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Nuclear Factor [Erythroid-derived 2]-like 2's (NFE2L2) Gene Expression in Feed Efficiency and Oxidative Stress in Avian Muscle

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Nuclear Factor [Erythroid-derived 2]-like 2's (NFE2L2) Gene Expression in Feed Efficiency and Oxidative Stress in Avian Muscle

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by

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ABSTRACT

Oxidative stress may play a role in the phenotypic expression of feed efficiency (FE). The transcription factor NFE2L2 (nuclear factor erythroid-derived 2-like 2) coordinates antioxidant response to oxidative stress and its activity is tightly regulated in part by KEAP1 (Kelch like-ECH protein 1) and the E3 ligase CUL3 (Cullin3). Thus, one objective was to determine mRNA expression of NFE2L2, KEAP1, and CUL3 as well as three antioxidant targets [glutathione peroxidase (GPx-1), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2)] in breast muscle of immature pedigree broiler males (8 wk), immature Japanese quail males (4 wk) divergently selected for high or low susceptibility to restraint stress, and mature Japanese quail (30 wk) exhibiting high or low FE. The second objective was to determine effects of 4-hydroxy 2-nonenal (4-HNE, a secondary lipid peroxide), on NFE2L2, KEAP1, CUL3, SOD1, SOD2, and GPx-1 mRNA expression in an avian muscle cell line (Quail Muscle 7, QM7). High FE pedigree broiler males exhibited increased KEAP1 and SOD1 mRNA expression in breast muscle compared to the low FE phenotype Quail from the HS (high stress) with low FE exhibited increased mRNA expression in all the genes except SOD1. In contrast, the immature high FE quail from the LS (low stress) line had higher levels of NFE2L2, SOD2, and GPx-1. The mature high FE Japanese quail had higher mRNA expression of SOD1, SOD2 and GPx-1 compared to the low FE phenotype, but there were no differences in mRNA expression of NFE2L2, KEAP1 and CUL3 between the high and low FE mature quail. The effects of 4-hydroxynonenal (4-HNE) (0, 10, and 20 μM) on mRNA expression in QM7 cells

was determined at 30, 120, and 240 min post 4-HNE treatment. After 30 min, NFE2L2, SOD1, and GPx1 mRNA expression was lower and KEAP1 levels higher ($P < 0.07$) in 20 μM compared to 0 μM 4-HNE. At 120 min, CUL3 was higher in 10 μM compared to 0 and 20 μM 4-HNE treated cells; SOD1 expression was lower and GPx-1 expression higher in 20 μM treated compared to controls. There were no differences in mRNA expression at 240 min of treatment with the exception that 20 μM 4-HNE raised CUL3 mRNA expression compared to the 10 μM 4-HNE treated cells.

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DEDICATION

This thesis is dedicated to my family and Kyler, who have always encouraged me to keep dreaming. Also to the members of my committee and their respective lab members who have been a tremendous amount of help, especially Dr. Bottje who made this all possible and pushes me to be the best researcher possible. He has been a tremendous friend and mentor throughout this entire process.

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CHAPTER 1

I. Overview of NFE2L2's Diverse Roles and Regulation

To maintain cellular homeostasis, eukaryotic cells must coordinate defense mechanisms that protect against stressors or toxins. Eukaryotic cells have developed diverse and complex signaling pathways to metabolize potentially harmful molecules and maintain cellular redox homeostasis. Cellular oxidative stress cascades have been the topic of many diabetic, cancer, and ageing research studies (Yu et al., 1994). This is mainly due to the pathways ability to release certain cellular components such as detoxifying enzymes or antioxidant proteins. A key transcription factor involved in coordinating the cellular antioxidant response is NFE2L2 (Nuclear factor, erythroid 2-like 2).

NFE2L2 is a basic leucine zipper (bZIP) transcription factor (Moi et al., 1994) that is ubiquitously expressed with highly conserved domains known as NFE2L2-ECH homology (Neh) domains. Deficiency of NFE2L2 can result in embryonic lethality and severe oxidative stress (Leung et al., 2003). Many different stimuli have the ability to induce this protein, such as oxidative, inflammatory, xenobiotic, and hypoxic stress on a cell (Osburn et al., 2008). There are also many different molecules that have the ability to induce and activate this protein (i.e. polyenes, heavy metals, peroxides, and quinones) (Li et al., 2012). NFE2L2 has the ability to induce cytoprotective proteins and enzymes that help maintain adequate or appropriate antioxidant protection, thus protecting the cellular components that are sensitive to oxidative state changes (i.e. DNA) (Hayes et al., 2000).

