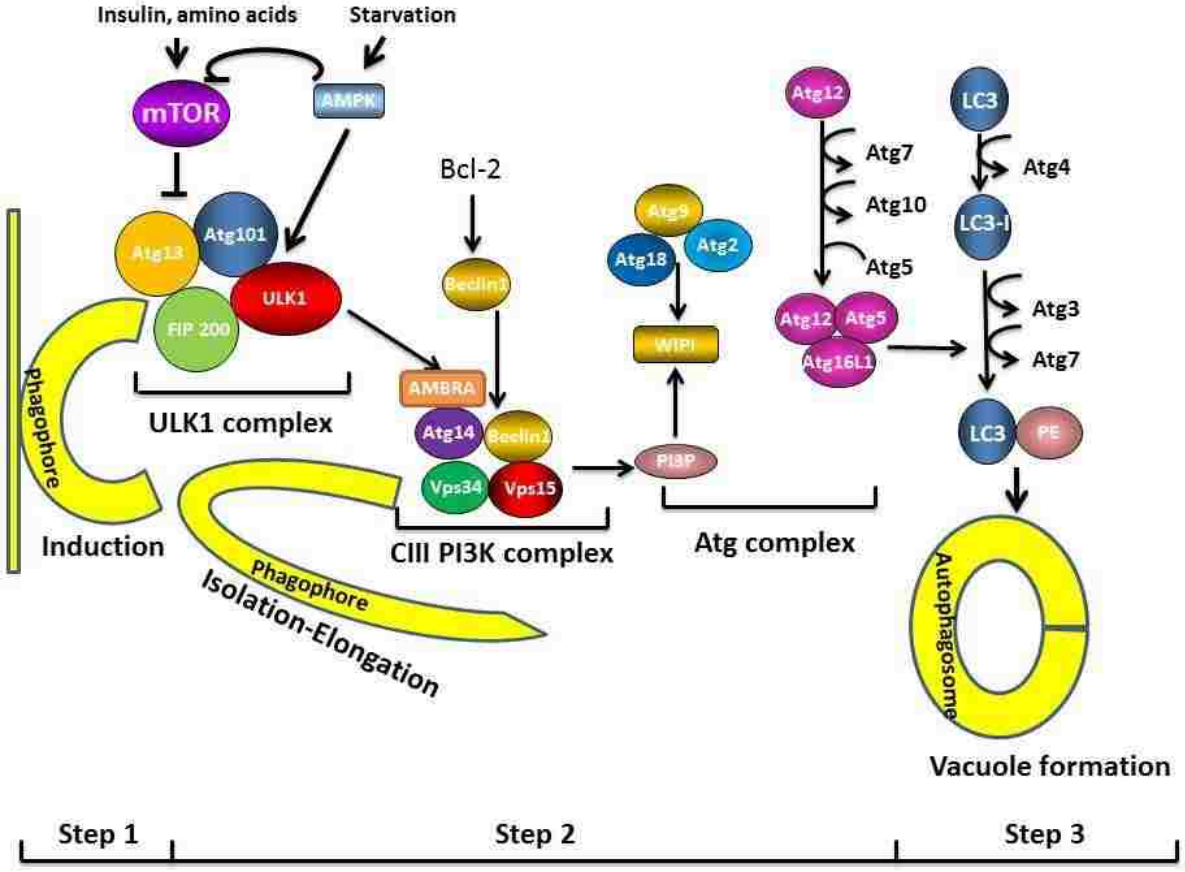

Figure 1 Autophagosome formation in the autophagy pathway (Piekarski et al., 2015). Autophagy is initiated by mTOR inhibition or AMPK activation in response to increased energy demand. Three steps in autophagy include induction, elongation, and autophagosome formation. Activation of ULK1 phosphorylates Autophagy genes (Atg13), Atg101 and FIP200. Beclin 1 is liberated from Bcl-2 and forms a complex with Vps34, Vps15 and Atg14 and AMBRA. Activated AMBRA a component of the PI3K CIII complex enables it to relocate from the cytoskeleton to the isolation membrane. Vps34 activation generates PI3P that catalyzes the first of two ubiquitination-like reactions that regulate membrane elongation; 1) Atg5 and Atg12 are conjugated in the presence of Atg7, 10, and 2. Attachment of the Atg5-Atg12-Atg16L1 complex on the isolation membrane induce covalent conjugation of PE to LC3 that facilitates isolation membrane closure. The Atg9-Atg2-Atg18 complex cycles between endosomes (Golgi and phagophore), and carry lipids for membrane expansion. LC3-II is formed by LC3 conjugation to PE and Atg4 removes LC3-II from the outer surface of newly formed autophagosomes. Finally, LC3 on the inner surface is degraded when the autophagosome fuses with lysosomes. Abbreviations: AMBRA, autophagy/beclin-1 regulator 1; Atg, autophagy-related genes; Beclin (ortholog of yeast Atg 6); LC3, microtubule-associated protein light chain; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3 kinase; PIP3, phosphatidylinositol 3-phosphate; ULK1, UNC51-like kinase

Figure 2

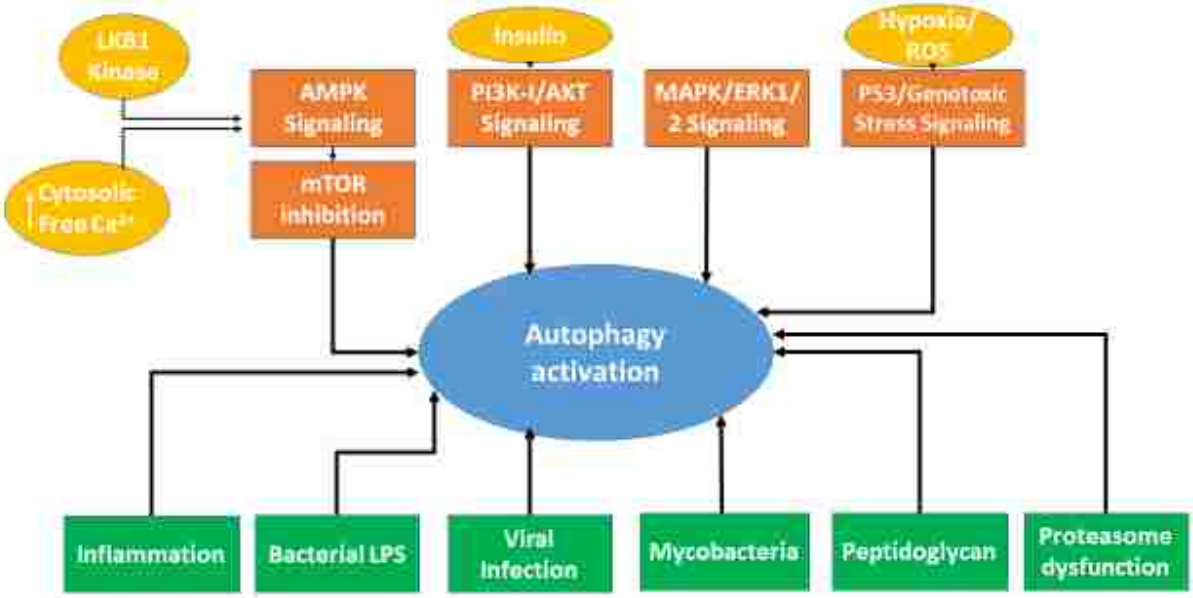

Figure 2 Signaling pathways as well as other activators of the autophagy pathway. There are four major pathways by which autophagy is most regularly initiated. Inhibition of mTOR (through AMPK signaling) or AMPK activation (increase in cytosolic Ca2+ and LKB1 kinase) in response to increased energy demand are the two most common. However, there are other pathways and activators outside of these two. PI3K-1/Akt signaling pathway activates mTOR by inhibiting TSC1/2 which activates mTOR. MAPK/ERK1/2 signaling activates mTOR inhibiting autophagy, but recent studies show that this pathway may regulate the induction autophagy by glucose (Moruno Manchón, et al., 2013). The fourth major pathway, p53/genotoxic stress induces autophagy by phosphorylation of AMPK which then activates TSC1/TSC2 complex that then leads to the inhibition of the mTORC1 pathway. Bcl2 family of proteins acts indirectly to inhibit autophagy through inhibition of Beclin1 (important in membrane nucleation), whereas the BH3-only family of proteins can indirectly activate autophagy.

CHAPTER 3

Tissue distribution, gender- and genotype-dependent expression of autophagy-related genes in avian species

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3.1 ABSTRACT

As a result of the genetic selection of broiler (meat-type breeders) chickens for enhanced growth rate and lower feed conversion ratio, it has become necessary to restrict feed intake. When broilers are fed *ad libitum*, they would become obese and suffer from several health-related problems. A vital adaptation to starvation is autophagy, a self-eating mechanism for recycling cellular constituents. The autophagy pathway has witnessed dramatic growth in the last few years and extensively studied in yeast and mammals however, there is a paucity of information in avian (non-mammalian) species. Here we characterized several genes involved in autophagosome initiation and elongation in Red Jungle fowl (*Gallus gallus*) and Japanese quail (*coturnix coturnix Japonica*). Both complexes are ubiquitously expressed in chicken and quail tissues (liver, leg and breast muscle, brain, gizzard, intestine, heart, lung, kidney, adipose tissue, ovary and testis). Alignment analysis showed high similarity (50.7 to 91.5%) between chicken autophagy-related genes and their mammalian orthologs. Phylogenetic analysis demonstrated that the evolutionary relationship between autophagy genes is consistent with the consensus view of vertebrate evolution. Interestingly, the expression of autophagy-related genes is tissue- and gender-dependent. Furthermore, using two experimental male quail lines divergently selected over 40 generations for low (resistant, R) or high (sensitive, S) stress response, we found that the expression of most studied genes are higher in R compared to S line. Together our results indicate that the autophagy pathway is a key molecular signature exhibited gender specific differences and likely plays an important role in response to stress in avian species.

Key words: autophagy, gene expression, chicken, quail, tissue distribution

3.2 INTRODUCTION

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 [1]. Based on their mechanisms and functions, there are various types of autophagy, including micro- and macro-autophagy, as well as chaperone-mediated autophagy [2,3]. The first two types have the capacity to engulf large structures through both selective (specific organelles such as mitochondria or endoplasmic reticulum referred to as mitophagy or reticulophagy, respectively [4,5]) and non-selective mechanisms (bulk cytoplasm), whereas chaperone-mediated autophagy degrades only soluble proteins [6]. Micro-autophagy refers to the sequestration of cytosolic components directly by lysosomes through invaginations in their limiting membrane. However macro-autophagy refers to the sequestration 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 [7].

A number of protein complexes (more than 30) and signaling pathways that regulate autophagy have been identified in yeast and many of these have mammalian orthologs (for review see [8]). These proteins can be grouped according to their functions at key stages of the autophagy pathway. The beclin-Vps34 complex is involved in the initiation of autophagosome formation. Beclin-1 enhances Vps34 activity [9] and binds to several partners that induce autophagy including ambra-1 [10] , UVRAG [11], and bif-1 [12]. The second complex implicated in the initiation step of autophagosome formation is the ULK1/Atg1-Atg13-FIP200 complex [13]. Indeed, Atg13 binds ULK1 and when they are dephosphorylated they activate ULK1 that phosphorylates FIP200 to

induce autophagosome formation [14-16]. For the autophagosome elongation, two ubiquitin-like systems are involved. The E1 ubiquitin activating enzyme-like, Atg7, activates Atg12 that is transferred to Atg10. Atg12 binds to Atg5 and then form a conjugate with Atg16L1 resulting in an 800-kDa complex [17] that is essential for the elongation of the pre-autophagosomal membrane. The second ubiquitin-like complex involves the protein microtubule-associated protein 1 light chain 3 (LC3/Atg8). LC3 is cleaved by Atg4B to form the cytosolic isoform LC3-I [18]. LC3-I is conjugated to phosphatidylethanolamine in a reaction involving Atg7 and Atg3 to form LC3-II which in turn targeted to elongating autophagosome membrane [19]. For the maturation and fusion stage, autophagosome moves, via dynein motor proteins [20,21] towards the microtubule organizing center where the lysosomes are enriched. Autophagosome fuses with lysosome in a reaction involving several proteins including ESCRT [22], SNAREs [23,24], Rab7 [25,26], and the class C Vps proteins [27]. Recently, it has been reported that beclin-1 functions in the maturation of autophagosome through interaction with Rubicon [28].

Autophagy is essential for maintaining cellular homeostasis and autophagy malfunction is associated with diverse diseases such as neurodegeneration [29], cancer [30], immunity [31] and metabolic syndrome [32]. The amount of research focused on the autophagy pathway has witnessed dramatic growth in the last few years and the bulk of data are mainly originated from yeast and mammals. There is, however, a paucity of information on avian (non-mammalian) species. Therefore, the present study aimed firstly to characterize autophagy-related genes and their tissue distribution in chicken and quail, and secondly to determine their regulation by gender and genotype.

3.3 MATERIALS AND METHODS

3.3.1 ETHICS STATEMENT

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Animal Care and Use Committee under protocols 13039 and 10025.

3.3.2 ANIMALS

Chickens. Red Jungle fowl male and female chickens (*Gallus gallus*) (body weight average 1049 ± 52 and 1654 ± 67g for female and male, respectively) were reared in floor pen under environmentally controlled facilities and under standard poultry rearing conditions (22 ± 3°C for temperature and 50 ± 5% relative humidity) . Chickens were supplied with food (12.6 MJ·kg−1, 22% protein) and water available *ad libitum*.

***Japanese quail*.** In order to assess whether the expression of autophagy-related genes is regulated by genotype, two lines of male Japanese quails (*coturnix coturnix Japonica*) were used. These two lines were established by long-term divergent selection for circulating corticosterone response to restraint stress, after which the low stress line (resistant, R) had 66% low plasma corticosterone levels compared to their high stress (sensitive, S) counterpart [33]. Quails of each genetic line were reared separately in floor pen under environmentally controlled facilities and were allowed *ad libitum* access to water and food (12.6 MJ·kg−1, 22% protein).

Animals were killed by cervical dislocation and tissues (liver, leg and breast muscle, brain, gizzard, intestine, heart, lung, kidney, adipose tissue, ovary and testis) were removed, immediately snap frozen in liquid nitrogen, and stored at -80°C until use.

3.3.3 RNA ISOLATION, REVERSE TRANSCRIPTION, AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from chicken and quail tissues by Trizol reagent (catalog # 15596018, Life Technologies) according to manufacturer's recommendations, DNAse treated and reverse transcribed (catalog # 95048-100, Quanta Biosciences). RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (catalog # 4312074, Life Technologies). Oligonucleotide primers used for avian autophagy-related genes are summarized in Table 1. The qPCR cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of a two-step amplification program (95 °C for 15 s and 58 °C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method [34].

3.3.4 MULTIPLE ALIGNMENT AND MOLECULAR EVOLUTION

Sequence alignments and percentage of amino acid conservation were assessed with the Clustal W and MUSCLE multiple alignment algorithms [35,36] using chicken (non-mammalian) and mammalian autophagy-related gene (beclin1, Atg3, Atg5, Atg9a, Atg10, Atg12, Atg14, Atg13, Atg7, Atg4b, Atg4a, Atg16L1, UVRAG, Ambra1) sequences from database (see Table 3.1

for GenBank accession numbers). The phylogenetic tree based on these nucleotide sequence alignments was constructed using the neighbor-joining method of the MEGA6 program [37].

3.3.5 STATISTICAL ANALYSES

Data were analysed by two-factor ANOVA with tissue and gender (for chicken) and tissue and genotype (for quail) as classification variables. If ANOVA revealed significant effects, the means were compared by Tukey multiple range test using the Graph Pad Prism version 6.00 for Windows, Graph Pad Software, La Jolla California USA. Differences were considered significant at $P<0.05$.

3.4 RESULTS

3.4.1 TISSUE DISTRIBUTION OF AUTOPHAGY-RELATED GENES IN CHICKENS AND QUAIL

Since the role of autophagy-related genes is still unknown in avian species, we classified them in the following sections based on their roles in yeast and mammals. Gene complexes involved in autophagosome initiation (beclin1, Ambra1, UVRAG, Atg9a, Atg13 and Atg14) and elongation (Atg3, Atg4A, Atg4B, Atg5, Atg7, Atg10, Atg12 and Atg16L1) were ubiquitously expressed in chicken and quail. Only one band of the predicted size for each gene was observed in liver, leg and breast muscle, brain, gizzard, intestine, heart, lung, kidney, adipose tissue, ovary and testis (Figure 3.1a,b and Figure 3.2a,b). The sequences of the fragments were identical (100%) to these previously described in GenBank (for accession number, see Table 3.1 and 3.2).

3.4.2 EXPRESSION OF AUTOPHAGOSOME INITIATION COMPLEX IN DIFFERENT TISSUES OF FEMALE AND MALE CHICKENS

The autophagosome initiation complex was expressed in all tissues examined in female and male Red Jungle fowl chickens. In female chickens, beclin 1 was highly expressed in the heart,

brain and leg muscle, followed by the ovary (Figure 3.3a). Ambra 1 mRNA abundance was higher in the ovary followed by kidney, lung, heart, brain and the liver (Figure 3.3b). UVRAG and Atg13 genes were highly expressed in the brain and the liver (Figure 3.3c and d). Atg9a mRNA levels were greater in the brain followed by breast muscle, ovary and liver (Figure 3.3e). The highest amount of Atg14 mRNA was found in the ovary followed by the liver, brain, kidney, heart and the breast muscle (Figure 3.3f). In males, however, the highest levels of beclin1 mRNA were observed in brain and testis, followed by leg and breast muscle and liver (Figure 3.3a). The greatest expression of Ambra 1 and Atg13 genes was found in kidney and testis (Figure 3.3b). UVRAG gene was highly expressed in liver and testis followed by the brain (Figure 3.3c). Atg9a mRNA abundance was high in the liver, brain, leg and breast muscle followed by the testis (Figure 3.3e). Atg14 gene expression was high in kidney followed by testis, intestine, brain, liver and breast muscle (Figure 3.3f).

Interestingly, when tissues from the two genders were plotted together, female chickens exhibited greater hepatic abundance of beclin 1 (3.57 fold, $P<0.05$), UVRAG (1.5 fold, $P<0.01$), Atg13 (6.25 fold, $P<0.01$), and Atg9a mRNA (1.35 fold, $P<0.05$) compared to males (Figure 3.3a, c, d and e). Females also exhibited significant higher expression of the following genes: beclin1 in leg muscle (4.25 fold), brain (2 fold) and heart (6.24 fold, Figure 3.3a), Ambra 1in lung and heart (3.67 and 4.72 fold respectively, Figure 3.3b), UVRAG in leg muscle, brain , and heart (4.4, 4.75, and 8.75 fold respectively, Figure 3.3c), Atg13 in brain (3.24 fold, Figure 3.3d), Atg9a in breast muscle, brain and ovary (2.71, 5.42, and 1.87 fold respectively, Figure 3.3e) and Atg14 in ovary (1.94 fold, Figure 3.3f) compared to males. However, in male chickens, Ambra 1 and Atg14 gene expression was higher in kidney (1.62 and 2.76 fold respectively, $P<0.01$, Figure 3.3d and f) and Atg13

mRNA levels were higher in testis and kidney (4.4 and 14.6 fold respectively, $P<0.01$, Figure 3.3d) compared to female.