Following Hayes et al. (2000), Miao et al. (2005) determined that NFE2L2 can initiate direct cross talk between phase I and II drug metabolizing enzymes such as cytochrome P450s. While cytoprotection is considered to be a primary role of NFEL2, it is also linked to many other

cellular processes such as cellular differentiation, proliferation, growth, and death (apoptosis) (Li et al., 2012). While some roles of NFE2L2 are not clear (e.g. mitochondria morphology), it is possible that some of the roles may differ from species to species. Itoh et al. (1995) demonstrated that NFE2L2 is highly regulated in chicken hematopoietic cells, suggesting that it could have originally played a crucial role in erythropoiesis in an avian system but not necessarily in a mammalian system. Although Chan et al. (1996) reported that NFE2L2 was not essential for the erythropoiesis in mammalian cells, it does not mean that the amount of reactive oxygen species (ROS) in circulation and production by red blood cells are not impacted by NFE2L2 activation, or lack thereof, in mammalian cells. The NFE2L2 protein also has been shown to regulate various proteins involved in the lipid and fatty acid metabolism. The NFE2L2 pathway can be induced by certain triterpenoids such as CDDO-lm and CDDo-Me [imidazole and methyl ester derivative of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO)] , that have been shown to reduce the amount of fatty acid accumulation in the livers of mice on a high fat diet (Shin et al., 2009).

As shown below in Fig. 1.1, NFE2L2 is regulated by proteosomal degradation when there is no cellular stress, or it can be transactivated to the nucleus in response to oxidative stress. Among the conserved Neh domains found in NFE2L2, the Neh2 domain mediates the cytosolic suppression of NFE2L2 when it binds to KEAP1 (Kelch-like-ECH protein 1) and forming a homodimer (Itoh et al., 1999). KEAP1 is a cysteine rich protein that attaches to the actin cytoskeleton (Kang et al., 2004) to anchor the NFE2L2 protein in the cytosol, thus preventing activation. The human KEAP1 protein has 27 cysteine residues that are highly reactive towards electrophiles and ROS.

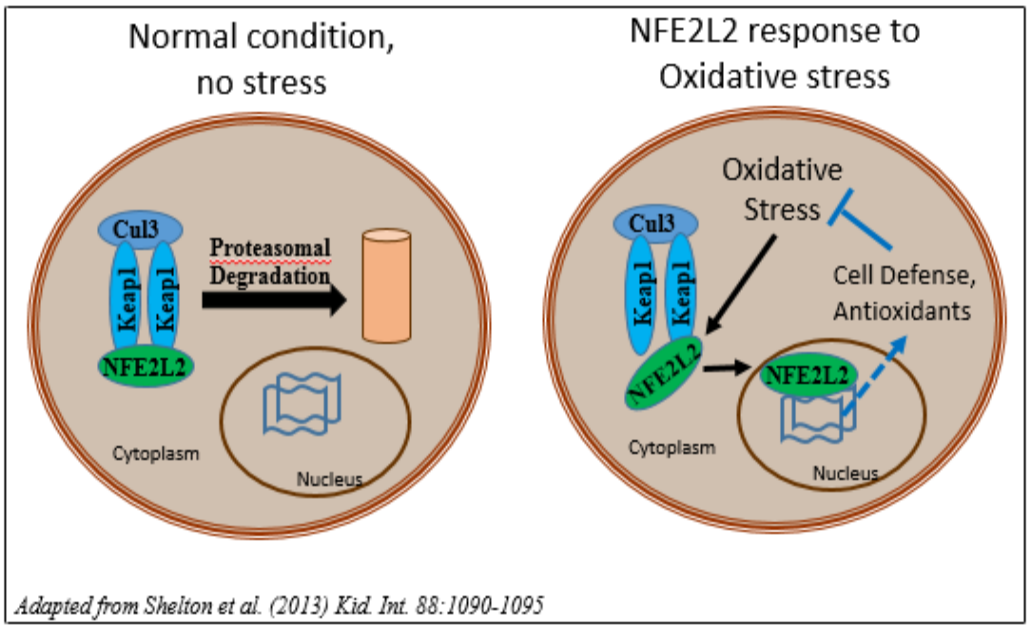

Fig. 1.1: Cellular Regulation of NFE2L2: A simple depiction of regulation of NFE2L2 in the cell is provider in Fig. 1 and discussed in detail below.

One proposed model indicates that two important peptide regions are involved in Neh2 and KEAP1 binding: namely [1]29DLG31 and 79ETGE82 respectively (Kobayashi et al., 2006). These protein motifs within the Neh2 domain of NFE2L2 are thought to be able to approach an alpha helix region of KEAP1 (known as the DGR site) that contains multiple lysine residues and attach. It is thought that 79ETGE82 amino acid sequence (motif) has a higher affinity for the KEAP1 subunit than the 29DLG31 motif, thus allowing it to act as somewhat of a "hinge" while 29DLG31 acts as a "latch" (Kobayshi et al., 2006). This allows KEAP1 to act in various redox states of the cell. KEAP1 has the primary responsibility of sequestering NFE2L2 in the absence of stress stimuli, but it does not do this without aid from another protein: Cullin-3 (CUL3).

[1]29DLG31, 79ETGE82, and DGR denote single letter abbreviations for amino acids and respective position within the protein.

KEAP1 facilitates the degradation of NFE2L2 via the 26s proteasome by serving as an adapter protein for CUL3 (Kobayashi et al., 2004).

CUL3 is a subunit of the E3 ligase complex that interacts with KEAP1 *in vivo* (Geyer et al., 2003). KEAP1 is one of many different cellular proteins that interact with CUL3, all of them using the CUL3 protein as a tag for degradation by the 26S proteasome (Geyer et al., 2003). Because CUL3 acts as a crucial degradation "tag" at times for multiple proteins (involved in different cellular pathways), it has made it a novel target in combating diverse pathologies. The association between KEAP1 and CUL3 is crucial for NFE2L2 ubiquitination, but the details of how the proteins interact is still not completely understood (Eggler et al., 2009). Various molecular interactions of KEAP1 and CUL3 have been proposed and studied. In response to stimuli (e.g. ROS) the cysteine residue(s) in KEAP1 undergo a conformation change and the E3 ubiquitin ligase CUL3 is unable to attach to KEAP1. This important covalent modification is happening primarily at the Cys151 residue of KEAP1 (Rachakonda et al., 2008; Eggler et al., 2009). This triggers NFE2L2 to detach from KEAP1 and translocate to the nucleus, thus preventing a polyubiquitin tag for proteosomal degradation. Another proposed mechanism states that when KEAP1 does not have the CUL3 ubiquitin tag attached, it will simply become saturated with NFE2L2, thus allowing newly synthesized NFE2L2 to accumulate and translocate to the nucleus (Katoh et al., 2001). Once NFE2L2 reaches the nucleus, the Neh4 and Neh5 domains are responsible for the transactivation of NFE2L2, and the Neh1 domain will bind to the antioxidant response element (ARE) within the DNA (Katoh et al., 2001). This binding to the ARE is made possible by NFE2L2 heterodimerization with small Maf proteins (transcriptional activating proteins) (Itoh et al., 1995) and induces the expression of various cell defense genes that will work to restore the cell to its basal state.

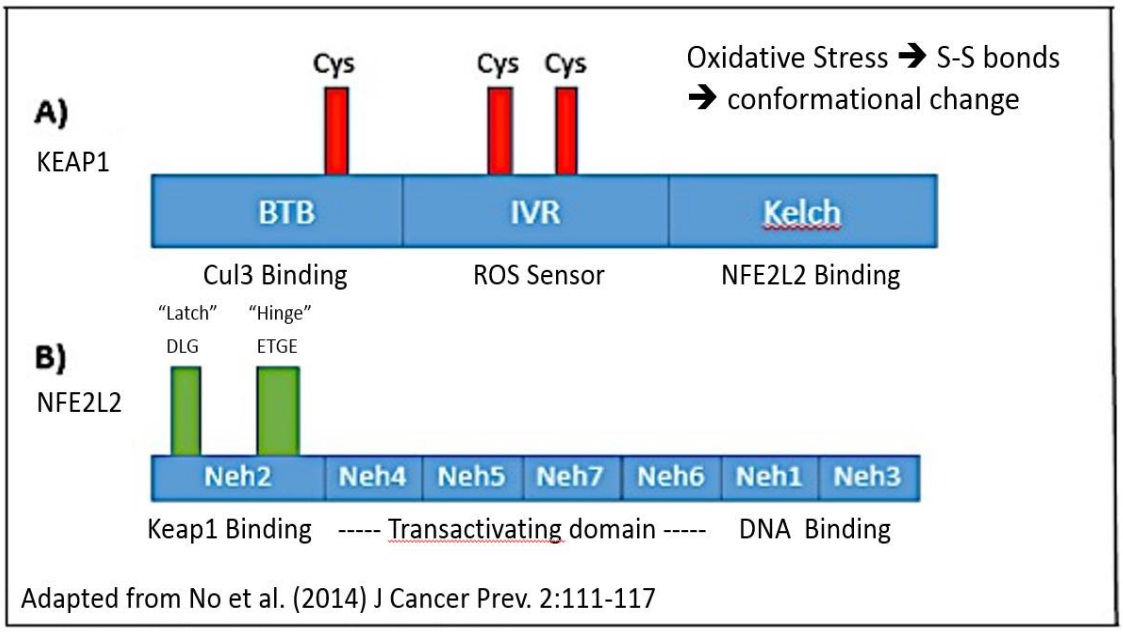

Figure 1.2: Conserved Domains of KEAP1 and NFE2L2: Structures of Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor E2-related factor 2 (Nrf2). BTB, Bric-a-Brac domain; IVR, intervening region; CUL3, Cullin E3 ubiquitin ligase; ROS, reactive oxygen species; DLG and ETGF, binding sites for Keap1.