3.4.3 EXPRESSION OF AUTOPHAGOSOME ELONGATION COMPLEX IN DIFFERENT TISSUES OF FEMALE AND MALE CHICKENS

As the initiation complex, the autophagosome elongation complex is ubiquitously expressed in both male and female chickens. In females, the highest amount of Atg3 mRNA was found in the ovary followed by brain and kidney. Atg4A mRNA was abundant in the liver, heart, brain, leg and breast muscle. Atg5 gene expression was higher in the liver, brain, gizzard, heart and breast muscle (Figure 3.4a, b and d). Atg4B gene was highly expressed in brain, followed by ovary and liver (Figure 3.4c). Atg7 and Atg12 mRNA were abundant in liver followed by ovary, brain, lung, kidney and leg muscle for Atg7 and ovary and brain for Atg12 (Figure 3.4e and g). Atg16L1 mRNA levels were high in heart followed by breast muscle, brain, liver, ovary and leg muscle (Figure 3.4h). In males, however, the highest amount of Atg3, Atg4B, Atg7, Atg10, Atg12 and Atg16L1 mRNA was found in testis followed by kidney, brain and liver for Atg3, intestine, brain, liver and kidney for Atg4B, brain, liver and kidney for Atg7, kidney, liver, brain, intestine and heart for Atg10, liver, brain and kidney for Atg12, and kidney, breast muscle, intestine, brain and liver for Atg16L1 (Figure 3.4a, c, e, f, g and h). Atg4A and Atg5 mRNA levels were high in kidney and intestine followed by testis, liver, brain and breast muscle for Atg4A and testis, liver and brain for Atg5 (Figure 3.4b and d). Importantly, when we profile the autophagosome elongation complex for each tissue within the two genders, only a few genes showed gender- and tissue-dependent pattern. Female chickens displayed significant high expression of Atg4A in leg muscle, heart and ovary (4.18, 2.85 and 1.68 fold, respectively, Figure 3.4b), Atg4B in the brain (3.41 fold, Figure 3.4c), Atg7 in liver, brain and lung (5.88, 2.59 and 14 fold, respectively, Figure

3.4e), and Atg16L1 in liver, leg muscle, brain and heart (1.69, 5, 1.85 and 5.24 fold, respectively, Figure 3.4h) compared to males.

However, male chickens exhibited significant higher levels of Atg4A mRNA in kidney (3.12 fold, Figure 3.4b), Atg4B mRNA in intestine (13.11 fold, Figure 3.4c), Atg5 mRNA in kidney and intestine (3.43 and 4.61 fold, respectively, Figure 3.4d), Atg7 mRNA in testis (2.17 fold, Figure 3.4e), Atg10 in testis and kidney (4.61 and 4.65 fold, respectively, Figure 3.4f), Atg12 in testis (9.25 fold, Figure 3.4g), and Atg16L1 in testis and kidney (1.52 and 5.31 fold, Figure 3.4h). Atg3 gene expression did not differ between male and female in every studied tissue (Figure 3.4a).

3.4.4 EXPRESSION OF AUTOPHAGOSOME INITIATION COMPLEX IN DIFFERENT TISSUES OF S AND R QUAIL LINES

The autophagosome initiation complex was expressed in all tissues examined in quail. Beclin 1 mRNA levels were abundant in testis, heart and leg muscle of R line and in adipose tissue and testis of S line (Figure 3.5a). The highest amount of Ambra1 was found in lung, heart and leg muscle of R line and in intestine, lung, heart, kidney, and breast muscle of S line (Figure 3.5b). The UVRAG expression was high in lung and adipose tissue of R quail and in lung followed by testis, lung, heart and intestine in S line (Figure 3.5c). The highest amount of Atg13 was found in leg muscle and kidney of R line and in intestine followed by kidney and testis in S line (Figure 3.5d). Atg9a gene was highly expressed in leg and breast muscle followed by intestine in R line and in intestine and adipose tissue in S line (Figure 3.5e). Atg14 gene expression was found to be high in lung, adipose tissue and intestine in both lines (Figure 3.5f). When tissues from the two lines were plotted together, R line exhibited significant higher mRNA abundance of beclin 1 in leg muscle, heart, and testis (2.76, 2.72 and 1.46 fold, respectively), Ambra 1 in lung and adipose tissue (1.46 and 2.37 fold, respectively), UVRAG in gizzard and adipose tissue (17.3 and 7.1 fold,

respectively), Atg13 in liver, leg muscle, brain, heart, lung and kidney (2.56, 46.5, 11.9, 3.4, 11.4 and 3.2 fold, respectively), Atg9a in lung, leg and breast muscle (3.86, 8.8 and 8.3 fold, respectively), and Atg14 in in lung (1.77 fold) compared to S line (Fig. 5a, b, c, d, e and f). However, S line exhibited significant higher levels of beclin 1 in adipose tissue (17.8 fold), Ambra 1 in breast muscle and intestine (9 and 12.5 fold, respectively), UVRAG in intestine and heart (17 and 1.7 fold, respectively), Atg13 in breast muscle, intestine and adipose tissue (7.5, 4.8 and 4.6 fold, respectively), Atg9a in intestine and adipose tissue (3.4 and 4.2 fold, respectively), and Atg14 in adipose tissue (2.6 fold) compared to R line (Figure 3.5a, b, c, d, e and f).

3.4.5 EXPRESSION OF AUTOPAGOSOME ELONGATION COMPLEX IN DIFFERENT TISSUES OF R AND S QUAIL LINES

Atg3 gene was highly expressed in adipose tissue of both lines followed by testis, heart, leg muscle, gizzard and liver in R line and by testis, lung and heart in S line (Figure 3.6a). The highest amount of Atg4a mRNA was found in leg muscle and adipose tissue of R line and in intestine of S line (Figure 3.6b). Atg4b mRNA levels, however, was high in adipose tissue, leg muscle, brain and lung of R line and in intestine and brain of S line (Figure 3.6c). Atg5 was highly expressed in intestine, heart, adipose tissue and lung of R line and its expression remain unchanged between the examined tissues of S line (Figure 3.6d). Atg7 mRNA abundance was found to be high in adipose tissue, leg muscle, brain and lung of R line and in brain, adipose tissue and intestine of S line (Figure 3.6e). Atg10 was highly expressed in leg muscle in R line and in brain of S line (Figure 3.6f). The highest amount of Atg12 mRNA was found in lung of R line but did not differ between tissues in S line (Figure 3.6g). Atg16L1 gene expression was high in adipose tissue followed by leg muscle and lung in R line and in leg and breast muscle of S line (Figure 3.6h). Interestingly, when the two genotypes are plotted together, R line displayed significant high levels of Atg3 in liver, leg muscle, gizzard, heart, and testis (6.6, 7.4, 10, 2.7,

and 3.3 fold, respectively), Atg4a in leg muscle and adipose tissue (9.4 and 5.6 fold, respectively), Atg4b in leg muscle, lung and adipose tissue (13.9, 7, and 8.7 fold, respectively), Atg5 in intestine, heart and adipose tissue (33, 8, and 5 fold, respectively), Atg7 and Atg10 in leg muscle (9.7 and 12.3 fold, respectively), Atg12 in lung (77 fold), and Atg16L1 in liver, leg muscle, brain, lung, and adipose tissue (6.25, 3.89, 5.2, 25, and 39 fold, respectively) compared to S line (Figure 3.6a-h). However S line exhibited higher mRNA levels of Atg3 in intestine (4.8 fold), Atg4a in kidney (8.4 fold), and Atg16L1 in breast muscle (2.6 fold) (Figure 3.6a-h).

3.4.6 ALIGNMENT AND PHYLOGENETIC TREE ANALYSIS OF CHICKEN AUTOPHAGY-RELATED GENES WITH OTHER SOURCES

Comparison of the nucleotide sequences of autophagosome initiation and elongation-related genes between chickens and other species showed low to high similarity (52.6%-91.5%) (Table 3.2). Phylogenetic analysis indicates that chicken Atg4b, Atg7, Atg9, Atg10, Atg14, Atg16L1, and Ambra1 are more closely related to the mouse orthologs however Atg3 and Atg4a are closely related to the pig orthologs, beclin1 is closely related to the horse ortholog, UVRAG is closely related to the rat ortholog and Atg5 is closely related to the bovine ortholog (Figure 3.7).

3.5 DISCUSSION

Autophagy is an evolutionary conserved catabolic process regulating the degradation of a cell's own components through the lysosomal machinery [38]. It plays a key homeostatic role in every cell type to preserve the balance between the synthesis, degradation, and subsequent recycling of cellular components [39]. Currently, more than thirty different autophagy-related genes have been identified by genetic screening in yeast, and many of these genes are conserved in plants, flies and mammals [40]. However, data in birds are scarce. Here, we report for the first time the characterization of fourteen avian genes involved in the autophagosome initiation and

elongation. All genes had high basal expression levels in every examined tissue from chicken and quail maintained under normal (low stress) physiological conditions. These data indicate that avian cells are also equipped with the autophagy system which may be involved in numerous vital cell processes including cellular homeostasis, tissue development and a defense mechanism against aggregated proteins, damaged organelles and infectious agents.

The MUSCLE alignment and the phenogram construction of nucleotide sequences of chicken autophagy-related genes and their mammalian orthologs show high homology and indicate that the evolutionary relationship is consistent with the consensus view of vertebrate evolution. Although the evolutionary conservation of the autophagy pathway, many of the mechanistic breakthroughs in delineating how autophagy is regulated and executed at the molecular level have been made in yeast [41]. Here, our quantitative real-time PCR analysis revealed that the expression of avian autophagy-related genes is tissue specific suggesting a tissue-dependent modulation mechanism of autophagy under physiological conditions and corroborating results from previous studies in rats [42] and mice [43]. This may reflect fundamental differences in the fate of the tissue and/or cells, the role of the autophagy-related genes and their interactions in rapidly dividing versus post-mitotic cells. For instance, inhibition of autophagy using Atg7 small interfering RNA inhibited cell death during starvation in neuronal cells, but increased cell death in fibroblasts [42]. Most likely there is no single autophagy pathway across all tissues, and even within the same tissue, multiple effectors and mediators may exist.

We have also demonstrated that avian autophagy-related gene expression is gender dependent. Although the underlying mechanism(s) for this apparent sexual dimorphic expression is (are) unknown, the results are not surprising because sex-dependent differences in the activation of the autophagic cytoprotection pathway have been reported in mammals [42,44,45]. The gender-

associated differences in autophagy-related genes observed in our study could be a result of physiological, morphological, and hormonal differences between both sexes. Indeed, variations in androgen levels have been shown to be an important factor for the development of autophagy [44]. Sobolewska and co-workers showed that 17 beta-estradiol and progesterone exerted stimulatory effects on autophagy in bovine mammary epithelial cells [46]. Furthermore, a sexual dimorphism of estrogen receptor (ERα and ERβ) expression was observed in basal vascular smooth muscle cells (VSMC) and their regulation by oxidative stress was also found to be gender-dependent (alteration in female but not in male VSMC) [47]. Additionally, The downstream cascades mediating the cardio-protective effects of ERβ in tumor necrosis factor receptor-2 (TNFR2) knockout mice has been shown to be gender-dependent with activation and translocation of signal transducer and activator of transcription 3 (STAT3) in female and decrease of c-jun N-terminal kinase (JNK) in male [48]. Because STAT3 activation has been associated with autophagy processes [49], and JNK phosphorylates B-cell lymphoma 2 (Bcl-2) triggering its release from beclin 1 in response to various stimuli [50], the existence of sexual dimorphic autophagy signaling cascades is very likely. As autophagy is tightly linked to starvation and fatty acid metabolism [42], it is possible that other sex-dependent hormones known to be involved in the regulation of energy homeostasis and lipid metabolism such as leptin, insulin and ghrelin, may affect autophagy-related gene expression as previously reported in mammals [51-54].

Interestingly, we also found that the expression of autophagy-related genes is tissue- and genotype-dependent in male Japanese quails. These quail lines were divergently selected for circulating corticosterone response to restraint stress [33]. The high stress or sensitive line had, in general, high plasma corticosterone levels, high mortality, increased bacterial colonization, high fearfulness, low sexual activity and high stress-induced osteoporosis compared to their low stress

(resistant) counterpart [33,55-58]. Although the role and the regulation of the autophagy-related genes are still unknown in avian species, the high expression of most studied genes in the R line suggest that the autophagy might be a protective mechanism in response to stress and might be involved in the aforementioned behavior and physiological differences between the two quail lines. For instance, inhibition of autophagy has been shown to aggravate the effect of glucocorticoid on cell viability of chicken primary osteocytes [59] which may explain the high stress-induced corticosterone levels and osteoporosis in S lines. Furthermore, sperm quality has been linked to autophagy (LC3B) processing [60] which may support the heightened reproductive efficiency of male R quails which are characterized by increased testis expression of beclin1 and Atg3 genes. Since autophagy has been, recently, reported to be associated with the development of learning and memory in fear conditioning [61], the low expression of Atg13 and Atg16L1 in the brain of S line might be involved in their high fearfulness. Intriguingly, the expression of autophagosome initiation (Ambra 1, UVRAG, Atg13, Atg9a) and elongation genes (Atg3 and Atg4a), except Atg5, was higher in the intestine of S line compared to the R line. The biological significance of this differential expression is not known at this time and further studies are warranted.

3.6 CONCLUSION

In conclusion, the characterization herein of several genes involved in autophagosome initiation and elongation will open new research avenues to understand the regulation and the roles of autophagy in avian species maintained under physiological and pathophysiological conditions. Further studies are warranted to identify and characterize genes involved in autophagosome maturation in birds. The present study also provides proof of principle evidence supporting gender- and genotype-dependent differences in autophagy in avian species and better insight into the underlying mechanisms may ultimately help to develop new management tools for poultry

production improvement. The quail lines may be a useful model to study stress-related disorder in human and develop therapeutic strategies.

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Table 3.1 Oligonucleotide PCR primers

Gene	Accession number[a]	Primer sequence (5' → 3')	Orientation	Product size (bp)
Beclin 1	NM_001006332	TGCATGCCCTTGCTAACAAA	Forward	61
		CCATACGGTACAAGACGGTATCTTT	Reverse	
Atg3	NM_001278070	GAACGTCATCAACACGGTGAA	Forward	65
		TGAGGACGGGAGTGAGGTACTC	Reverse	
Atg5	NM_001006409	TCACCCCTGAAGATGGAGAGA	Forward	66
		TTTCCAGCATTGGCTCAATTC	Reverse	
Atg9A	NM_001034821	AGTATGCCTCCACTGAGATGAGTCT	Forward	65
		GGCATGCTGCTTGTGCAA	Reverse	
Atg10	XM_424902	CATCTCACCAGATCTCAAGAAGGA	Forward	62
		CGACATGCGTAAGCAACGTT	Reverse	
Atg12	XM_003643073	GCACCCGCACCATCCA	Forward	61
		GAGGCCATCAGCTTCAGGAA	Reverse	
Atg14	XM_426476	GCGCTGCGAGGGTGTTAAT	Forward	61
		TTCTGTTACAAAAGCGTTCCTTGA	Reverse	
Atg13	XM_003641387	GGTCCCCCGAGCCAAATA	Forward	55
		ATGAGGTGCGGGAGCTGTAG	Reverse	
Atg7	NM_001030592	ACTGGCAATGCGTGTTTCAG	Forward	57
		CGATGAACCCAAAAGGTCAGA	Reverse	
Atg4B	NM_213573	CCCCGATGAAAGCTTCCA	Forward	56
		GCTCAGCGATGCTCATTCTG	Reverse	
Atg4A	NM_001271986	CACAGCAGTGCACATTTGCA	Forward	62
		CAGAGTCCTGCTGCGTTCCT	Reverse	
Atg16L1	XM_003641751	TGCATCCAGCCAAACCTTTC	Forward	57
		CGACGCTGGTGGCTTGTC	Reverse	
UVRAG	NM_001030839	GGGCTCATGGTCAGATGTGA	Forward	57
		CTTTGGAACGGGAATTGCA	Reverse	
Ambra 1	XM_001233288	GGGATGTTGTGCCTTTGCA	Forward	67
		CCTGGTGTGGGAAGAGAGAAGA	Reverse	

[a] Accession number refer to Genbank (NCBI).

Table 3.1 Multiple alignment of the amino acid sequences of chicken autophagy-related genes with their mammalian orthologs. Genbank accession number is indicated for each gene and each species between brackets.

Table 3.2	SPECIES					
GENE	**Human**	**Mouse**	**Rat**	**Horse**	**Pig**	**Bovine**
Chicken Beclin 1 (NM_001006332)	75.97 (NM_003766)	73.87 (NM_019584)	74.03 (NM_001034117)	75.26 (XM_005597370)	72.28 (XM_005668792)	75.36 (NM_001033627)
Chicken Atg3 (NM_001278070)	81.84 (NM_001278712)	81.6 (NM_026402)	79.7 (NM_134394)	75.57 (XM_005601995)	80.33 (XM_003132682)	91.53 (NM_001075364)
Chicken Atg5 (NM_001006409)	80.11 (NM_004849)	75.55 (NM_053069)	72.09 (NM_001014250)	68.96 (XM_005596852)	68.84 (NM_001037152)	79.53 (NM_001034579)
Chicken Atg9A (NM_001034821)	71.97 (BC_065534)	71.37 (NM_001288612)	66.47 (NM_001014218)	74.7 (XM_001493040)	80.01 (NM_001190275)	73.46 (NM_001034706)
Chicken Atg10 (XM_424902)	59.76 (NM_001131028)	64.64 (NM_025770)	65.6 (NM_001109505)	62.35 (XM_005599592)	69.42 (NM_001190281)	59.12 (NM_001083531)
Chicken Atg12 (XM_003643073)	65.87 (NM_004707)	52.69 (NM_026217)	54.48 (NM_001038495)	75.89 (XM_003362836)	61.67 (NM_001190282)	66.99 (NM_001076982)
Chicken Atg14 (XM_426476)	65.13 (NM_014924)	64.74 (NM_172599)	65.33 (NM_001107258)	74.53 (XM_001914860)	65.34 (XM_001924990)	64.05 (NM_001192099)
Chicken Atg13 (XM_003641387)	65.84 (NM_001205119)	67.14 (NM_145528)	75.47 (NM_001271212)	69.47 (NM_001242529)	61.84 (XM_003122826)	63.58 (NM_001076812)
Chicken Atg7 (NM_001030592)	75.77 (NM_006395)	71.52 (NM_001253717)	76.28 (NM_001012097)	74.05 (XM_005600372)	76.15 (NM_001190285)	69.17 (NM_001142967)
Chicken Atg4B (NM_213573)	79.19 (NM_013325)	78.26 (NM_174874)	50.76 (NM_001025711)	78 (XM_005610806)	75.8 (NM_001190283)	76.06 (NM_001001170)
Chicken Atg4A (NM_001271986)	69.32 (NM_052936)	66.82 (NM_174875)	65.76 (NM_001126298)	73.15 (XM_005614404)	72.51 (XM_005657911)	78.03 (NM_001001171)
Chicken Atg16L1 (XM_003641751)	67.77 (NM_030803)	68.31 (NM_001205391)	70.67 (NM_001108809)	65.24 (XM_005610723)	78.4 (NM_001190272)	67.59 (NM_001191389)
Chicken UVRAG (NM_001030839)	77.23 (AB.12958)	76.16 (NM_178635)	73.96 (NM_001107536)	76.6 (XM_001917231)	----------	75.73 (NM_001193026)
Chicken AMBRA1 (XM_001233288)	70.69 (NM_001267782)	73.38 (NM_172669)	73.4 (NM_001134341)	77.09 (XM_005598075)	74.5 (XM_003122844)	72.66 (NM_001034522)

Table 3.2 Multiple alignment of the amino acid sequences of chicken autophagy-related genes with their mammalian orthologs.