It has been shown that a lack of the KEAP1 protein can cause hyperactive signaling and a decreased half-life of NFE2L2 (McMahon et al., 2003) but the exact mechanisms and roles of KEAP1 are still heavily debated. KEAP1 itself has shown to be affected by the autophagy related protein Sequestosome1 (p62) (Fan et al., 2010). Sequestosome1 acts as a scaffold protein that activates the NFE2L2 stress response by inactivation of KEAP1 (Copple et al., 2010). There are theories that KEAP1 may have either an independent or dependent mechanism of regulation for NFE2L2. The independent regulation theories suggest that while KEAP1 plays an important role in the regulation, it can also be heavily regulated by factors at the transcriptional and post-transcriptional level. Multiple proteins have been identified as NFE2L2 binding partners, and each could affect binding affinity, localization, or degradation. In 2008, Jain et al., reported that phosphorylation plays a role in the nuclear export of NFE2L2. *In vivo* proof that modification of one of the critical cysteine residues in KEAP1 automatically acts as a biological trigger of

NFE2L2 to translocate to the nucleus has not been demonstrated (Eggler et al., 2005). However, KEAP1 has a significant number of sulfhydryl groups from its cysteine residues that collectively play a role in conformational changes and function. There is no doubt that KEAP1 acts as a fundamental sensor of oxidative or electrophilic stress for the NFE2L2 pathway, but by varying degrees depending on conditions in the cell. The many cysteine residues of KEAP1 lower the predicted pKa value of NFE2L2 by binding with more basic amino acids that are a part of the NFE2L2 inducers. Dinkova-Kostova et al. (2002) noticed that the majority of NFE2L2 inducers react with thiol groups even though they are structurally dissimilar. Cysteine modification as one of the primary means of NFE2L2 transactivation is not clear, as the Neh2 region of NFE2L2 does not contain cysteine residues itself. This could be a result of a chain reaction of various cysteine residues in KEAP1 started by the Bric-a-Brac region of KEAP1 that is responsible for the dimerization of two KEAP1 molecules (Stogios et al., 2005). This Bric-a-Brac region dimerization is required to sequester NFE2L2 in the cytoplasm and has been the key feature of many models that try to explain NFE2L2 stabilization (Zipper et al., 2002). Upon induction, the BTB (Broad complex, Tramtrack, and Bric-a-Brac) region of KEAP1 may undergo covalent modification and create somewhat of a steric hindrance when it tries to interact with CUL3.

Recently, microRNAs have emerged as new players in managing redox homeostasis (Cheng et al., 2013) and may be related to the age of the cell or organism. A factor to consider when it comes to the expression and activation of NFE2L2 is the age of the organism in which NFE2L2 is being studied. NFE2L2 protein expression in tissues declines with age (Suh et al., 2004). This decrease in NFE2L2 expression has been demonstrated in mouse liver, which coincides with a decrease in glutathione synthesis (Suh et al., 2004). This decrease, however, may have more to do with translation, rather than by KEAP1- mediated inhibition. Recently

Smith et al. (2015) demonstrated that KEAP1 expression also declines with age. This suggests that NFE2L2 degradation is not to blame for this age-related decreased expression, and the decrease in KEAP1 expression might even be a way that the cell mechanistically copes with a lack of the NFE2L2 protein being synthesized. In general, a decrease in protein translation appears to be a trait of aging. Given that NFE2L2 has a relatively short half-life compared to other cellular proteins, it may be particularly sensitive to the adverse effects that come with aging. These direct mechanisms that contribute to the age-related decline in NFE2L2 expression are still not yet defined but it could be in part due to the expression of specific microRNAs such as miRNA-144, 153, 27a, and 142-5p (Cheng et al., 2013). These specific miRNA binds to NFE2L2 mRNA to attenuate the amount of NFE2L2 and its activity. Likewise, miRNA-200a has shown to cause attenuation of the KEAP1 protein, as shown by using miRNA mimicking techniques (Yang et al., 2014). With both NFE2L2 and KEAP1 showing signs of declining that correlate with certain mi-RNA, more studies are clearly needed to define which mi-RNAs can inhibit or promote the translation of these proteins.

II. Antioxidants and Oxidative Stress

Upon NFE2L2 activation of ARE, there follows a first line of antioxidant defense that sets out in the cell to metabolize ROS. In this first line of defense are superoxide dismutases (SODs) that are a family of enzymes that work to catalyze the dismutation of superoxide anions (Liochev et al., 2007). There are multiple isoforms of SODs that have been molecularly characterized. CuZn-SOD or superoxide dismutase 1 (SOD1), is a copper and zinc-containing homodimer that is found almost exclusively in the cytoplasm (Parge et al., 1992). CuZn-SOD converts naturally occurring, but harmful superoxide radicals to hydrogen peroxide (H_20_2). After this has occurred, the H_20_2 can then be metabolized by the enzyme catalase (Alscher et al., 2002).

Within the SOD1 protein, the Cu/Zn site that holds the superoxide ion is crucial for the conversion of superoxide to H_20_2. It has been implicated that SOD1 plays an important role in apoptotic signaling and cell death by means of its regulation of the amount of ROS within the cytoplasm (Liang et al., 2009). Another family member of the superoxide dismutases is Mn-SOD or superoxide dismutase 2 (SOD2). This protein will bind one manganese ions per subunit and is found predominately in the mitochondria. Mn-SOD serves to protect the electron transport chain from superoxide produced by electron leak during oxidative phosphorylation. Like SOD1, SOD2 also plays a role in apoptotic signaling and protection against ischemia-reperfusion injury (Schneider et al., 2010). Given that it has tremendous cytoprotective effects, overexpression of SOD1 or SOD2 has been linked to increased tumor metastasis (as has NFE2L2).

This first evidence of a relationship between NFE2L2 and SOD1 was reported by Kirby et al. (2005) who showed that the presence of a mutated SOD1 caused a reduction in NFE2L2 mRNA expression. Milani et al. (2013) reported that SOD1 was shown to converge in the NFE2L2 pathway with the DJ-1 (Parkin7) protein. DJ-1 is a ubiquitously expressed protein that is activated by the NFE2L2 pathway and translocates from the cytoplasm to the mitochondria or nucleus hours after the cell is exposed to oxidative stress. The essential role of DJ-1 is to protect the cell from apoptotic death triggered by high levels of ROS by activating other genes such as SOD1. DJ-1 is considered to be a crucial protein in protecting against neurodegeneration and thus is the focus of numerous Parkinson's disease studies (Hiroyoshi et al., 2013). This crosstalk between the antioxidant and apoptosis pathway helps maintain mitochondrial integrity, thus further proving NFE2L2's importance to an array other antioxidant defenses that interact with different cellular pathways.

The glutathione peroxidase family also plays a big role in fighting ROS. Glutathione peroxidase 1 (GPx-1) is another oxidative stress related enzyme found in the mitochondria. GPx-1 is one of only a few proteins in higher vertebrates that contains selenocysteine moieties. Selenocysteine is the 21st proteogenic amino acid and is coded by UGA, which normally functions as a translation termination codon (Lubos et al., 2011). GPx-1 catalyzes the reduction of H_20_2 to water. Unlike SOD, GPx-1 will also reduce other organic hydroperoxides such as lipid peroxides, to alcohols, thus making it one of the most important antioxidant enzymes with respect to lipid peroxides and H_2O_2 metabolism in eukaryotic organisms (Lubos et al., 2011). Another notable difference is the fact catalase has a much higher Km for H_2O_2 than GSH peroxidase, so often catalase functions in a pathophysiological environment as to where GSH peroxidase will act at physiological basal levels (Chrissobolis et al., 2008). The link between NFE2L2 to GPx-1 activity is due in part to NFE2L2 mediated-induction of GSH. However, it has been shown that NFE2L2 knockout mice can still retain some GPx-1 function (Zhu et al., 2005). These enzymes, SOD1, SOD2, and GPx-1 are all crucial antioxidant enzymes that can be affected by NFE2L2 activity.

III. 4-HNE and QM7 Cells

A consequence of oxidative stress in lipids is lipid peroxidation that can result in the formation of a stable secondary lipid peroxide. The α,β-unsaturated hydroxyalkenal, 4-hydroxy-2-nonenal (4-HNE), is a stable secondary lipid peroxide that is produced during the oxidative lipid breakdown of biological membranes. The deleterious effects of 4-HNE are related to amounts and the ability of the cell to metabolize or succumb to it once a critical threshold is reached: making it a double edged sword. Lower intracellular concentrations of 4-HNE can be beneficial to cells promoting pathways concerning differentiation, proliferation, and antioxidant

defense (Chen et al., 2005). In mitochondria formation of 4-HNE has been shown to induce uncoupling that lowers superoxide production (Schulechev et al., 1996; Miwa et al., 2003). Higher concentrations, however, induce caspase enzymes and trigger apoptosis or necroptosis pathway in various cell lines. The threshold at which 4-HNE induces cellular death varies from cell to cell. 4-HNE has shown to be a NFE2L2 inducer at sublethal concentrations, thus allowing NFE2L2 to activate and exert its cytoprotective effects (Chen et al., 2005).

IV. Feed Efficiency and Stress in Relation to NFE2L2

NFE2L2 has been shown to be an ortholog gene between many different species, meaning it has evolved from a common ancestral gene. It is believed to share many of the same functions between mouse, chicken, and even aquatic animals such as the zebrafish (Gacesa et al., 2015). Recently, Zhou et al. (2015) reported that NFE2L2 was predicted to be activated in commercial broilers exhibiting high feed efficiency (FE). In recent years, the scientific community has been able to use invaluable bioinformatic tools such as pathway analysis software to assess biological differences between two groups of interest. Kong et al. (2016) have also observed that NFE2L2 was predicted to be activated in male broilers with high FE based on expression of downstream target molecules in a proteomics dataset. Thus, NFE2L2 could play a role in the phenotypic expression of FE by orchestrating antioxidant protection within cells.

Previous studies have revealed a link between mitochondrial function and FE in broiler chickens. Lower electron transport chain coupling and greater H_2O_2 production were observed in mitochondria of low feed efficient birds (Bottje et al., 2002). Recently, NFE2L2 disruption led to decreased oxidative phosphorylation efficiency (Holmstrom et al., 2013). Despite its role in antioxidant response to oxidative stress, little is known about NFE2L2 in avian species. Perhaps what makes it such an attractive gene or protein to study is its ability to impact so many different

factors concerning the mitochondria including biogenesis, fatty acid oxidation, and integrity (Dinkova-Kostova et al., 2015).