Figure 3.1

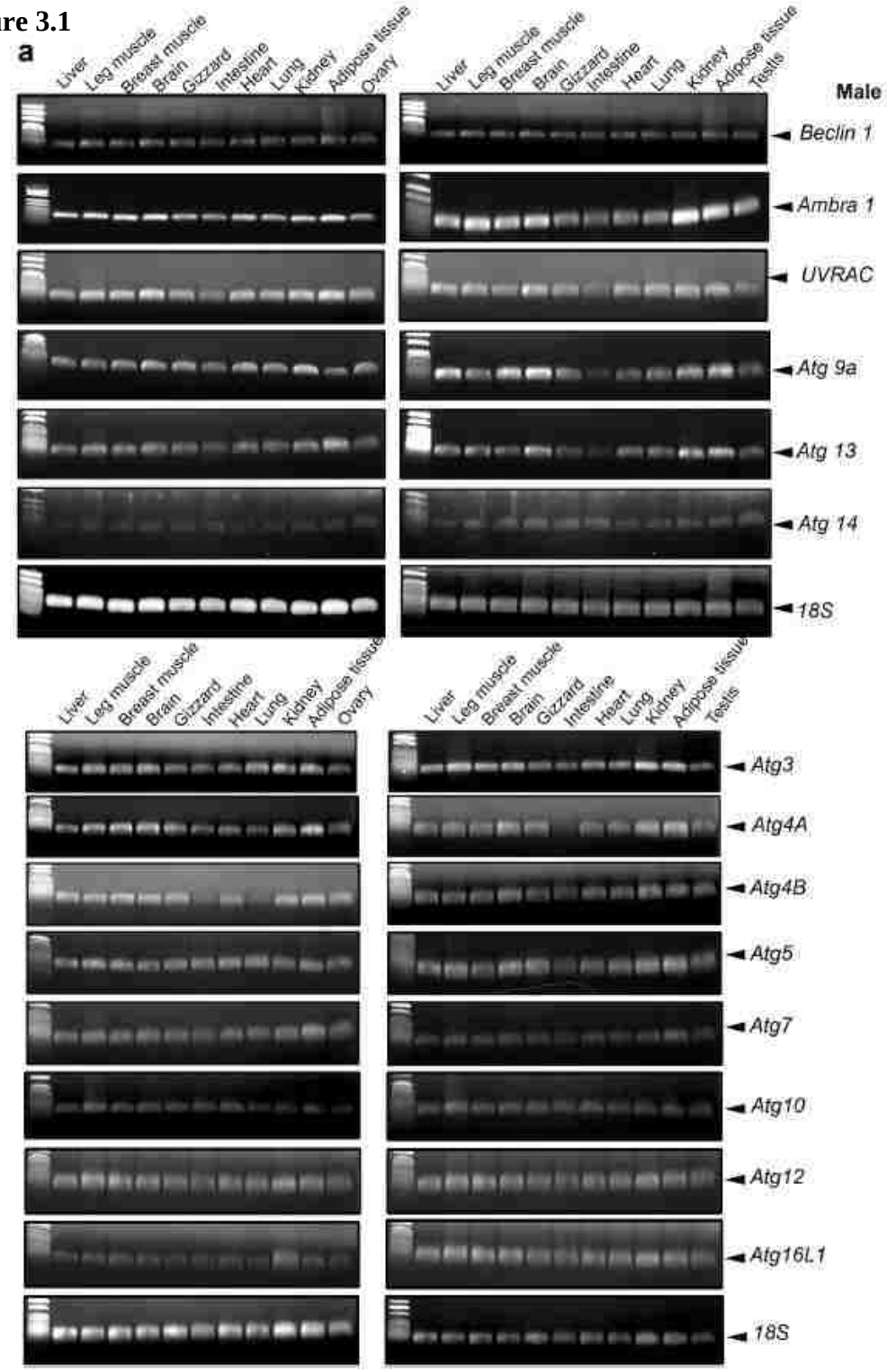

Figure 3.1 Characterization of autophagosome initiation (a) and elongation-related genes (b) in various tissues of male and female Red Jungle Fowl (Gallus gallus) by RT-qPCR as described in materials and methods. Signals were visualized by agarose gel electrophoresis.

Figure 3.2

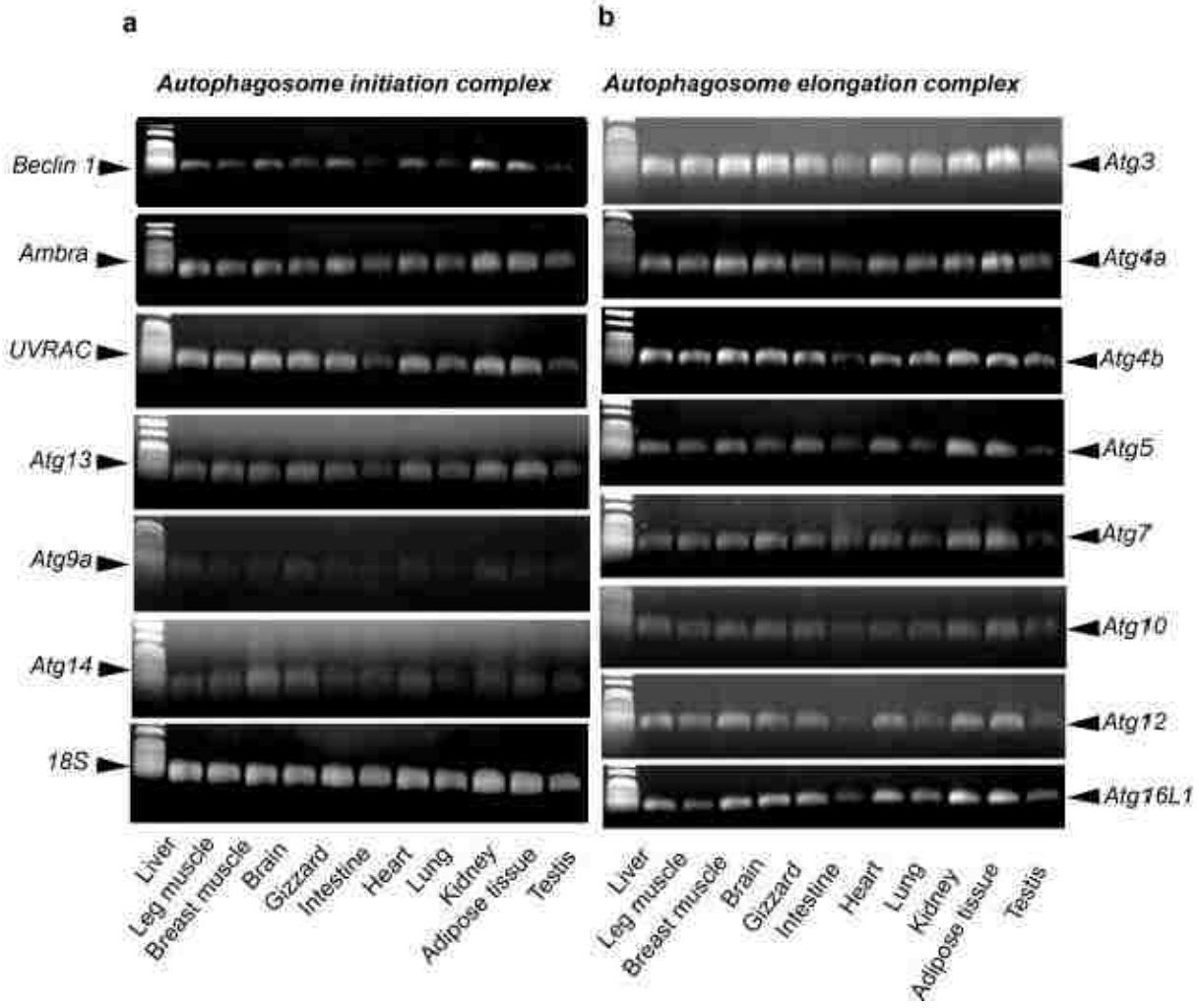

Figure 3.2 Characterization of autophagosome initiation (a) and elongation-related genes (b) in various tissues of stress-sensitive (S) and stress-resistant (R) male Japanese quail (Coturnix coturnix Japonica) using RT-qPCR. Signals were visualized by agarose gel electrophoresis.

Figure 3.3

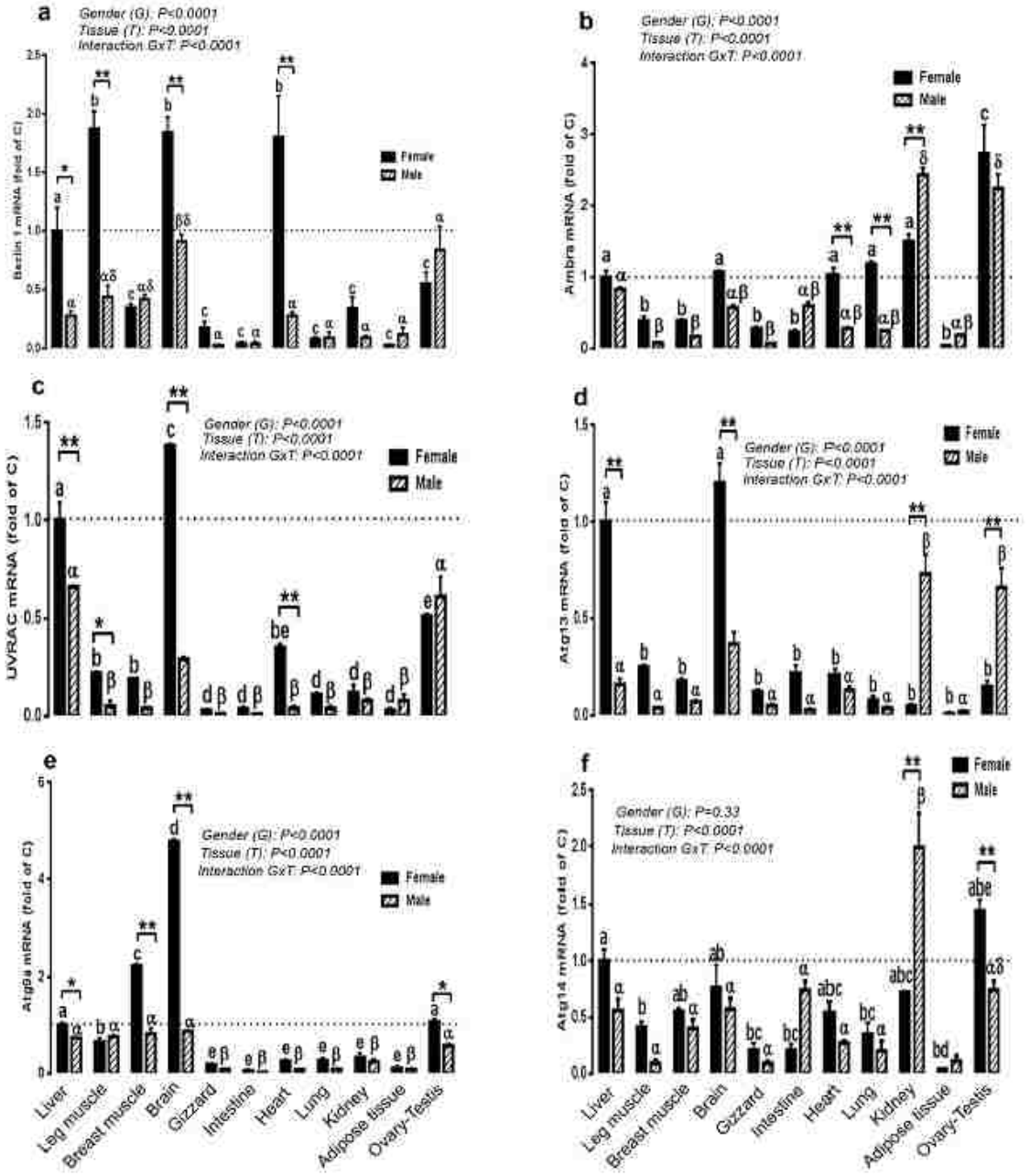

Figure 3.3 Comparison of relative expression of autophagosome initiation-related genes in various tissues of male and female Red Jungle Fowl. Total RNA from each tissue was DNAse-treated, reverse transcribed, and subjected to real-time quantitative PCR as described in material and methods. Samples were run in duplicate, and the average threshold cycle (Ct) values were determined for the target and houskeeping genes. Relative quantity of autophagy genes was determined by the 2-ΔΔCt method [58]. Data are presented as mean ± SEM (n=6 for each gender and each tissue). * Sex-matched differences among tissues (*P<0.05 and **P<0.01). Different letters indicate tissue-matched differences among gender (a-e, difference between tissues within female and α-δ indicate differences between male tissues).

Figure 3.4

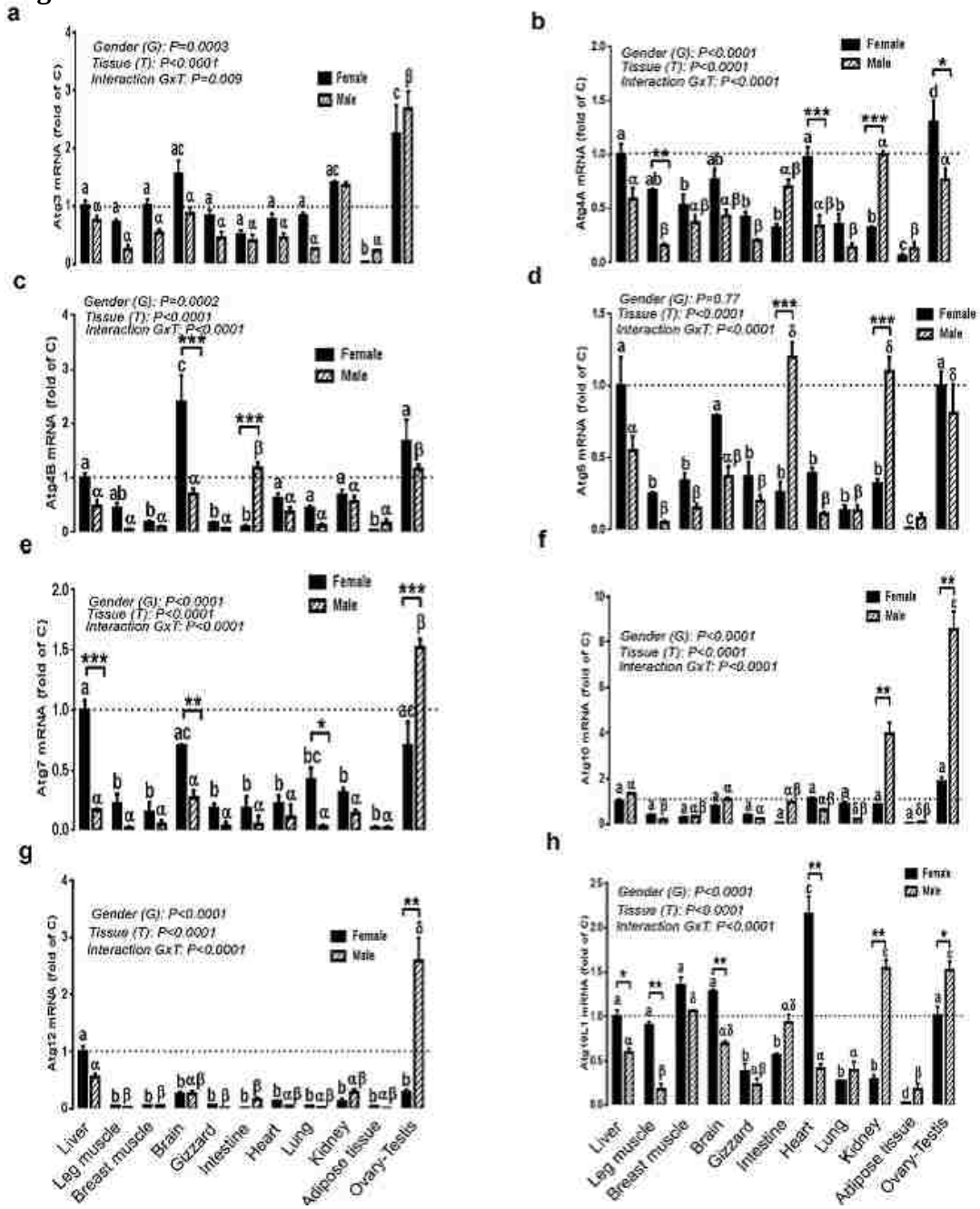

Figure 3.4 Comparison of relative expression of autophagosome elongation-related genes in various tissues of male and female Red Jungle Fowl. Total RNA from each tissue was DNAse-treated, reverse transcribed, and subjected to real-time quantitative PCR as described in material and methods. Sample were run in duplicate, and the average threshold cycle (Ct) values were determined for the target and houskeeping genes. Relative quantity of autophagy genes was determined by the 2-ΔΔCt method [58]. Data are presented as mean ± SEM (n=6 for each gender and each tissue). * Sex-matched differences among tissues (*P<0.05 and **P<0.01). Different letters indicate tissue-matched differences among gender (a-e, difference between tissues within female and α-δ indicate differences between male tissues).

Figure 3.5

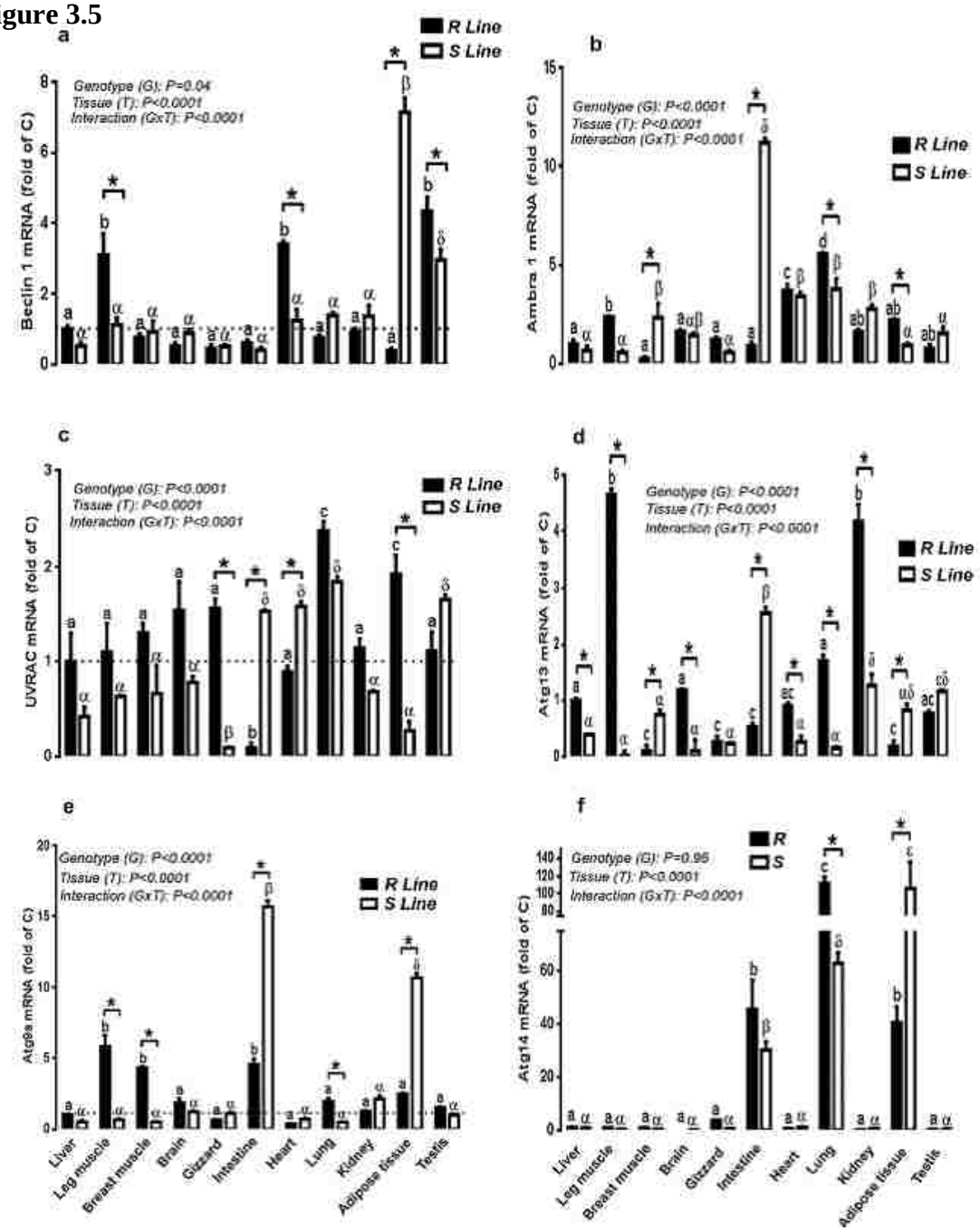

Figure 3.5 Relative expression of autophagosome initiation-related genes in various tissues of R and S male *Japonica* quail lines. Total RNA from each tissue was DNAse-treated, reverse transcribed, and subjected to real-time quantitative PCR. Sample were run in duplicate, and the average threshold cycle (Ct) values were determined for the target and housekeeping genes. Relative quantity of autophagy genes was determined by the 2-ΔΔCt method [58]. Data are presented as mean ± SEM (n=6 for each line and each tissue). * Line-matched differences among tissues (*P<0.05). Different letters indicate tissue-matched differences among Lines (a-c, difference between tissues within R line and α-ε indicate differences between tissues within S line).