Given the different conditions broilers are raised in, it is important to examine the connection NFE2L2 might have with an animals phenotypic expression of FE. It has been proposed that the NFE2L2/ARE signaling pathway is activated under times of cold stress to aid liver cells in fatty acid storage to protect the cell against the cold environment (Chen et al., 2015). During cold stress, increased free radical production can cause oxidation of polyunsaturated fatty acids and compromise the integrity of the cell membrane. The cell protects against this by means of up-regulating the expression of L-FABP (Fatty acid-binding protein 1) to shut down lipid peroxidation of the cell membrane (Tanaka et al., 2008). L-FABP has shown to be directly involved in the NFE2L2/ARE downstream signaling pathway (Chen et al., 2015). Interestingly, Kong et al. (2016) showed that FABP was upregulated in high FE broilers. Heat stress in poultry has not shown to up-regulate of NFE2L2 without the addition of certain dietary supplements such as lycopene (Sahin et al., 2016). This is somewhat intuitive as heat stress also can produce oxidative stress on the cell, but at the same time damage the transcription and translation going on within the cell to combat this stress.

V. Objectives

Understanding the relationship that oxidative stress plays in the phenotypic expression of FE will be crucial in moving forward with selective breeding for future generations to insure that the global population has a sustainable amount of food. Given that NFE2L2 plays a crucial role in activating the ARE and thus many phase II detoxifying enzymes, it is important to understand what is occurring with this gene at the transcriptional level. Thus, the first major objective of this study is to examine NFE2L2 mRNA expression as well as its regulators (e.g. KEAP1 and

CUL3) and three downstream targets (e.g. SOD1, SOD2, and GPx-1) in avian breast muscle that has been phenotyped for low and high FE.

The second objective will be to examine the mRNA expression of the genes listed above in Quail muscle clone 7 (QM7) cells that have been treated with sub lethal concentrations of 4-HNE for different time periods. QM7 cells is an immortal cell line derived from Japanese quail muscle tissue with fibrosarcoma. Moscovici et al. (1977) originally isolated tumor cells that developed in a bird treated with methylcholantherene. These cells replicated as myoblasts in medium containing serum, and form large multinucleated myotubes when switched to a medium without serum. QM7 cells are commonly used in studies due to their ability to transfect with high efficiency, and express consistent phenotypes through many passages. These traits make them a novel cell line for studying gene expression. To our knowledge, no definitive relationship has been established between NFE2L2 and SOD1, SOD2, or GPx-1 in avian breast muscle.

CHAPTER 1 – LITERATURE CITED

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CHAPTER 2

Nuclear factor [Erythroid-derived 2]-like 2's (NFE2L2) gene expression in feed efficiency and oxidative stress in avian muscle [1]

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ABSTRACT

Oxidative stress may play a role in the phenotypic expression of feed efficiency (FE). The transcription factor NFE2L2 (nuclear factor erythroid-derived 2-like 2) coordinates antioxidant response to oxidative stress and its activity is tightly regulated in part by KEAP1 (Kelch like-ECH protein 1) and the E3 ligase CUL3 (Cullin3). Thus, one objective was to determine mRNA expression of NFE2L2, KEAP1, and CUL3 as well as three antioxidant targets [glutathione peroxidase (GPx-1), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2)] in breast muscle of immature pedigree broiler males (8 wk), immature Japanese quail males (4 wk) divergently selected for high or low susceptibility to restraint stress, and mature Japanese quail (30 wk) exhibiting high or low FE. The second objective was to determine effects of 4-hydroxy 2-nonenal (4-HNE, a secondary lipid peroxide), on NFE2L2, KEAP1, CUL3, SOD1, SOD2, and GPx-1 mRNA expression in an avian muscle cell line (Quail Muscle 7, QM7). High FE pedigree broiler males exhibited increased KEAP1 and SOD1 mRNA expression in breast muscle compared to the low FE phenotype Quail from the HS (high stress) with low FE exhibited increased mRNA expression in all the genes except SOD1. In contrast, the immature high FE quail from the LS (low stress) line had higher levels of NFE2L2, SOD2, and GPx-1. The mature high FE Japanese quail had higher mRNA expression of SOD1, SOD2 and GPx-1 compared to the low FE phenotype, but there were no differences in mRNA expression of NFE2L2, KEAP1 and CUL3 between the high and low FE mature quail. The effects of 4-hydroxynonenal (4-HNE) (0, 10, and 20 μM) on mRNA expression in QM7 cells was determined at 30, 120, and 240 min post 4-HNE treatment. After 30 min, NFE2L2, SOD1, and GPx1 mRNA expression was lower and KEAP1 levels higher ($P < 0.07$) in 20 μM compared to 0 μM 4-HNE. At 120 min, CUL3 was higher in 10 μM compared to 0 and 20 μM 4-HNE

treated cells; SOD1 expression was lower and GPx-1 expression higher in 20 µM treated compared to controls. There were no differences in mRNA expression at 240 min of treatment with the exception that 20 µM 4-HNE raised CUL3 mRNA expression compared to the 10 µM 4-HNE treated cells.

Key Words: broiler, quail, feed efficiency, NFE2L2, antioxidants

INTRODUCTION

The projected doubling of the human population by 2060 will require a 100% increase in food production coming from plants and animals with most of this increase coming from new technology and greater efficiency (Simmons, 2010). In animal production agriculture, feed is the highest input cost (50 to 70% of total) in raising an animal to market weight and feed costs can spike as was observed in drought conditions in the central US in 2012 (Nixon et al., 2012). Great strides have been made in animal agriculture production efficiency by selecting animals for feed efficiency (FE, gain to feed), feed conversion ratio (FCR, feed to gain), or residual feed intake (RFI, the actual amount of feed intake that is above or below predicated feed intake in a group of animals). These methods are effective but are labor intensive procedures that require measuring feed intake and body weight gain on individual animals. Development of biomarker selection tools to be used in commercial breeding programs can contribute significantly towards increasing animal production efficiency.

NFE2L2 (Nuclear factor erythroid-derived 2-like 2) is a molecule that coordinates cellular response to oxidative stress (e.g. Shelton et al., 2013). As shown in Fig. 1.1 (p.5), under normal (non-stressful conditions), NFE2L2 remains in combination with CUL3 (Cullin3) and Keap1 (Kelch like-ECH protein 1) and is rapidly directed to proteasomes for degradation. However, in response to oxidative stress, NFE2L2 is transported into the nucleus where it

stimulates antioxidant gene expression and various other protection mechanisms through the antioxidant response element (ARE). Recently, NFE2L2 was predicted to be activated based on downstream target molecules in commercial broilers (meat chickens) and in pedigree male broilers exhibiting high FE (Zhou et al., 2015; Kong et al., 2016). One of these target molecules upon which the prediction was based was SOD2 (Mn-SOD Superoxide Dismutase 2). SOD2 is found predominately in the mitochondria where it acts to protect the electron transport chain and mitochondria from oxidative stress from superoxide radicals. Given that SOD2 was upregulated in the high FE broilers, it warrants investigation into other downstream antioxidant enzymes such as SOD1 (Superoxide Dismutase 1, CuZn-SOD), which is predominantly found in the cytosol of a cell, as well as another antioxidant enzyme that localizes to the mitochondria: GPx-1 (Glutathione Peroxidase 1). To our knowledge, the relationship between NFE2L2 mRNA expression and FE has not been clearly defined. Thus, NFE2L2 could play a role in the phenotypic expression of FE by orchestrating antioxidant protection within cells. Oxidative stress biomarkers remain elusive to a degree (Beaulieu et al., 2014) and may prove crucial in solving the global food shortage in the years to come.

MATERIALS AND METHODS

Avian Lines Selected for Feed Efficiency

Three different lines of birds were used for the *in vivo* studies to investigate relationships of NFE2L2 and FE. The first line examined was a male pedigree broiler breeder line that had been selected through many generations for the high FE phenotype. Muscle tissue was harvested at 8 wk after animals were individually phenotyped for FE between 6 and 7 wk (Table 2.1). The second line examined were male Japanese quail that were phenotyped for FE between 3 and 5 wk and with tissues obtained at 5 wk. Both the pedigree broilers and Japanese quail were

considered sexually immature (Table 2.1). These Japanese quail were the progeny of a line of Japanese quail initially phenotyped for the purpose of distinguishing high stress (HS) and low stress (LS) lines based on corticosterone. This was primarily done by measuring blood corticosterone levels in response to restraint stress (Satterlee et al., 1988). The birds used in this study were male progeny from both the HS and LS line. The final line examined was a line of Japanese quail that were phenotyped for FE between 5 and 6 wk and harvested at 30 wk (Table 2.1). These Japanese quail selected from this line were both male and female and were considered sexually mature when tissue were obtained. These birds weighed significantly more than the quail described in the HS and LS line. This larger body mass is due to selecting for a heavier quail over many generations of this line to closer resemble the mass and body proportions of a broiler chicken. All animal care and handling was conducted with approval by the Institutional Animal care Committee (IACUC), protocol # 14012 and # 13039.

RNA Isolation, cDNA Preparation and Quantitative Real-Time PCR

After humanely killing animals, a portion of breast muscle was quickly removed, flash frozen in liquid nitrogen and stored at -80 C. Total RNA was extracted by TriZOL reagent (Life Technologies) according to the manufacturer's recommendations, DNase-treated, and reverse-transcribed (Quanta Biosciences, Gaithersburg, MD). RNA concentrations and purity were determined for each sample on a Take 3 microvolume plate using Synergy HT multimode microplate reader (BioTek, Winooski, VT). The RNA was converted into cDNA with oligo-dT primer and Superscript II reverse transcriptase (Invitrogen Life Technologies) following the manufacturer's instructions. The RT products (cDNAs) were subject to a real-time quantitative reverse-transcription PCR (qRT-PCR) (Applied Biosystems, 7500 real-time PCR system) with Power SYBR Green Master Mix. The conditions of qRT-PCR amplification were 1 cycle at

95°C for 2 min, 40 cycles at 95°C for 30 s, 58°C for 1 min, 72°C for 30 s, and 1 cycle at 72°C for 10 min. The chicken 18S ribosomal RNA (18s) gene was used as the internal control. Dissociation curves were performed at the end of amplification for validating data quality. All qRT-PCR reactions were performed 2 times and the values of average cycle threshold (Ct) were determined for each sample, and these $2^{-\Delta\Delta}$Ct values were used for relative quantification by fold change and statistical significance. 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 confirmed by agarose gel and showed only one specific band of the predicted size. Relative expressions of target genes were determined by the $2^{-\Delta\Delta}$ Ct method. All forward and reverse primer sequences are shown in Table 2.2.