Figure 3.6

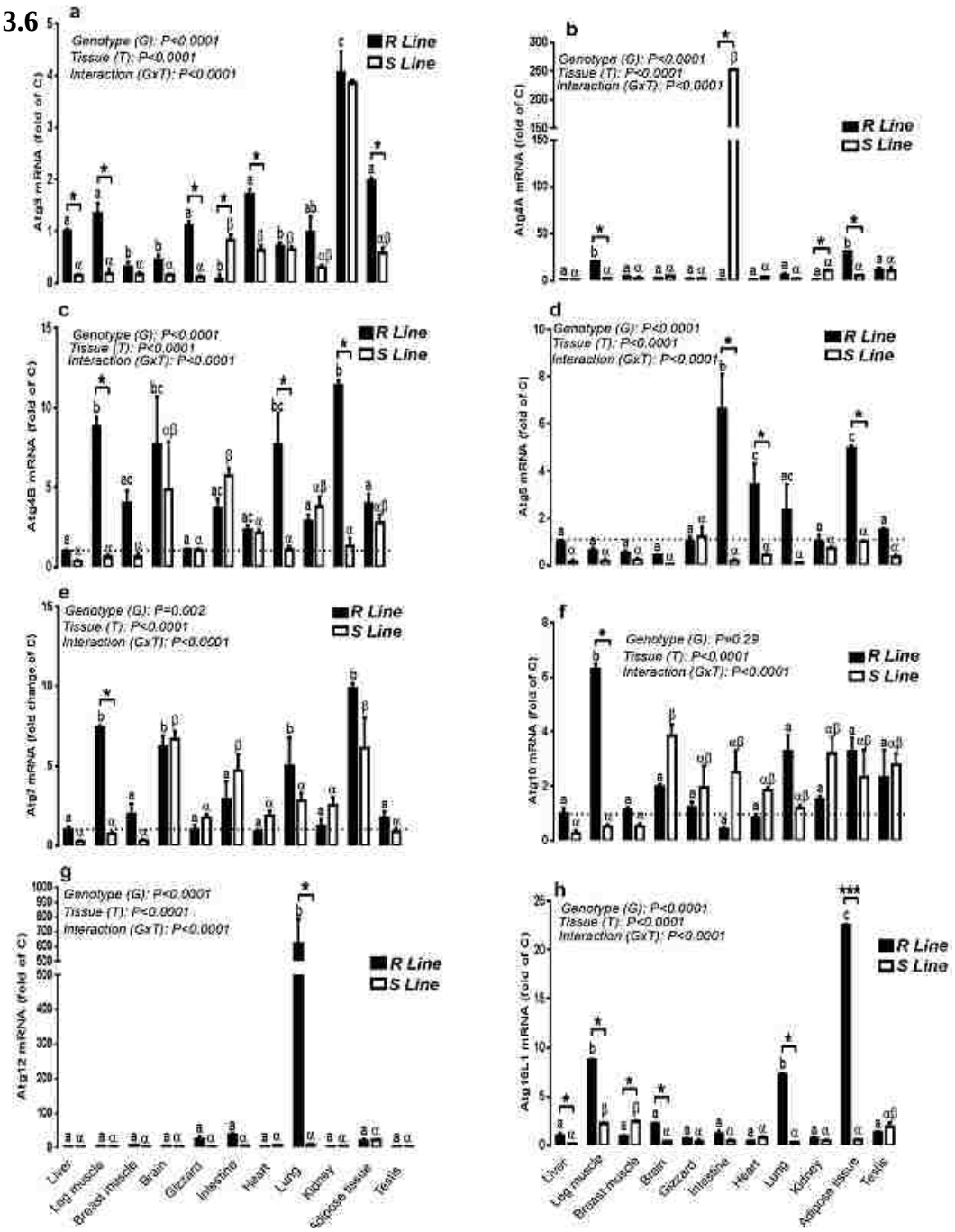

Figure 3.6 Comparison of relative expression of autophagosome elongation-related genes in various tissues of R and S male Japonica quail lines. Total RNA from each tissue was DNAse-treated, reverse transcribed, and subjected to real-time quantitative PCR. Sample were run in duplicate, and the average threshold cycle (Ct) values were determined for the target and housekeeping genes. Relative quantity of autophagy genes was determined by the 2-ΔΔCt method [58]. Data are presented as mean ± SEM (n=6 for each line and each tissue). * Genotype-matched differences among tissues (*P<0.05 and ***P<0.001). Different letters indicate tissue-matched differences among genotype (a,b, difference between tissues within R line and α-β indicate differences between tissues within S line).

Figure 3.7

Figure 3.7 Phylogenetic relationships among chicken autophagy-related genes and their mammalian orthologs were inferred using the neighbor-joining method in MUSCLE alignment and MEGA6. Scale bar indicates the substitution rate per residue. Genbank accession numbers are included in the phenogram and in Table 3.1.

CHAPTER 4:
Effect of Oxidative and Heat Stress on Autophagy Related Genes in Avian Model

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4.1 ABSTRACT

Whereas the process of autophagy (self-digestion) has been well characterized in yeast and mammals, relatively little information is known regarding the autophagy pathway in avian species. Therefore, experiments were conducted to determine the effect of heat and oxidative stress on expression of autophagy-related genes and protein expression in quail muscle cells (QM7 cells) *in vitro* and the effect of heat stress in Japanese Quail *in vivo*. To induce oxidative stress, QM7 cells were treated with 0, 32, and 64 μM 4-hydroxy 2-nonenal (4HNE) (a stable secondary lipid peroxide). After 30 min, an increase in LCB3 (define, an indication of autphagosome formation) was observed using immunofluorescence. Increased expression of several autophagy pathway proteins (e.g. mTOR, p-mTOR, Beclin 1, Atg 3, LC3B) was also observed 30 min after 64 uM 4-HNE treatment in comparison to control treated cells. Next, autophagy expression was analyzed in QM7 cells exposed to heat stress (45 C) or control (37 C) conditions. After 7 h of heat stress at 45 C followed by 1 h recovery at 37 C, HSP70, AMPKa1 and FOXO1 expression was elevated in comparison to Control treated cells. In a second heat stress study *in vitro*, time course changes in autophagy pathway expression was monitored at 0.5. 1, 2, and 4 h of heat stress at 45 C. In this time course study, there was an increase in mRNA expression of all genes studied (AMPKa1, Beclin1, Atg3, Atg7, Atg16L1 and LC3B) at 1 h followed by a decline at 2 and 4 h of heat stress. To assess the effect of heat stress *in vivo*, Japanese Quail selected for resistance (R) or susceptibility (S) to restraint stress, were exposed to 75 min of heat stress at 42 C with control birds maintained at 25 C. The S Japanese Quail line exhibited up-relation of 5 of 6 autophagy-related genes in response to heat stress whereas the R line exhibited an increase in expression of only 1 autophagy related gene in the liver with down-regulation of two other genes. The results of this study indicate that heat and oxidative stress can

alter the activity (expression) of the autophagy pathway in avian cells and that there may differences in autophagy response to heat stress in Japanese Quail selected for resistance and susceptibility to restraint stress.

KeyWords: autophagy, heat stress, oxidative stress, Japanese Quail

4.2 INTRODUCTION

Stress, whether external (e.g. temperature stress, disease, crowding) or internal (e.g. endogenous oxidative stress) reduces animal production efficiency. A major source of oxidative stress in cells is mitochondrial reactive oxygen species (ROS). Mitochondria are responsible for 90% of cellular energy (ATP) production and also a major site of ROS production. Low levels of ROS modulate translation and transcription processes and high levels can oxidize proteins, lipids and DNA. Heat stress has been shown to increase mitochondrial ROS production in broilers and layers (Mujahid et al., 2007a; Mujahid et al., 2007b; Azad et al., 2010) which could lead to mitochondrial autophagy (mitophagy) if radical generation becomes excessive (Levine and Kroemer, 2008).

A relatively new field in cell biology is autophagy in which cells digest damaged components (e.g. proteins, organelles) to maintain optimal cell viability and function (Klionsky, 2005; Levine and Kroemer, 2008). It is a highly conserved pathway (from yeast to humans) with many novel findings coming from laboratories every year in many fields of study. Figure 1 (pg. 43) shows the characterization of several genes involved in the autophagy pathway, of which, many genes were characterized for the first time in avians (Chapter 3) (Piekarski et al., 2015). When energy sources are limiting, cells initiate autophagy to help maintain mitochondrial ATP production (Alers et al., 2012). Autophagy involves a network of proteins regulated by rapid and reversible post-translational modifications. In autophagy, damaged cytosolic components (e.g. proteins, cytoskeleton) and entire organelles (e.g. mitochondria) are transported to lysosomes via autophagosomes where hydrolytic enzymes rapidly degrade sequestered material (Chen and Klionsky, 2011; Cuervo and Macian, 2012). Autophagy is critical for homeostasis, but the process must be tightly controlled to avoid extensive self-digestion. A recent characterization of the

autophagy pathway revealed tissue- and gender-specific differences in jungle fowl (*Gallus gallus*) as well as tissue and genotype differences in quail (*Coturnix coturnix japonica*) (Piekarski et al., 2015). By maintaining optimal functionality of proteins and organelles, as well as providing an alternative to cell death by necrosis or apoptosis, autophagy likely has important roles at the organismal level ranging from generalized stress, feed efficiency, and nutrient limitation, to disease response and toxin insult.

Animal agriculture is facing substantial challenges, including a steep projected increase in demand and the need to adapt to changing environmental conditions. Due to a predicted increase in world population to 9 and 10 billion, United Nations FAO estimates that by 2050 there will be a 73% increase in meat and egg consumption and a 58% increase in dairy consumption over 2011 levels; with heat wave frequency and intensity projected to rise during the next century (Alexandratos et al., 2006). Reducing the impact of climate change and cost of animal protein production is essential to achieve a sustainable, affordable, and secure animal protein supply. To do so, mechanistic understanding (at molecular and cellular levels) of heat stress is necessary and of uppermost interest. The current study aimed to determine the effects of heat and oxidative stress on the autophagy pathway in avian species using *in vitro* and *in vivo* models.

4.3 MATERIALS AND METHODS

4.3.1 ANIMALS

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas (Fayetteville, AR 72701) protocol #13039.

In order to assess the expression of autophagy-related genes during times of heat stress, two lines of male Japanese quail (*Coturnix coturnix Japonica*) were used. These two lines were established by long-term divergent selection for circulating corticosterone response to restraint

stress, after which the low stress line (resistant, R) had 66% low plasma corticosterone levels compared to their high stress (sensitive, S) counterpart (Satterlee and Johnson, 1988). Quail of each genetic line were reared separately in floor pens under environmentally controlled facilities and were allowed *ad libitum* access to water and food (12.6 MJ·kg−1, 22% protein). Quail were heat stressed in environmental chambers (Conviron 18L) for 75 min at 42°C with control birds maintained at 25°C.

Animals were humanely killed by cervical dislocation and tissues (liver, leg muscle, and breast muscle) were removed, immediately snap frozen in liquid nitrogen, and stored at −80°C until use. For these studies we wanted to look at highly metabolically active tissue and so selected muscle and liver tissue to work with for heat stress studies.

4.3.2 CELL LINE

Avian muscle cells (Quail Muscle Clone 7, QM7) were obtained from ATCC (Manassas, VA) cultured, and were maintained in 10 cm petri dishes (BD Biosciences, East Rutherford, NJ) M-199 media (Life Technologies, Grand Island, NY) complemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY), 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO2 and 95% air. At 80-90% confluence, cells were subcultivated and treatment began at the 12^{th} passage with n = 3. Heat stress was imposed by 7h exposure to 45°C followed by a 1h recovery at 37°C. Control cells were maintained at 37°C. For time-course studies QM7 cells were heat stressed at 45°C, with no recovery period, for 4h with measurements taken at 0.5, 1, 2, 4h. Oxidative stress was chemically induced by treating cells with 0, 32, or 64 µM 4-HNE (4-hydroxy-2-nonenal) (Billerica, MA). Cells were removed form plates using a cell lysis

buffer (Na_3SO_4, NaF, PMSF, Aprotinin, Leupeptin, and Pepstatin). Cells were isolated for either protein or RNA and stored at -80°C.

4.3.3 IMMUNOFLUORESENCE

Cells were grown on chamber slides (Lab-Tek, catalog #1773801) and, after 50-60% confluency was reached, were treated with the same concentrations of 4-HNE. After 30 min, media was removed and cells were fixed for immunofluorescence using Alexafluor 488 (Invitrogen) for LC3B detection. Immunofluorescence was performed as previously described (Dridi, 2012). Briefly, cells were grown to 50-60% confluence in chamber slides (Lab-Tek, Hatfield, PA) and fixed in methanol for 10 min at –20°C. Cells were blocked with protein block serum free blocking buffer (Dako, Carpinteria, CA) and incubated with rabbit LC3B (Cell Signaling, Danvers, MA) primary antibody overnight at 4°C and visualized with Alexa Fluor 488-conjugated secondary antibody (Molecular probes, Life Technologies, grand Island, NY). After DAPI counterstaining, slides were cover slipped in Vectashield (Vector Laboratories, Burlingame, CA). Images were obtained using the Zeiss Imager M2 with a 20X Plan-APOCHROMAT 20x/0.8 objective and a 100X EC PLAN-NEOFLUOR 100x/1.3 oil objective. Alexa Fluor 488 fluorophore was observed through filter set 38 1031-346 with an excitation of BP 470/40, beam-splitter of FT 495, and emission spectrum of BP 525/50. Differential interference contrast images were collected using DIC M27 condensers. The Alexa Fluor 488 fluorophore was excited for 500 ms prior to capturing each image using an Axio Cam MR3 camera. All analysis was performed using AxioVision SE64 4.9.1 SP1 software (Carl Zeiss Microscopy GmbH 2006-
2013).

4.3.4 QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR methods were performed as previously described (Lassiter, 2015). Briefly, total RNA was extracted from chicken and quail tissues by Trizol reagent (catalog #15596018, Life Technologies) according to manufacturer's recommendations, DNAse treated and reverse transcribed (catalog #95048-100, Quanta Biosciences). The integrity and quality of RNA was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (catalog #4312074, Life Technologies). Oligonucleotide primers were used for avian autophagy-related genes determined as previously described in Table 3.1 (p. 64) of Chapter 3. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

4.3.5 STATISTICAL ANALYSES

Data were analyzed by two-factor ANOVA for quail with genotype (R vs. S) and environment (HS vs. TN) as classification variables. If ANOVA revealed significant effects, the means were compared by Tukey's multiple range test using the Graph Pad Prism version 6.00 for Windows, Graph Pad Software, La Jolla California USA. Differences were considered significant at $P \leq 0.05$.

4.4 RESULTS AND DISCUSSION

4.4.1 4-HNE TREATMENT AFFECTS AUTOPHAGY-RELATED GENES IN QM7 CELLS

Eukaryotic organisms depend on homeostatic mechanisms which ensure overall maintenance and cellular viability. Autophagy plays a major role in cellular maintenance through sequestration and lysosomal degradation of large protein aggregates as well as damaged organelles that are inaccessible to smaller proteolytic systems in the cell. Internal stress, such as oxidative stress, can reduce an animal's production efficiency; with the major source of oxidative stress in cells presenting in the form of mitochondrial reactive oxygen species (ROS). Mitochondria are responsible for 90% of cellular energy (ATP) production and also a major site of ROS production. Low levels of ROS modulate translation and transcription processes and high levels can oxidize proteins, lipids and DNA. Heat stress has been shown to increase mitochondrial ROS production in broilers and layers (Mujahid et al., 2007a; Mujahid et al., 2007b; Azad et al., 2010) which could lead to mitochondrial autophagy (mitophagy) if radical generation becomes excessive (Levine and Kroemer, 2008). With mitochondrial ROS, a secondary lipid peroxide is formed (4-hydroxy 2-nonenal, 4HNE) that can cause significant toxicity (Skulachev, 1997; Brand et al., 2004). Through immunofluorescence and protein expression of autophagy genes exposed to the stable secondary lipid peroxide 4-HNE, we showed that the autophagy pathway is indeed active during times of oxidative stress (Figures 4.1 and 4.2 respectively).

Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein that is distributed ubiquitously in tissues and cultured cells and an important component of autophagy (formation of the autophagosome) (Asanuma et al., 2003). LC3 contains two isoforms: a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), and then is recruited to autophagosomal membranes. Autophagosomes then fuse with lysosomes (autolysosomes), and intra-

autophagosomal components are degraded by lysosomal hydrolases. During this time, LC3-II in the autolysosomal lumen is degraded and when the autolysosome lyses, it releases the now free proteins and amino acids that can be used as a new energy source for the starving or stressed cell in need of repair. Detecting LC3 by immunoblotting or immunofluorescence has become a reliable method for monitoring autophagy and autophagy-related processes, including autophagic cell death (Tanida et al., 2008). Observation of distinct puncta in images of the stressed QM7 cells validated autophagosome formation and, in turn, the confirmation of autophagy activation. Figure 4.1 shows increased LC3B expression in QM7 cells following exposure to 4-HNE for 2h using immunofluorescence. DAPI (4', 6-diamidino-2-phenylindole) strongly binds to nuclear DNA such that the merged image shows the presence of LC3B to be perinuclear (i.e. cytosol and membranous) in 4-HNE treated cells. The formation of LC3A and B isoforms in QM7 cells under 4-HNE oxidative stress has also been demonstrated (data not shown). This image, along with protein data from western blotting and data received from qPCR (Figure 4.2), indicates that autophagy is indeed progressing following acute oxidative stress.

4.4.2 WESTERN BLOT ANALYSIS OF AUTOPHAGY-RELATED GENES IN 4-HNE (QM7) AND HEAT STRESSED (QM7; QUAIL) SAMPLES

The effect of 4-HNE induced oxidative stress on autophagy related protein expression is shown in Figure 4.2. Densitometry analysis (Figure 4.2) suggests mTOR, p-mTOR, Beclin1, and Atg3 all were decreased in QM7 cells after a 30 min exposure to 64 μM 4-HNE (Figure 4.2). These results lend credence to the possibility that once mTOR has signaled downstream to initiate autophagy its expression decreases, the same with Beclin1 and Atg3.

The presence of molecular chaperone HSP70 (heat shock protein 70kDa), transcription factor FOXO1 (forkhead box protein O1), and p-AMPK (phospho-AMP activated protein kinase) were also measured through Western blot analysis. Heat stress (7 h 45°C and 1 h 37°C recovery)

induced the expression of Hsp70 and p-AMPK. In addition, FOXO1 expression (which plays a role in the regulation of autophagy) was also increased in response to HS. Increased levels of p-AMPK indicates that autophagy may be activated (Figure 4.3) in heat stressed QM7 cells and would be instrumental in enhancing expression of downstream target molecules of the autophagy pathway. A recent article showed that the autophagy pathway is mediated through the regulatory role of the chaperone activity of Hsp70 (Dokladny et al., 2013).

Protein expression analysis was conducted on liver and leg muscle of R and S Japanese quail under thermoneutral (TN) and heat stress (HS) conditions. Though the expression levels changed between tissues, with HSP70 significantly increased in liver and Beclin1 significantly increased in leg muscle, it is consistent with previous findings that autophagy is tissue specific in Japanese Quail (Figure 4.4) (Chapter 3). Another explanation for this difference come from studies with autophagy and the heat shock protein (HSP) family. Doklandy and colleagues have shown that the HSP family may regulate autophagy and, in fact, show evidence directly linking the HSP and autophagy systems as well as demonstrate a master regulatory role of HSP70 in controlling autophagy (2013).

4.4.3 ANALYSIS OF HEAT STRESS IN QM7 CELLS

Considering Hsp70 plays a key role in the protection of cells during times of heat stress, we decided to look also at mRNA expression levels of this gene under the same conditions (Figure 4.5). A significant increase can be seen in the levels of Hsp70 in heat stressed cells as compared to the control QM7 cells, clearly indicating signs of heat stress (Figure 4.5).

Using a time course study (30 min, 1h, 2h, 4h) QM7 cells were heat stressed AT 45°C with a 1h recovery and compared to control cells kept at 37°C. Autophagy gene expression of Beclin1, AMPKα1, LCB3, Atg7, 16L1, and 3 was analyzed and results show an interesting time course

response in all genes. All autophagy related genes (Atg) showed an increase at 1h of heat stress (Figure 4.6). This indicates that QM7 cells may utilize the autophagy pathway during times of heat stress up to 1h and then may reach a threshold in which the pathway shifts into apoptosis due to accumulation of autophagosomes. For example, if mitochondrial depolarization and release of proapoptotic factors exceed the autophagic capacity, apoptosis ensues (Kundu and Thompson, 2008). Cells from the present study were observed under a microscope for floating, rounded, or cytoplasm shrinkage all of which were largely not detected allowing the assumption of very little cell death at the end of the experiment that was due to heat stress exposure (data not shown). For further clarification, a cell viability assay, or apoptosis/necrosis markers, will need to be conducted in the future to determine actual number of cell death in relation to heat stress studies. Another possible explanation to this pattern may be that autophagy, now activated, has removed any organelles damaged by heat stress and provided new energy for cellular repair, with expression levels for these genes having returned to baseline by the fourth hour. The other three genes show a similar pattern, but with the highest expression levels seen at the 1 and 2h time points. An increase in AMPK signals an activation in the autophagy pathway, which can be seen by the increase in all subsequent autophagy genes tested. Beclin1 and LCB3 have been used as definitive biomarkers in the autophagy pathway (Meyer et al., 2013). We show here that there is a difference at the 1h time point in both AMPKα1 and Beclin1 with the highest levels of LC3B expression at both the 1 and 2h time points. Because LC3B is one of the last genes in the autophagy pathway, this may suggest that the autophagy pathway is sustained in these cells for up to 2h of heat stress before levels start to diminish.

4.4.4 EFFECT OF HEAT STRESS IN VIVO

Previously, differences in autophagy expression was observed between male and female Japanese quail (Piekarski et al., 2015, Chapter 3). The purpose of this experiment was to determine if differences in autophagy could be observed in R nad S quail under thermoneutral (TN) or heat stress (HS) conditions. The same genes (Beclin1, AMPKα1, LCB3, Atg7, Atg16L1, and Atg3) were analyzed in tissue (muscle) obtained from quail either held at thermoneutral (TN) levels or heat stress (HS) conditions in environmental chambers. When comparing a change under HS conditions between R and S quail in leg muscle, it can be seen that expression of LC3B, and Atg16L1 increase with HS, and are unchanged in Atg7, Atg3, AMPKα1, and Beclin1 (Figure 4.7). Under thermoneutral conditions (TN), in the low stress line, there was a significant increase in expression of Atg7, Atg3, and Atg16L1 with no difference in expression of LC3B, and Beclin1 and an increase in expression in the high stress line in AMPKα1 (Figure 4.7). In liver, it can been seen that there was an overall increase in expression in all genes (Atg3, Atg7, and Beclin1) while no change was observed in AMPK, Atg16L1, and LC3B (Figure 4.8). Also, under TN conditions, there is increased expression in Atg3, Atg7, and LC3B in the low stress line with no difference in expression of AMPKα1, Beclin1, and Atg16L1 (Figure 4.8). A recent study has shown that heat stress is related to autophagy (Dokladny et al., 2013). The expression of autophagy gene LC3B is indicative of autophagosome formation and many, including this group, look to its expression as a marker that autophagy is occurring in their experimental model. These results show that LC3B expression is active in both tissues but because it did not reach significance, it could be that autophagy is either defective in these animals, or that it detects a cellular inefficiency and was finishing autophagosome formation, and thus, autophagy. This shows there is indeed a difference between lines as well as that heat stress does affect the autophagy pathway and could mean that autophagy is activated in these animals as a compensatory mechanism, aiding in cell survival under

heat stress. Immunofluorescence and protein measurements will be measured in future studies to help to determine if this is the cytosolic, autophagosome form of LC3B or the nuclear form before it is involved in autophagosome formation.

Interestingly, the activation of LC3B, indicated by increased immunofluorescence as well as increased expression in the western blot analysis, was observed, showing that LC3B expression was high and autophagosome initiation was occurring in response to 4-HNE treatment (Figure 4.1). If samples were obtained at time points later than 30 min post 4-HNE treatment, one could expect to see LC3B eventually decrease as the autophagosome would lyse or this pathway would be no longer useful (with too high a level of stressor) and the cell would give into the apoptosis pathway. The results shown in Figure 4.2 suggest a possible cascade-like pathway, where once an upstream gene sends a signal to activate the next gene in the cascade, it then decreases its signaling and expression levels eventually return to baseline; this then continuing to occur with sequential genes in the pathway. More experiments will need to be performed to confirm this mechanism.

Due to animal agriculture facing substantial challenges from a projected increase in demand for high quality animal protein and the need to adapt to higher temperatures due to climate change, increasing production efficiency, especially during periods of heat stress, while reducing the environmental impact and cost of animal protein is essential to achieving a sustainable, affordable, and secure animal protein supply. At opposite ends of the homeostatic system spectrum are autophagy and heat shock response (Dokladny et al., 2013). As has been stated before, exposure to stressors such as radiation, heavy metals, and heat, can lead to protein denaturation, damage of nucleic acid, and even death (Dokladny et al., 2013). Pathways such as autophagy and the heat shock pathway have been developed by organisms to help them withstand the damage that can be come from these stressors. In the heat shock pathway, specific proteins

called heat shock proteins are activated to help decrease stress-related damage. Among these proteins, HSP70 offers protection against damaging factors at the macromolecular, single cell, and whole organism levels (Dokladny et al., 2013). Heat shock proteins are molecular chaperones that assist in folding of proteins as well as translocation, degradation, and reactivation of damaged proteins (Hendrick and Hartl, 1993). Due to their role in protection and degradation, the interaction of the heat shock and autophagy pathways became of interest to our laboratory and, specifically, my research. Although both pathways represent protein management alternatives for a stressed cell, there is not much literature on the relationship of these two pathways; Doklandy and colleagues having the majority of published works in this field, with recent additions by other laboratories. The laboratory of Doklandy has shown that the heat shock system is prioritized through its ability to interrupt activation of the autophagic response (Dokladny et al., 2013). We have seen similar responses in the stress resistant line of liver of heat stressed Japanese quail where HSP70 levels were highly expressed and autophagy gene Beclin1 showed no difference in expression between the stress susceptible and resistant lines under the same heat stress conditions (data not shown). Doklandy and colleagues have shown that the knockdown of HSF-1 increased the LC3 lipidation associated with autophagosome formation as well as the requirement for HSP70 and HSF1 in this formation (Dokladny et al., 2015). As was shown above, we found that during heat stress, QM7 cells exhibited an increase in LC3B expression at the 1h time-point. In addition, we saw an increase in expression of LC3B in the high stress line of quail when exposed to heat stress, possibly meaning there is an upregulation of autophagosome formation to help the cell cope with heat stress damage.

4.6 CONCLUSION

Reducing the impact of climate change and cost of animal protein production is essential to achieve a sustainable, affordable, and secure animal protein supply. To see this reduction come to fruition, a better understanding of the mechanistics underlying the causative effects of heat stress must be determined. Autophagy, upregulated during many different types of stress, is a pathway which may contribute greatly to alleviating this issue. Additional studies will need to be performed to further dissect these mechanisms and whether or not they have potential for alleviating or preventing heat stress in animal agriculture.

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Figure 4.1

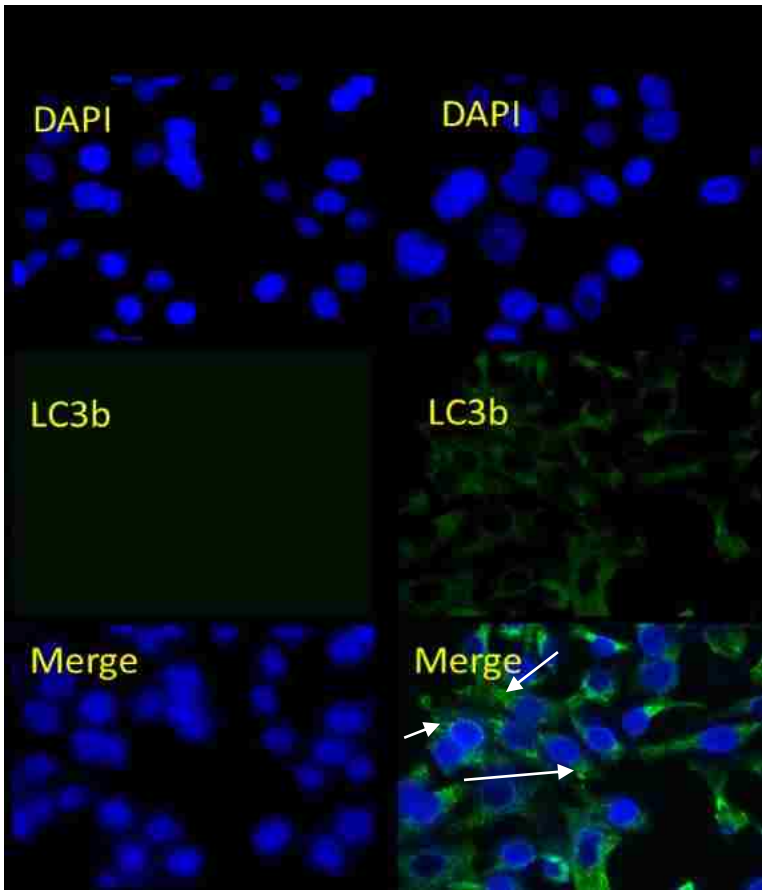

Figure 4.1 Autophagosome formation through the presence of puncta visualization using the fluorescent marker for LC3b after 30 min of exposure to 64 µM 4-HNE. White arrows show location of puncta.

Figure 4.2

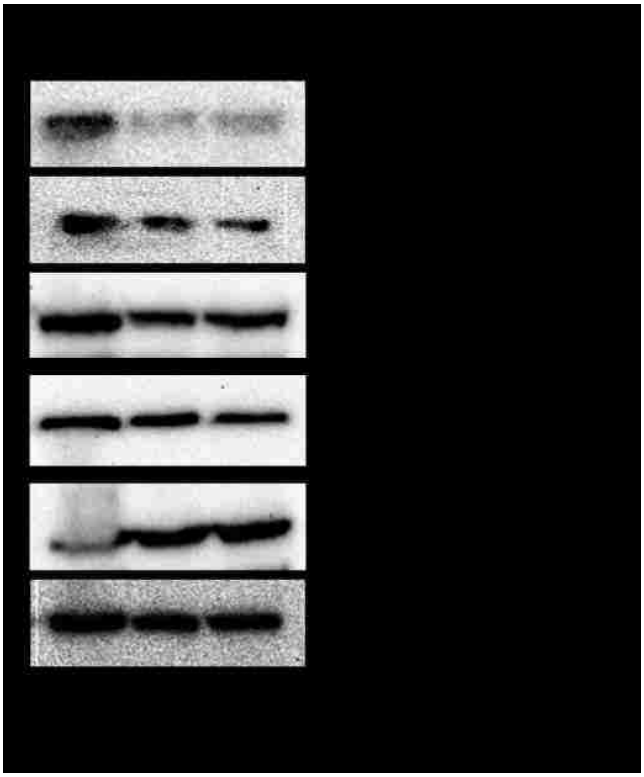

Figure 4.2 Western blot analysis of autophagic genes in response to 4-HNE treated QM7 cells (64 μM, 30 min). An increase in Atg3 and LCB3 was observed with a decrease in p-mTOR and total mTOR suggesting a cascade-like signaling pathway. (n=3 on 10mm plates)

Figure 4.3

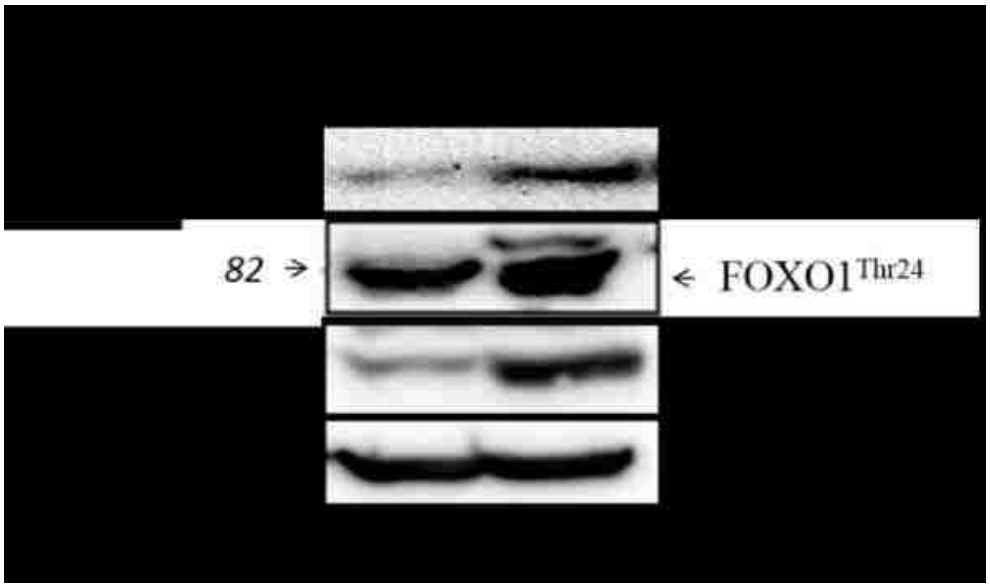

Figure 4.3 Western blot analysis showing heat stress (7 h 45°C and 1 h 37°C recovery) induced the expression of Hsp 70, p-AMPK, and protein expression in quail muscle (QM7) cells. (n=3 on 10mm plates)

Figure 4.4

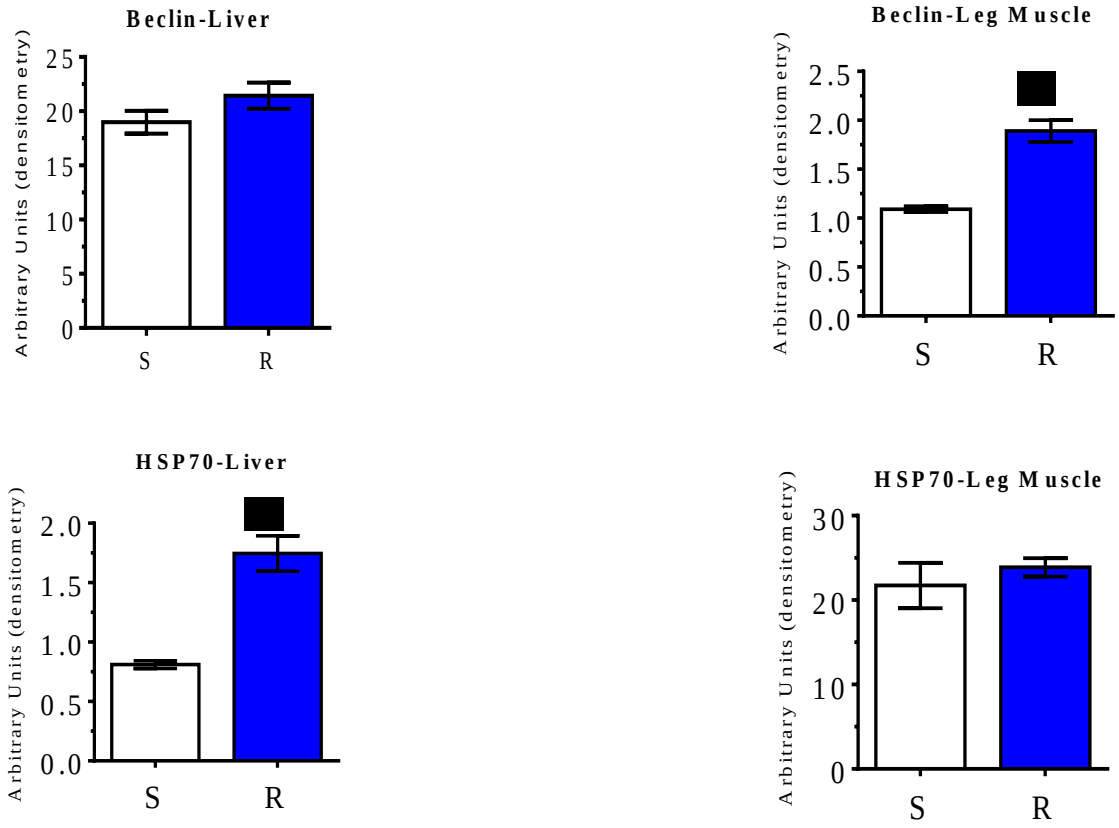

Figure 4.4 Western Blot analyses of HSP70 and Beclin1 expression in Japanese quail leg muscle and liver. Significant increases can be seen in the expression of Beclin1 in the leg muscle of the R line, with a significant increase in expression of HSP70 in the liver of the R line; S=susceptible, R=resistant. Bars represent mean $\pm$ SE of 6 observations/group.

Figure 4.5

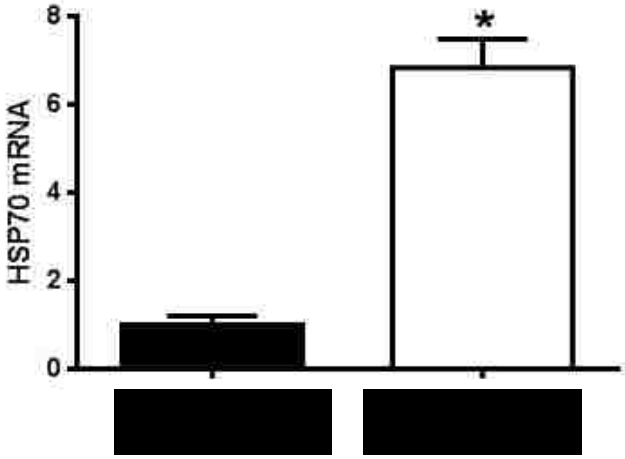

Figure 4.5 mRNA analysis showing heat stress (7 h 45°C and 1 h 37°C recovery) significantly induced the expression of Hsp70 as compared to control in quail QM7 cells. Bars represent mean ± SE on 3, 6mm plates/treatment.

Figure 4.6

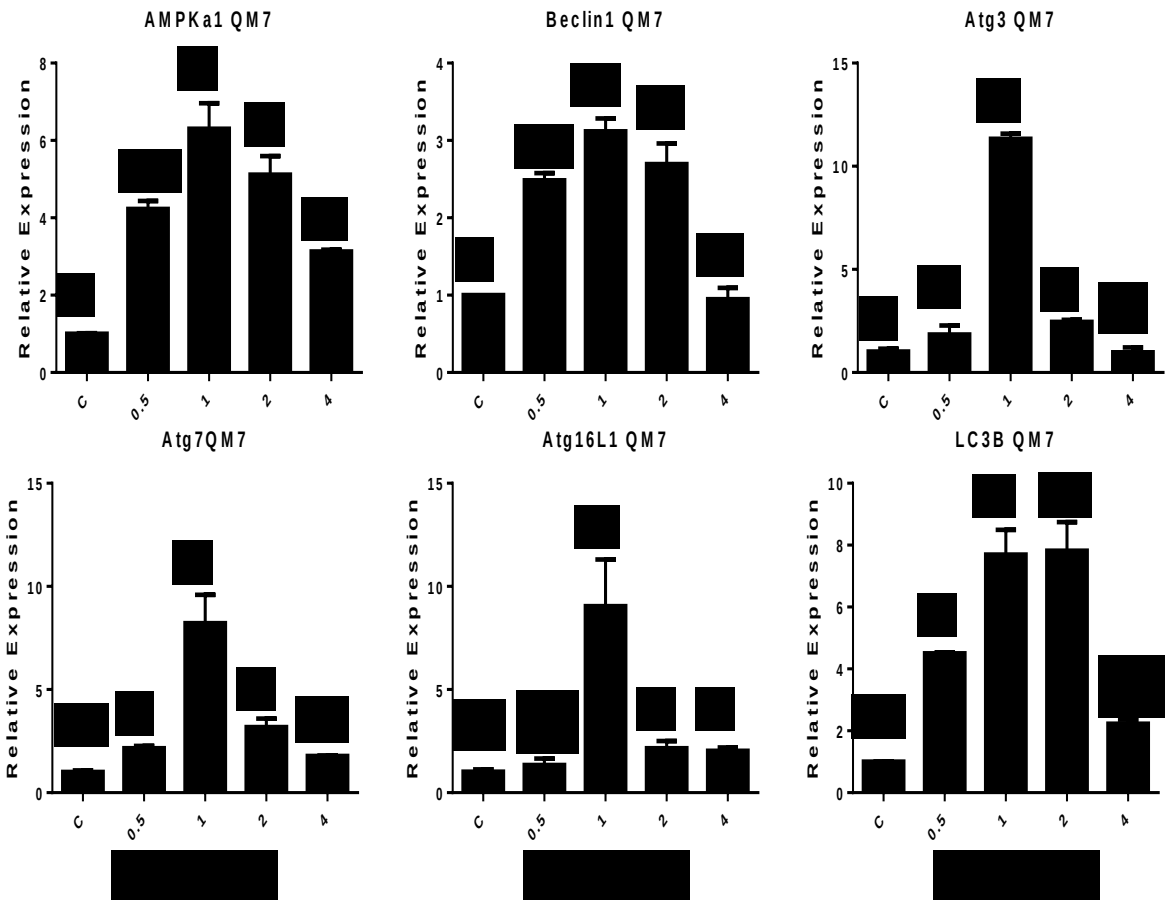

Figure 4.6 mRNA analysis of the effect of heat stress on autophagy pathway gene expression in QM7 cells. QM7 cells were exposed to heat stress (HS) at 45°C and thermoneutral (TN) at 37°C conditions. Bars represent mean ± SE of 3 observations/ 6mm plate. Letters (A,B,C,D) represent mean and SE and denote significance.

Figure 4.7

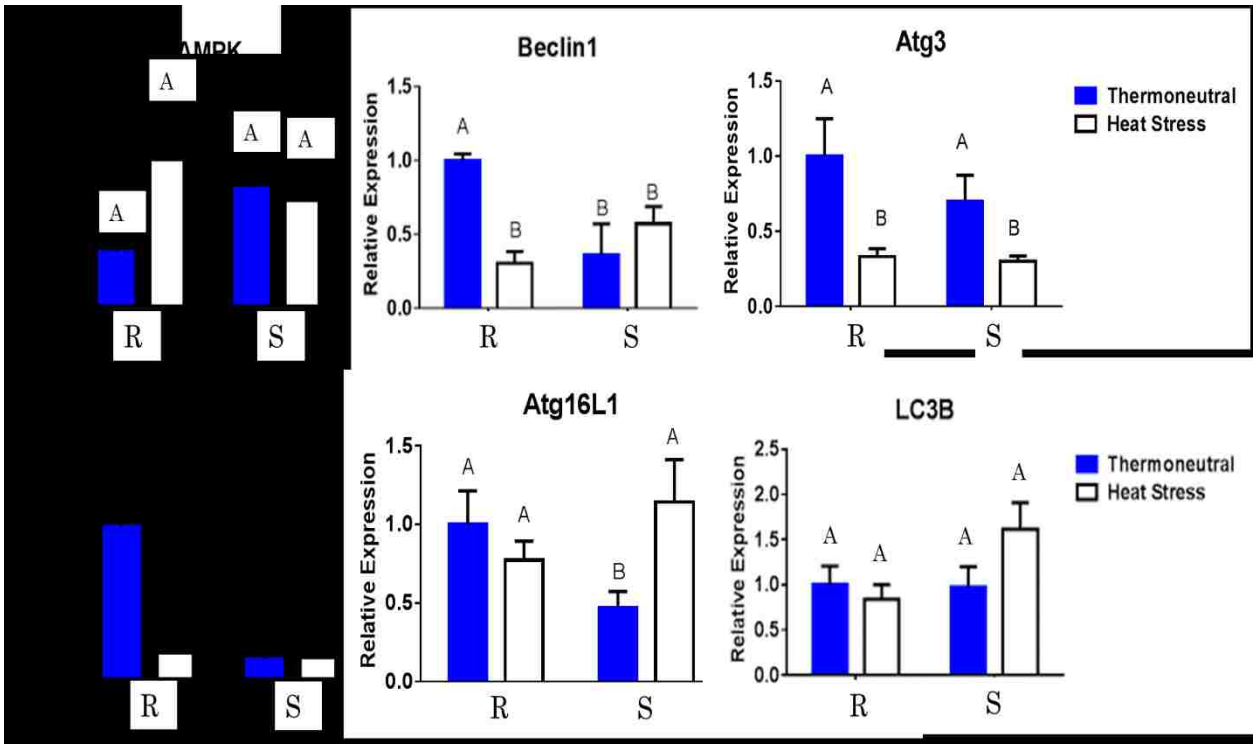

Figure 4.7 The effect of heat stress on autophagy pathway gene expression in leg muscle tissue of Japanese quail selected for susceptibility (high stress) or resistance (low stress) to restraint. Quail were exposed to heat stress (HS) or thermoneutral (TN) conditions. Bars represent mean $\pm$ SE of 6 observations. Letters (A,B,C,D) represent mean and SE and denote significance.

Figure 4.8

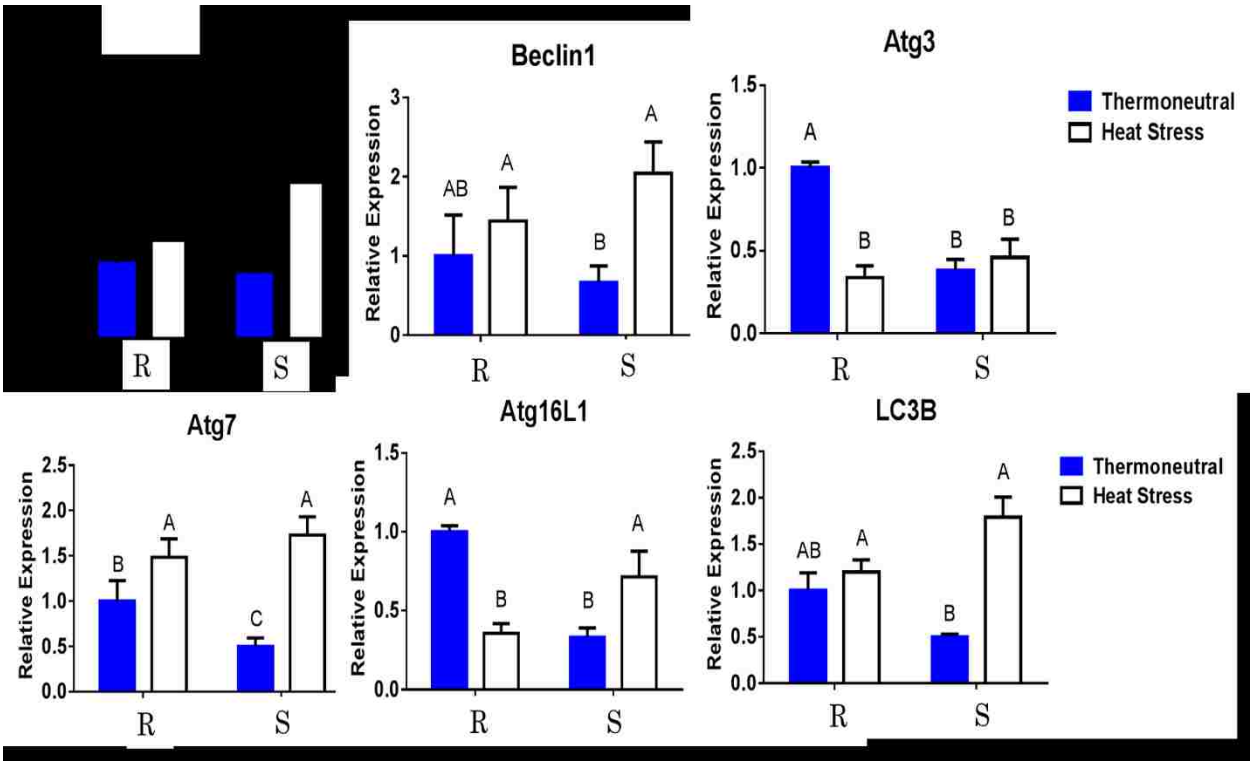

Figure 4.8 The effect of heat stress on autophagy pathway gene expression in liver tissue of Japanese quail selected for susceptibility (high stress) or resistance (low stress) to restraint. Quail were exposed to heat stress (HS) or thermoneutral (TN) conditions. Bars represent mean $\pm$ SE of 6 observations/group. Letters (A,B,C,D) represent mean and SE and denote significance.

CHAPTER 5:
The effect of Autophagy on Feed Efficiency in Two Avian Species

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5.1 ABSTRACT

Feed efficiency (FE) is a very important genetic trait in poultry and livestock that can be negatively impacted by stress of any kind. Autophagy expression of several genes (AMPKα1, mTOR, Atg16L1, and Atg7) were upregulated in breast muscle of broilers exhibiting a high FE phenotype compared to broilers exhibiting a low FE phenotype. This suggests that part of the cellular basis of FE may hinge on the ability of the cell to maintain optimal functionality by a more active endogenous repair system offered by the autophagy pathway. Furthermore, using two experimental Japanese quail lines selected for low feed efficiency (LFE) or high feed efficiency (HFE) it was found that the expression of several autophagy-related genes were increased in the low efficient line of 30 wk old quail with expression in the HFE group showing significant increases in only two genes (mTOR and Atg3). This suggests that there may be a difference in the pathway between poultry species as well as a possible age effect (autophagy is known to have a decrease in activity as an organism ages). This increase in mTOR would essentially block the autophagy pathway which is seen by the decrease in expression of all subsequent autophagy genes. Since autophagy has been shown to play a key role in stress (such as starvation), as well as fat metabolism, the differential expression of autophagy-related genes between the lines indicated that these birds, as well as future studies with stress resistant and susceptible quail, would be a very useful model to study the differences between LFE and HFE animals giving us greater insight into autophagy and feed efficiency.

Keywords: Autophagy, feed efficiency, broiler, quail, low feed efficiency, high efficiency, parent, second generation

5.2 INTRODUCTION

Autophagy has been shown to play an important role in elimination of damaged proteins and organelles from cells. Recently, Rolf and colleagues reported that autophagy may play a role in feed efficiency (determined by residual feed intake- RFI) in Angus cattle (Rolf et al., 2012). In another model, Morck and colleague indicated that the long-term starvation seen in *C. elegans* mutants that are "feeding-defective," activates autophagy, and leads to depletion of fat deposits, small cell size, and small body size (Morck et al., 2006). These experiments as well as others dealing with starvation, show that the autophagy pathway is involved in how these organisms cope with a certain feeding regime. The question to which this experiment hopes to address is: to what degree does autophagy affect the high or low feed efficiency trait, and could this be a potential aid in helping to solve the feed efficiency problem in the poultry industry.

Previous studies have also been conducted at the cellular level in regards to the mechanistics of feed efficiency. One of these studies aimed to determine the relationship between feed efficiency and mitochondrial function and biochemistry (Bottje et al., 2002). Due to the fact that mitochondria are the "powerhouse" of the cell, and 90% of the energy for the cell is produced them, it was proposed that some variations seen in broiler growth performance as well as phenotypic expression of feed efficiency (Emmerson, 1997) may be due to or related to mitochondrial function (Bottje et al., 2002). This group studied mitochondrial function, respiratory chain activity, and electron leak and found that they are all linked to feed efficiency in broiler breeder males that have either the high or low feed efficient trait (Bottje et al., 2002). Members of the same group published another study to assess proton leak kinetics (proton conductance) in breast muscle mitochondria from broiler breeder with the same high or low feed efficient trait (Bottje, et al., 2009). Proton motive force that develops from proton pumping

across the inner mitochondrial membrane is used to drive ATP synthesis in the electron transport chain during oxidative phosphorylation. It is also possible that these protons may flow back into the mitochondria and effectively short circuit the coupling of ATP synthesis in a process called proton leak (Brand, 1995). It has been show that this leak represents up to 30% of oxygen consumption in isolated liver cells and up to 50% of oxygen use in perfused muscle (Brand, 1990; Rolfe and Brand, 1996), therefore, proton leak could contribute as much as 25% of total basal metabolic rate of an animal (Rolfe and Brand, 1996, 1997; Rolfe et al., 1999). Because of this, the previously mentioned group hypothesized that proton leak could be involved in the phenotypic expression of FE in animals (Bottje et al., 2009). This study showed that subtle differences existed in proton leak kinetics in mitochondria from broilers exhibiting the high or low feed efficient trait (Bottje et al., 2009). They showed a lower activity of respiratory complexes in the low FE broiler which they believe as possibly from an increase in protein oxidation (Bottje et al., 2008). A higher ROS may mean more damage to cells which would lead to an increase in the autophagy pathway, specifically mitophagy, to clear out these damaged and dysfunctional mitochondria to try and save the cell from further damage. With studies such as these, it was suggested to view autophagy as another possible underlying factor in this trait and to determine whether or not it is beneficial to commercial animals.

An animal that produces either greater body mass with the same feed intake or the same body mass with less feed intake would be considered more efficient than its contemporaries. That having been said, birds that are considered to have better feed efficiency typically have a lower proportion of feed intake to body mass. Using this definition, one can determine the relative efficiency of several species of agriculturally important animals including poultry. Willems and colleagues have stated that generally accepted feed costs represent about 70% of the

cost of poultry production making a bird's ability to use feed efficiently very important (Willems et al., 2013). Changing methods in lighting, temperature, and nutrition with birds have all been ways the industry has tried to manage feed efficiency in meat producing poultry. Perhaps the most recent area, as well as the one most likely to produce a lasting effect on the industry, is genetic selection for feed efficiency. Originally bred for body weight gain, significantly larger birds were produced but, as feed costs began to increase, it became clear that, in order to be profitable, selection needed to include other traits (Willems et al., 2013). While there are a multitude of ways for measuring feed efficiency, the two most often used are feed conversion ratio (FCR) and residual feed intake (RFI).

FCR can be defined as the amount of feed consumed per unit of weight gain, and is a composite trait of starting and ending body weight and feed intake (Skinner-Noble and Teeter, 2003). RFI, on the other hand, is defined as the difference between actual and predicted feed intake based on the regression of requirements for production and body weight maintenance (Van Der Werf, 2004). Although these two methods are by far the most widely used and understood, there are alternative methods for measuring feed efficiency. Some alternatives include: residual maintenance energy (RME_m) that, unlike RFI or FCR, aims to measure energetic efficiency without being compounded by feed intake. Although each has its own advantages and disadvantages, usage of FCR, which can be used on a large scale, and RFI, which tends to be used on a smaller scale, are common, making these the top two choices for measuring feed efficiency that are widely accepted. RME_m is also becoming more favorable as it avoids confounding environmental effects and allows measurement standardization (Romero et al., 2011).

The cost of feed represents about 70% of the cost of production broilers, but feed utilization efficiency has not kept up with the growth rate of broilers (Aggrey et al., 2010). Due to biofuel policies and a growing global demand for animal protein, feed, fuel, and fertilizer costs have been on a steady rise leading to intensified focus on the development of selection strategies for the improvement of FE in poultry and livestock production (Steinfeld et al., 2006). These increases in feed cost have driven an increase in live production costs which, in turn, decreases profitability for the industry. To alleviate this problem and make the industry more profitable, FCR has been implemented to make these improvements possible. As stated earlier, feed intake is a heritable trait and, as Pym and colleagues demonstrated over a decade ago, genetic studies for FCR show that it could be improved by selection on growth (Pym et al., 1990). Studies such as this were performed because efficiency deteriorates overtime because the broiler has an ever-increasing body mass to maintain (Leeson, 2009). Genetic potential drove much of the change in feed efficiency values from 2.2 to 1.75 today, but due to changes in the industry, (range of bird weights, males and females grown separately) there is a range of dietary specifications and feeding programs and because of this, the poultry meat business is questioning the usefulness of classical feed efficiency (Leeson, 2009). The trend now is to consider energy efficiency rather than feed efficiency and because many are searching for new and improved ways of developing feed efficient/energy efficient animals by studying genes and pathways that may be of importance to this trait. RMEm, defined as the residual of estimated maintenance requirement as a function of energy intake, is another way of looking at efficiency in production birds. In the search for a more efficient bird, the RME_m methodology has been evaluated and applied to multiple studies as a refinement to the traditional measure of FCR (Romero et al., 2011). It is possible that selection for specific components of energy efficiency, such as utilizing mechanism

of the autophagy pathway, may have a positive effect on the productivity of broilers and broiler breeders (Romero et al., 2011). Feed efficiency is very important to the industry in these animals, using poultry as a model, we studied common genes involved in autophagy and compared how this pathway may be involved in birds that are selected for either high feed efficiency (HFE) or low feed efficiency (LFE).

Based on these earlier studies, we hypothesize that autophagy will be up-regulated in the LFE animals. To study this hypothesis we used muscle from:

a. Low and high feed efficient Broilers (LFE, HFE, respectively)

b. Low and high feed efficient Japanese Quail (LFE, HFE, respectively)

5.3 MATERIALS AND METHODS

5.3.1 INITITAL STUDIES

Japanese quail selected for susceptibility or resistance to restraint stress (Satterlee et al., 1988) were used to characterize autophagy genes in 11 tissues under thermoneutral conditions (Chapter 3).

5.3.2 FEED EFFICIENCY STUDIES

Three separate experiments were performed using twelve broilers within a single male line selected for either high feed efficiency (6 birds) or low feed efficiency (6 birds) characteristics; twelve male Japanese quail (*Coturnix coturnix Japonica*) selected for high or low feed efficiency (6 HFE, 6 LFE) from the parent line and; twelve (6 HFE, 6 LFE) from the second generation. Birds were used to compare autophagy in feed efficiency. Breast muscle tissue from

the broilers and Japanese quail parent line (30 wks) and second generation (6 wks) were collected. For all studies, tissues were flash frozen and stored at -80°C.

Feed efficiency, gain, and feed intake were calculated for broilers and Japanese quail and are shown in Tables 5.1 and 5.2, respectively.

5.3.3 QUANTITATIVE REAL-TIME PCR

Real-Time qPCR data was derived from Lassiter, 2015. Briefly, total RNA was extracted from chicken and quail tissues by Trizol reagent (catalog #15596018, Life Technologies) according to manufacturer's recommendations, DNAse treated and reverse transcribed (catalog #95048-100, Quanta Biosciences). RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (catalog #4312074, Life Technologies). Oligonucleotide primers were used for avian autophagy-related genes determined as previously described in Table 3.1 of Chapter 3. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

5.3.4 STATISTICAL ANALYSES

Data were analyzed by one-factor ANOVA with genotype as classification variable and Student's T-test was ran as a comparison. If ANOVA revealed significant effects, the means were

compared by Tukey's multiple range test using the Graph Pad Prism version 6.00 for Windows, Graph Pad Software, La Jolla California USA. Differences were considered significant at $P \leq 0.05$.

5.4 RESULTS AND DISCUSSION

5.4.1 GENE EXPRESSION IN LOW AND HIGH FEED EFFICIENT BROILER BIRDS

The body weight gain, feed intake and feed efficiency data for broilers in Exp. 1 is shown in Table 5.1. The high FE phenotype broilers exhibited greater weight gain on the same amount of feed as the low FE phenotype broiler, resulting in higher efficiency. In experiment 2, 6 wk old (second generation of selection) high FE Japanese Quail also exhibited greater body weight gain on the same amount of feed compared to the low FE phenotype quail (Table 5.2). However, first generation 30 wk old high FE quail males exhibited greater gain as well as lower feed intake (FI) compared to the low FE phenotype (Table 5.3).

In experiment 1, broilers selected for the LFE or HFE trait were used. Gene expression was measured on six autophagy related genes (Atg3, Atg16L1, Atg7, mTOR, AMPKα1, and Beclin1). Results show that there was increased expression of Atg16L1, Atg7, AMPKα1, and mTOR in the HFE compared to LFE broilers birds (Figure 5.1). Usually an increase in mTOR would shut down the autophagy pathway by signaling that the cell has had its energy demands met and that processes such as cell synthesis and maturations can proceed. Due to the fact that mRNA is being measured here, it is not clear what may be happening post-translationally. It is not uncommon to see expression of both mTOR and autophagy genes in mRNA, as well as with protein. In this experiment, we see that two genes (Atg7, Atg16L1) significantly expressed are genes towards the end of the autophagy pathway. This could mean that autophagy signaling travels a cascade-type route in which these latter signals may still be spiked, finishing an earlier signal. The autophagy pathway may also be mTOR independent, meaning that if AMPKα1 levels are

increased then Beclin1 levels would be as well to start the signaling cascade. Lastly, many of the genes involved in the autophagy pathway are parts of complexes. Atg7 and Atg16L1 happen to be two of these genes, the former involved as an E1-like enzyme initiating complexes to form and the latter is part of a complex where it is an integral gene that, if not present, can halt the autophagy pathway from continuing further. In figure 5.1, one can see the significance of expression in the HFE line where the chart shows the fold difference of LFE for all genes measured.

5.4.2 GENE EXPRESSION IN LOW AND HIGH FEED EFFICIENT JAPANESE QUAIL

Experiment 2 was conducted in breast muscle tissue of male Japanese quail phenotyped for high and low FE. The parent line was sampled and seven genes (AMPKα1, Beclin1, LC3B, mTOR, Atg3, Atg16L1, and Atg7) important in the autophagy pathway were selected for expression analysis. When AMPKα1 is decreased, mTOR is active, leaving it free for cell synthesis and other energy consuming processes, and the autophagy pathway is inhibited (Chapter 5). This seems to be the case with the parent line where all other autophagy genes, with the exception of Atg3, having a decreased expression in the HFE line (Figure 5.3). Though rare, the autophagy pathway can still be active with an increase in mTOR, especially when looking at mRNA levels. It has been seen that Beclin1 mRNA expression significantly increases with age in murine muscle models (Wohlgemuth et al., 2010). In this older line of quail, there is no significant difference in Beclin1 expression, although it is expressed higher in the LFE group than the HFE group. It is possible that this means the LFE group has a more active autophagy mechanism, possibly due to compensation for not being able to efficiently convert feed into usable energy as well as their HFE counterparts or that there is a possible defect in the autophagy pathway of these animals. There has also been evidence of an increase in autophagy protein expression in HFE versus LFE in a global protein expression experiment (unpublished observations).

Experiment 3 was conducted in breast muscle of second generation (6 wks) quail phenotyped for high and low feed efficiency. Results in the second generation show very little differences between expression of autophagic genes. Not much change can be seen with most autophagy genes in the second generation, showing that autophagy is active at this point (Figures 5.3 and 5.2).

Looking at the expression of Atg7 shows no change in the second generation between HFE and LFE, but a change could be seen in the parent line (Figure 6.1). A previous study showed that Atg7 mRNA expression did not change with age in rat muscle but expression was significantly elevated in old calorie restricted rats compared to old ab libitum fed rats (Wohlgemuth et al., 2010). LC3B expression, essential for expansion of the early autophagosome, showed an increase in expression in LFE in the second generation although the difference did not reach significance (Figure 6.1). This is similar to earlier results in rats that showed no change in mRNA levels in muscle of old rats compared to young rats, with a tendency to be lower in the older rats similar to what is seen in our parent line (Wohlgemuth et al., 2010). It seems as though gene expression for the upstream regulators are similar to what one would expect in normal autophagy. The second generation birds have no significant difference in the amount of AMPKα1 mRNA expression but the relation can be seen by looking at mTOR (Figure 6.1). The expression of mTOR shows a decrease in the HFE line of the second generation where the AMPKα1 is increased. This is an indicator that the autophagy pathway is activated and that the HFE line of the second generation is able to utilize the autophagy pathway efficiently. Although upstream regulators were upregulated or unchanged, the mRNA expression of LC3B that regulates this pathway downstream seemed to decrease.

It seems as though gene expression for the upstream regulator, Beclin1, is similar to what we would expect in normal autophagy. Although Beclin1 expression was unchanged, the mRNA expression of LC3B that regulate this pathway downstream also did not change (Figure 5.2). This could suggest a possibility that this pathway runs in a cascade-like fashion and these genes, at the time tissues were collected, had not yet received an upstream signal to be activated. It could also point to a possible impairment of autophagic degradation. Further studies, including protein measurement and testing other downstream regulatory proteins such as the receptor for chaperone-mediated autophagy LAMP-2 (Lysosome-associated membrane protein 2) must be performed to further define the current data.

Feed efficiency has been a major criterion in defining broiler performance influencing everything from diet energy level to health management (Leeson, 2009). Broilers use feed for two reasons: growth and maintenance with young birds using more for growth than maintenance having a high efficiency which dwindles over-time due to their ever-increasing body mass (Leeson, 2009). Interest in feed efficiency has always been of concern but in recent years, with a growing world population and increased cost of feed product, much emphasis has been placed on better understanding its underlying mechanisms and how to continue to improve feed efficiency numbers. Although feed efficiency has been improved over the years firstly, through changes in management (optimizing temperatures, lighting, and bird densities) and secondly, research such as nutrition and genetic selection for feed efficiency, there are still more avenues through which to explore improvement. Nutrition has been at the forefront for a long time, however genetics may be the next front runner in this line of research and autophagy in particular may have a larger role than most may have thought.

Data from this experiment shows that the autophagy pathway is expressed in high and low feed efficient broilers and quail. The broilers exhibited a significant increase in expression in nearly all genes of the HFE line. This could explain why these animals are more efficient at converting feed into usable energy than their LFE counterparts. The autophagy pathway is involved in the clearance of damaged cytosolic components, in turn making the cell better able to cope during times of stress while providing elements from the lysate of these dissolved components to aid in providing the cell with energy. The quail line showed expression of this pathway, but showed instead the activation of mTOR. Once activated, mTOR can inhibit the autophagy pathway, as shown in Figure 5.2, by the decrease in expression of autophagic genes. This may have happened in these birds because the autophagy pathway may have already been activated and performed its function of removing damaged cellular material. It should be noted that one can come to this conclusion because LC3B levels are slightly increased, though did not reach significance, in the HFE line (Figure 5.2). This means that autophagy was activated and autophagosomes formed. What is shown here may be the cells energy levels restored and the stressor "eliminated" enough that the cells can inhibit the autophagy pathway and activate the mTOR pathway which, in turn, activates energy-consuming processes such as cell synthesis and growth. The second generation showed that autophagy gene expression is activated in most genes studied, but no delineation could be made as to what "step" the pathway was performing in the breast muscle of these animals.

Autophagy is one of several different intracellular proteolytic systems that contributes to protein degradation. Because it is part of the lysosomal system, autophagy plays a role in conditions that require extensive cellular remolding such as cell differentiation, embryogenesis, and complete cellular destruction that occurs in some forms of cell death (Shintani et al., 2004;

Massey et al., 2006; Cuervo, 2004). It has been shown that feed restriction on chickens reduces incidence of sudden death syndrome (SDS) in poultry, and can attenuate the age-related impairment of autophagy, which is a possible mechanism by which it attenuates age-related cellular damage and death (Bowes et al., 1988; Wohlgemuth et al., 2010). The role of autophagy and feed efficiency, can be appreciated by its tight linked to feeding as some of the first studies identifying the pathway were performed in starvation models. Identifying how feed restriction acts in parallel to high and low efficient birds involving the autophagy pathway may be an important step in delineating some mechanistic features of this pathway and how, through understanding gene expression, it can aid in improving feed efficiency.

5.6 CONCLUSION

In conclusion, FE is a topic of uppermost importance in all aspects of animal production. The poultry industry has come a long way since the inception of the broiler and broiler breeder with many still searching for ways to improve upon these models. With a growing demand for high quality animal protein coupled with the cost of feed for poultry production, making a bird's ability to use feed efficiently has become a topic of interest the world over. The results found in this study aim to shed light on one of many pathways, and factors affecting this pathway, that may be involved in whether or not a bird is efficient.

The autophagy pathway is indeed activated in both generations of Japanese quail as well as the broilers studied. While the original objective in quail was to determine whether or not autophagy played a role in generational effects of feed efficiency, it became clear that too many factors could have had a role in the data received so all experiments were evaluated individually. However, this has now opened a new investigation into a possible age effect autophagy has on these lines as well as possible effects that different diets have on these animals. Future studies will

be aimed at delineating at what age the autophagy pathway is most efficient in these birds. Other studies will include observing differences in the pathway due to different feed (beginner, grower, and finisher) as well as continuing to look at a possible generational effect, comparing birds of the same age with the same diet over multiple age groups to determine if there is an optimal age and diet associated with an increase or decrease in autophagy activation.

More data needs to be collected to identify underlying mechanisms which play a potentially larger role in this trait. With further research, autophagy may yet become a key factor in determining and producing efficiency in production animals.

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Table 5.1

Broilers	Gain	FI	FE
High FE	629.3 ± 20.7 *	973.3 ± 30.5	0.645 ± 0.005 *
Low FE	462 ± 15.5	999 ± 37.8	0.463 ± 0.006

Table 5.1 Feed efficiency, feed intake, and gain data for high and low FE male broilers. Feed efficiency was measured as feed intake/weight gain (g/2wks). Mean ± SE of 6 observations and (*) represents P<0.05.

Table 5.2

Quail Second Gen.	Gain	FI	FE
High FE	148.8 ± 17.6	264.5 ± 11.5	0.461 ± 0.012 *
Low FE	91 ± 2.12 *	264.17 ± 5.67 *	0.345 ± 0.010 *

Table 5.2 Feed efficiency, feed intake, and gain data for high and low FE male Japanese quail. Feed efficiency was measured as feed intake/weight gain (g/2wks). Mean ± SE of 6 observations and (*) represents P<0.05

Table 5.3

Quail Parent	Gain	FI	FE
High FE	119.17 ± 3.12 *	242.17 ± 7.58 *	0.493 ± 0.010 *
Low FE	106 ± 4.36	269.17 ± 14.6	0.396 ± 0.010

Table 5.3 Feed efficiency, feed intake, and gain data for high and low FE male Japanese quail. Feed efficiency was measured as feed intake/weight gain (g/2wks). Mean ± SE of 6 observations and (*) represents P<0.05

Figure 5.1

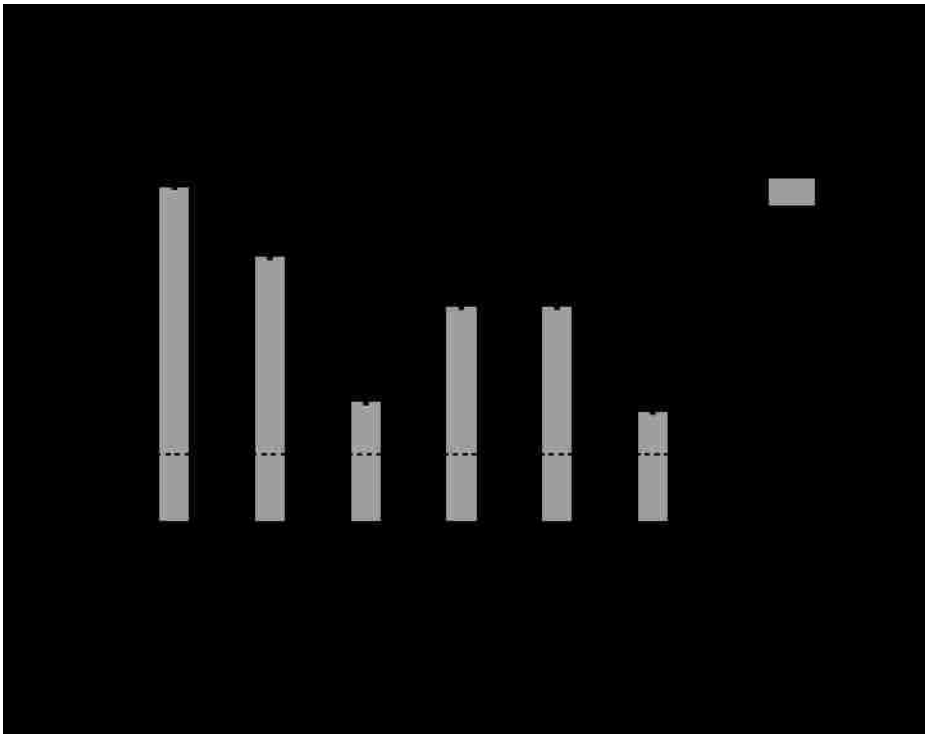

Figure 5.1 Expression of autophagic genes as a fold difference of FE in broiler line, including AMPKα1 (AMP activated protein kinase α1), mTOR (mechanistic target of rapamycin), Beclin1 (Bcl-2 interacting protein), Atg16L1, Atg7, and Atg3. Bars represent mean $\pm$ SE (n=6) and (*) represents $P<0.05$

Figure 5.2

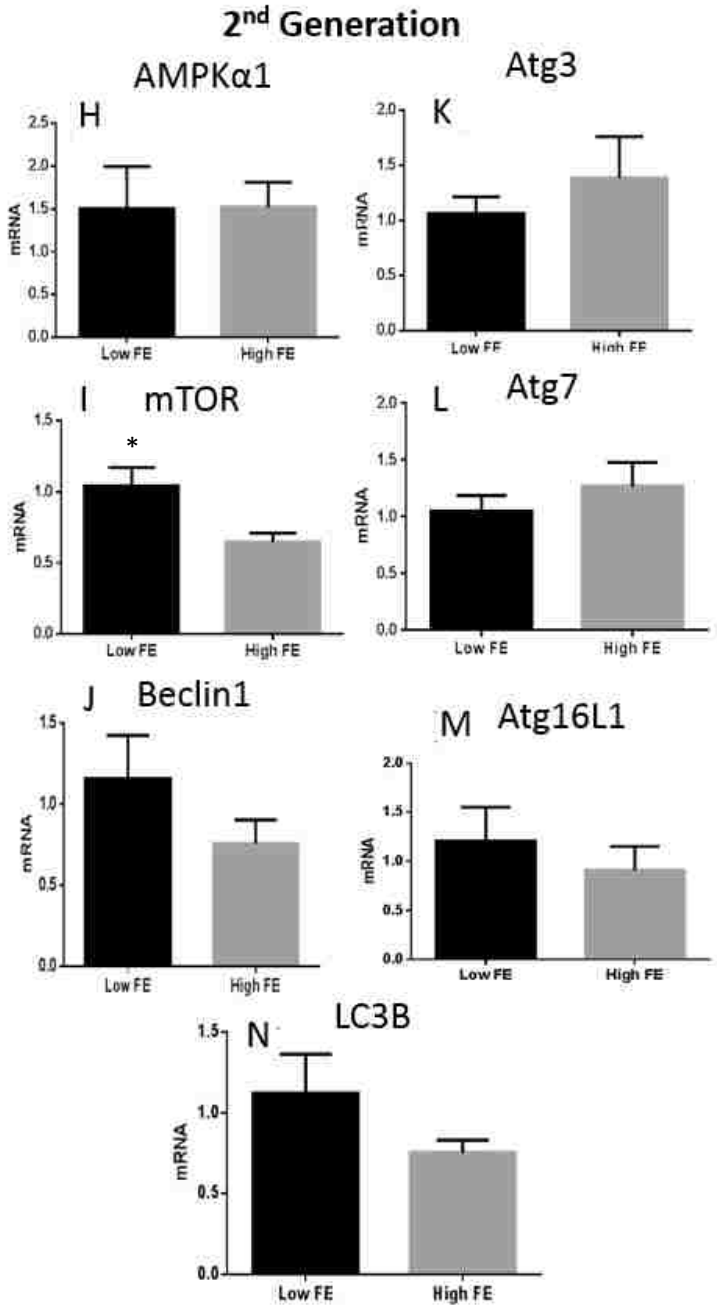

Figure 5.2 H-N: Autophagy gene expression in second generation Japanese quail. Data shows a significant increase mTOR of the LFE line with increases in Beclin1, Atg16L1, and LC3B although these did not reach significance (H-N). Bars represent mean $\pm$ SE (n=6) and (*) represents $P<0.05$.

Figure 5.3

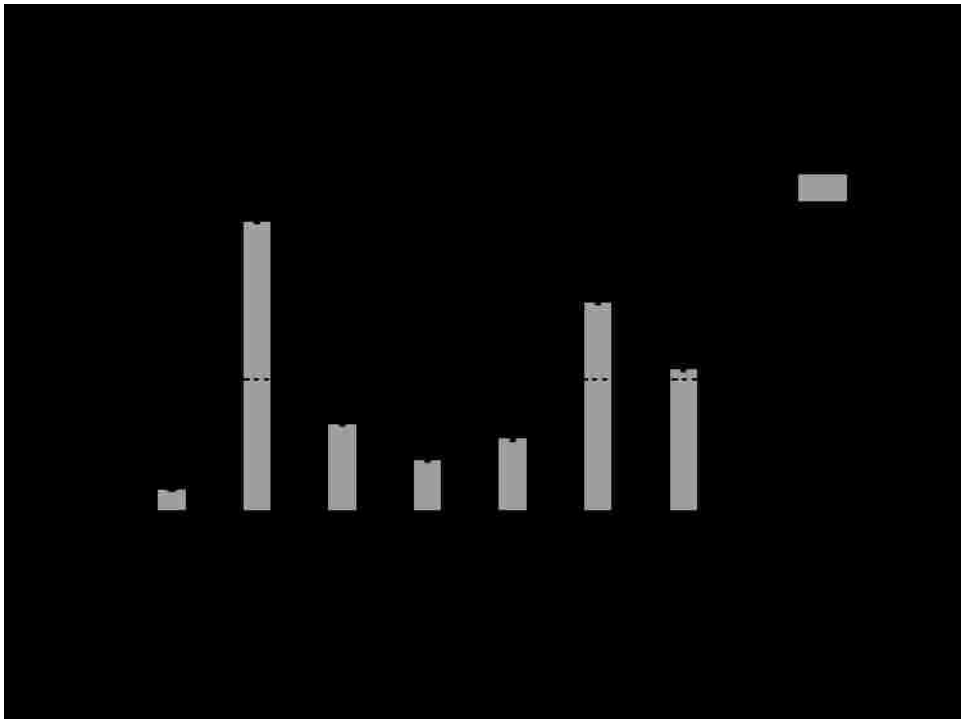

Figure 5.3 Expression of autophagic genes in Japanese quail line exhibiting HFE or LFE phenotypes. Values are shown as a fold difference of high compared to low FE. Bars represent mean $\pm$ SE (n=6) and (*) represents $P<0.05$.

CHAPTER 6:
Generational Effect of autophagy on Parent and Second Generation Feed Efficient Quail

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6.1 ABSTRACT

The primary objective of this study was to observe differences in autophagic genes, if any, between a high and low feed efficient (HFE, LFE, respectively) quail parent line and its second generation. Using twelve Japanese quail (*Coturnix coturnix Japonica*), mRNA of autophagy gene expression was measured. Data revealed a significant increase in autophagy genes of the parent line in Atg3, and mTOR with decreases in all other genes within the HFE group. The second generation yielded interesting results showing a significant increase in mTOR of the LFE line with increases in Beclin1, Atg16L1, and LC3B although these did not reach significance. In conclusion, there may be an age effect happening between these lines as well as a difference in feed that may have affected the outcome of this study. Further research will need to be performed in order to remove these underlying factors.

6.2 INTRODUCTION

The cost of feed represents about 70% of the cost of production broilers, but feed utilization efficiency has not kept up with the growth rate of broilers (Aggrey et al., 2010). Due to biofuel policies and a growing global demand for animal protein, feed, fuel, and fertilizer costs have been on a steady rise leading to intensified focus on the development of selection strategies for the improvement of FE in poultry and livestock production (Steinfeld et al., 2006). With autophagy playing an important role in cell survival and turning damaged organelles and cellular components into usable energy, it is clear why it would be studied for its use in energy efficiency. This highly conserved cellular mechanism is responsible for the degradation and recycling of damaged organelles, and has been shown to play critical roles during overall development of the organism as well as degradation. Using feeding-type studies performed in cattle (Rolf et al., 2012) and *Caenorhabditis elegans* (Mörck et al., 2006), focus was placed on how this pathway may contribute to the feed efficiency trait between generational lines of HFE and LFE Japanese quail. In addition, previous studies from this laboratory have showed a lower activity of respiratory complexes in the low FE broiler which is believed to be from a possible increase in protein oxidation (Bottje et al., 2009). A higher ROS may mean more cellular damage which could lead to an increase in the autophagy pathway, specifically mitophagy, to clear out damaged and cells and try to save the cell from further damage. After observing the results from this study, it was decided to research autophagy as another possible underlying factor in the feed efficient trait and to determine whether or not there is a generational effect.

In addition, it has been noted in invertebrates and higher organisms that a decline in autophagy occurs with age as well showing a decrease in chaperone-mediated autophagy (Wohlgemuth et al., 2010). It has been shown that levels of LAMP2a were significantly lower in

lysosomes from old rats and later delineated that it is the number of binding sites that declined with age, but binding affinity was not altered (Cuervo, et al., 2000). Looking at these factors could be essential to understanding how to create a more feed efficient/energy efficient bird, satisfying the growing demand for food while simultaneously decreasing costs for industry.

6.3 OBJECTIVE

Therefore the objective of this study was to determine whether or not autophagy has a generational effect on the parent and second generation HFE and LFE Japanese quail lines.

6.4 MATERIALS AND METHODS

6.4.1 ANIMALS

Twelve male Japanese quail (*Coturnix coturnix Japonica*) selected for high or low feed efficiency (6 HFE, 6 LFE) were used to compare autophagy in feed efficiency. A parent line (30wks of age) was compared against the second generation (6wks of age). Breast muscle tissue from the Japanese quail parent and second generation lines were collected. For all studies, tissues were flash frozen and stored at -80°C.

Feed efficiency, gain, and feed intake were calculated for and Japanese quail and are shown in Tables 5.2 and 6.1 for the parent and second generation, respectively.

6.4.2 QUANTITATIVE REAL-TIME PCR

Real-Time qPCR data was performed as previously described (Lassiter, 2015). Briefly, total RNA was extracted from chicken and quail tissues by Trizol reagent (catalog #15596018, Life Technologies) according to manufacturer's recommendations, DNAse treated and reverse transcribed (catalog #95048-100, Quanta Biosciences). RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for

each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (catalog #4312074, Life Technologies). Oligonucleotide primers were used for avian autophagy-related genes determined as previously described in Table 3.1 of Chapter 3. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. Relative expressions of target genes were determined by the 2–ΔΔCt method (Schmittgen and Livak, 2008).

6.4.3 STATISTICAL ANALYSES

Data were analyzed by two-factor ANOVA with genotype as classification variables. If ANOVA revealed significant effects, the means were compared by Tukey's multiple range test using the Graph Pad Prism version 6.00 for Windows, Graph Pad Software, La Jolla California USA. Differences were considered significant at $P<0.05$.

6.5 RESULTS

This experiment was conducted in male Japanese quail phenotyped for high and low FE (Table 6.1). The parent line, and second generation were sampled and seven genes (AMPKα1, Beclin1, LC3B, mTOR, Atg3, Atg16L1, and Atg7) important in the autophagy pathway were selected for expression analysis. Results showed a decrease in AMPKα1 expression of HFE in the as well as a significant increase in mTOR and Atg3. When AMPKα1 is decreased, mTOR is active, leaving it free for cell synthesis and other energy consuming processes, and the autophagy pathway is inhibited (Chapter 5). This seems to be the case with the parent line where all other

autophagy genes, with the exception of Atg3, having a decreased expression in the HFE line (Figures 5.3 and 5.4). Though rare, the autophagy pathway can still be active with an increase in mTOR, especially when looking at mRNA levels. It has been seen that Beclin1 mRNA expression significantly increases with age in murine muscle models (Wohlgemuth et al., 2010). In this older line of quail, there is no significant difference in Beclin1 expression, although it is expressed higher in the LFE group than the HFE group. It is possible that this means the LFE group has a more active autophagy mechanism, possibly due to compensation for not being able to efficiently convert feed into usable energy as well as their HFE counterparts.

Looking at the expression of Atg7 shows no change in the second generation between HFE and LFE, but a change could be seen in the parent line (Figure 6.1). A previous study showed that Atg7 mRNA expression did not change with age in rat muscle but expression was significantly elevated in old calorie restricted rats compared to old ab libitum fed rats (Wohlgemuth et al., 2010). LC3B expression, essential for expansion of the early autophagosome, showed an increase in expression in LFE in the second generation although the difference did not reach significance (Figure 6.1). This is similar to earlier results in rats that showed no change in mRNA levels in muscle of old rats compared to young rats, with a tendency to be lower in the older rats similar to what is seen in our parent line (Wohlgemuth et al., 2010). It seems as though gene expression for the upstream regulators are similar to what one would expect in normal autophagy. The second generation birds have no significant difference in the amount of AMPKα1 mRNA expression but the relation can be seen by looking at mTOR (Figure 6.1). The expression of mTOR shows a decrease in the HFE line of the second generation where the AMPKα1 is increased. This is an indicator that the autophagy pathway is activated and that the HFE line of the second generation is able to utilize the autophagy pathway efficiently. Although upstream regulators were

upregulated or unchanged, the mRNA expression of LC3B that regulates this pathway downstream seemed to decrease. This could suggest a possibility that this pathway runs in a cascade-like fashion and these genes, at the time tissues were collected, had not yet received an upstream signal to be activated. It could also point to a possible impairment of autophagic degradation.

Further studies, including protein measurement and testing other downstream regulatory proteins such as the receptor for chaperone-mediated autophagy LAMP-2 (Lysosome-associated membrane protein 2) as well as feed evaluation and comparison must be performed to further define the current data. As it currently shows, there seems to be an age effect happening between these two lines. Only further research into the effects of autophagy on different age groups of these quail lines can help clarify how the autophagy pathway is affecting the feed efficiently trait.

6.6 DISCUSSION

Autophagy, which becomes active when the cells energy stores decline, has gained much interest from groups studying cancer to aging. This pathway seems to be part of many cellular processes as well as central to many diseases. Because it is part of the lysosomal system, autophagy plays a role in conditions that require extensive cellular remolding such as cell differentiation, embryogenesis, and complete cellular destruction that occurs in some forms of cell death (Shintani et al., 2004; Massey et al., 2006; Cuervo, 2004). The role of this catabolic pathway and feed efficiency, can be appreciated by its tight linked to feeding as some of the first studies identifying the pathway were performed in starvation models. Identifying how feed restriction acts in parallel to high and low efficient birds involving the autophagy pathway may be an important step in delineating some mechanistic features of this pathway and how, through understanding gene expression, it can aid in improving feed efficiency.

Due to the fact that animal agriculture is facing substantial challenges, including a steep projected increase in demand and the need to adapt to changing environmental conditions, identifying factors that could aid in understanding feed efficiency has become an important research topic in the agricultural field. Due to a predicted increase in world population to 9 and 10 billion, United Nations FAO estimates that by 2050 there will be a 73% increase in meat and egg consumption and a 58% increase in dairy consumption over 2011 levels (Alexandratos et al., 2006). Reducing the cost of animal protein production is essential to achieve a sustainable, affordable, and secure animal protein supply. To do so, mechanistic understanding (at molecular and cellular levels) of heat stress and feed efficiency response are necessary and of uppermost interest.

This study aimed to begin an understanding of autophagy and how it effects feed efficiency over generations. It was found that the parent line had already utilized the autophagy pathway due to the increase in expression levels of mTOR with the concomitant decreased expression in all other autophagy genes with the exception of LC3B. Expression of this gene led to the possibility that autophagy had occurred earlier in the cells of this tissue, but had since been inhibited due to clearance of the damaged material and alleviation of cellular damage. In the second generation birds it seems as though the HFE line was currently utilizing autophagy by showing an increase in AMPKα1 compared to mTOR under both LFE and HFE conditions. This shows that the autophagy pathway is activated and, although mTOR is expressed, it is not significant with respect to the amount AMPKα1. It should also be noted that because this is mRNA, there will be levels of mTOR expressed at the same time levels of AMPKα1 and other autophagy genes are expressed. Future work will need to utilize protein data to better understand upregulation or downregulation

of this pathway under these conditions. With a better mechanistic understanding, autophagy may yet prove to be an important cellular determinant of this desirable trait.

6.7 CONCLUSION

In conclusion, this study reveals that the autophagy pathway is indeed activated in both generations of Japanese quail. While the original objective was to determine whether or not autophagy played a role in generational effects of feed efficiency, it became clear that too many factors could have had a role in the data received. However, this has now opened a new investigation into a possible age effect autophagy has on these lines as well as possible effects that different diets have on these animals. Future studies will be aimed at delineating at what age the autophagy pathway is most efficient in these birds. Other studies will include observing differences in the pathway due to different feed (beginner, grower, and finisher) as well as continuing to look at a possible generational effect, comparing birds of the same age with the same diet over multiple age groups to determine if there is an optimal age and diet associated with an increase or decrease in autophagy activation.

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Figure 6.1

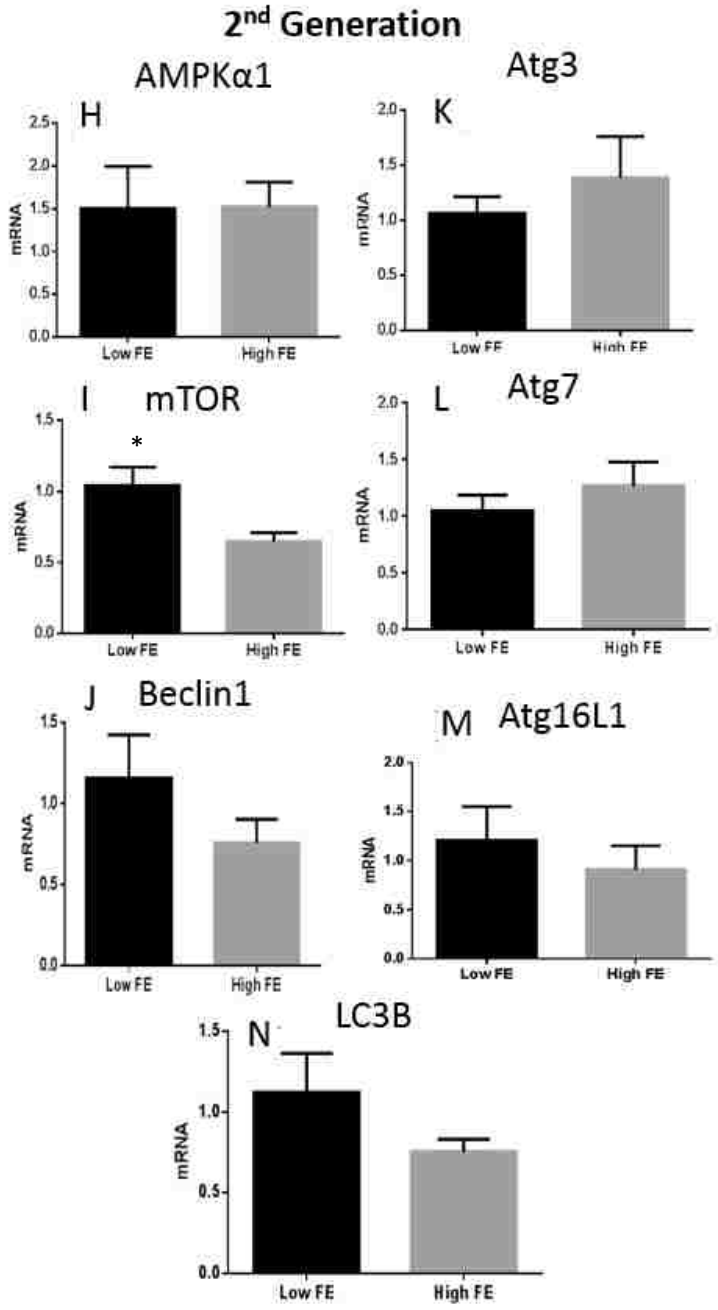

Figure 6.1 H-N: Autophagy gene expression in second generation Japanese quail. Data shows a significant increase mTOR of the LFE line with increases in Beclin1, Atg16L1, and LC3B although these did not reach significance (H-N). Bars represent mean $\pm$ SE (n=6) and (*) represents $P<0.05$.

7. CONCLUSION

In this dissertation, the expression of autophagy-related genes were determined in two avian species maintained under different physiological, genetic, and environmental conditions. The autophagy pathway is a very important pathway linked to many different conditions, such as starvation and stress which could impact avian species development and function. Despite this importance, very little information is available on the autophagy pathway in birds or in how various types of stress or management conditions could affect the autophagy pathway. The overall goal of research in this dissertation was to characterize the autophagy pathway in jungle fowl, to determine the effect of different types of stress on autophagy, and finally to determine if autophagy may contribute to the phenotypic expression of feed efficiency in broilers.

We found that the autophagy pathway is tissue, gender, and genotype specific and this pathway is indeed found in avian species. This characterization can open new research avenues to understand the regulation and the roles of autophagy in avian species maintained under physiological and pathophysiological conditions. Further studies are warranted to identify and characterize genes involved in autophagosome maturation in birds. This study gives insight into autophagy in avian species as well as into the underlying mechanisms that may ultimately help to develop new management tools for poultry production improvement. The quail lines may also be a useful model to study stress-related disorders in humans and, from this study, develop further therapeutic strategies.

Due to the aid or progress autophagy lends to certain diseases in human and murine models, we decided to see whether to not this pathway played any role in heat stress on birds. We saw that high stress quail showed an upregulation of two key genes involved in the final stages of

autophagy, Atg16L1 and LC3B. These genes are indicative of autophagosomes formation and the degradation of damaged particles. There was also an upregulation of autophagy genes in the low stress line with a possibility that these genes, which are expressed at a higher level in the low stress line, could be involved in the development of this trait. Reducing the impact of climate change and cost of animal protein production is essential to achieve a sustainable, affordable, and secure animal protein supply. To see this reduction come to fruition, a better understanding of the mechanistics underlying the causative effects of heat stress must be determined. Autophagy, upregulated during many different types of stress, is a pathway which may contribute greatly to alleviating this issue. Further studies will need to be performed to determine these exact mechanisms as well as studies to determine the roles of the genes involved in autophagy during heat stress and whether or not they have potential for alleviating or preventing heat stress in animal agriculture.

Our next research topic of interest was to perform experiments on high and low feed efficiency in male broilers and male Japanese quail. The high feed efficient line of broilers seemed to have an increase in overall autophagy genes expressed. This, again, may be due to the genotype of these birds with the high feed efficiency birds able to produce new forms of energy from nutrients released via the autophagy pathway breaking down damaged organelles. High and low feed efficient Japanese quail were used as well where we see a possible age effect occurring with the expression of autophagic genes in parent (30 wks) versus second generation (6 wks). It seems only two genes were significantly higher in the high feed efficient line in the parent group whereas no significant difference was found in the second generation group. Feed efficiency is a topic of uppermost importance in all aspects of animal production. The poultry industry has come a long way since the inception of the broiler and broiler breeder with many

still searching for ways to improve upon these models. With a growing demand for high quality animal protein coupled with the cost of feed for poultry production, making a bird's ability to use feed efficiently has become a topic of interest the world over. The results found aim to shed light on one of many pathways, and factors affecting this pathway, that may be involved in whether or not a bird is efficient. More research will have to go into the mechanisms behind these differences such as age, and feed composition, with future experiments to determine those mechanisms currently underway.

APPENDIX

1. IACUC approval for heat stress studies

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Sami Dridi

FROM: Craig N. 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 3 years 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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2. IACUC approval for feed efficiency studies

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Walter Bottje

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: September 17, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : September 17, 2016

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#14012-** "**Molecular signatures and mechanistic modeling for improving feed efficiency in broilers**". 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 **09-17-2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years 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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