QM7 Cells

QM7 cells were plated in a 6 well plate and grown in M199 medium (Life Technologies, Grand Island, NY) complemented with 10% FBS (Life Technologies), 10% tryptose phosphate (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, the cells were then treated 4-hydroxy-2-nonenal (4-HNE) at different concentrations. 0 µM 4-HNE (Control), 10 µM 4-HNE, or 20 µM 4-HNE for 30 min, 120 min, and 240 min in different passages of the cells (Passages 13,14,15). After washing with ice cold PBS twice, TriZOL reagent was added directly to the tissue culture plate and the cells were detached from the plate using a sterile scraper. Total RNA extraction was performed following the same protocol as the breast muscle.

Statistical analysis

Values in graphs and tables represent the mean ± SE. Differences of mean values were determined by two-tailed Student's T-test (or analysis of variance) with a P value of ≤ 0.05 being considered statistically significant.

RESULTS AND DISCUSSION

The main goal of this study was to determine relationships of NFE2L2 mRNA expression, along with regulators of NFE2L2, and downstream antioxidant gene expression in muscle associated with feed efficiency *in vivo* and 4-HNE induced oxidative stress *in vitro*.

In Vivo Studies

Although the expression of NFE2L2 and CUL3 mRNA in the broilers was not different between the high and low FE phenotype (Figure 2.1, A), expression of KEAP1and SOD1 mRNA was higher ($P < .05$) in the high FE phenotype. With KEAP1 being the primary sensor of oxidative stress in the NFE2L2 pathway (Shelton et al., 2013), this may suggest the high FE broilers show a tighter regulation when compared to the low FE phenotype. Given that KEAP1 is the primary negative regulator of NFE2L2, an increase in KEAP1 transcription could help regulate an increased NFE2L2 protein expression. The results concerning KEAP1 expression may point to a more diverse function of KEAP1 than just regulation of NFE2L2. Although it is known that avian NFE2L2 has the same primary functions as mammalian NFE2L2, it shares only 67% homology (Maher et al., 2010). The cysteine rich nature of KEAP1 makes it an ideal target for other proteins found in the cell and therefore could be affecting its translation in some capacity apart from NFE2L2. This higher expression of KEAP1 in the high FE broilers may also just simply suggest high FE birds tightly regulate the NFE2L2 pathway. In a proteomics study, conducted on the same group of birds they observed that high FE broilers had a higher

expression of the Kelch-like-40 protein (KLH40) compared to low FE (Kong et al., 2016). This protein shares many of the same conserved domains that KEAP1 contains, such as the BTB and POZ regions (Dhanoa et al., 2013). Sahin et al. (2016) examined the effects of heat stress and dietary supplements on NFE2L2 expression in broilers. Increasing the dietary intake of lycopene led to a decrease in KEAP1 protein expression, and increase NFE2L2 protein expression in heat stressed broilers (Sahin et al., 2016), indirectly improving FE. Similarly, Zhang et al. (2015) showed that increasing dietary intake of curcumin in heat stressed broilers improved mitochondrial function and FE, thus inhibiting heat stress impaired growth performance. Possibly, curcumin may upregulate NFE2L2, leading to a more efficient antioxidant defense system or mitochondrial biogenesis. Although a high expression of NFE2L2 mRNA was not observed, it is possible that NFE2L2 protein expression or activity could be different due to interaction with KEAP1, CUL3, or difference in proteosomal degradation. It has been shown that NFE2L2 undergoes various post transcriptional and post translational modifications (Li et al., 2010; Kobayashi et al., 2004; Furukawa et al., 2005) and is an extremely unstable protein. These modifications can affect the amount of NFE2L2 protein produced, as well as how much of it is actually activated or degraded. No difference was shown in CUL3 expression between the high and low FE group although it has been shown that ubiquitin expression in low FE broilers was higher (Bottje and Carstens, 2009). Bottje and Carstens (2009) were not targeting the specific ubiquitin CUL3, but rather differences in ubiquitin levels in general between high and low FE broilers by means of immunoreactivity assays. Similar to most cellular proteins, it has been shown in past studies that NFE2L2 mRNA expression can be a good predictor for protein expression despite the post transcriptional modifications it undergoes.

In the LS line of quail individually phenotyped for FE, NFE2L2 and SOD2 were higher and SOD1 levels lower in the high FE compared to low FE (Figure 2.1, B). This contrasts with the results in the HS line of Japanese quail, with the low FE group having higher mRNA expression of all the genes except SOD1 (Figure 2.1, C). Given that these quail were selected from a line that showed elevated corticosterone levels in the blood after a period of handling stress, this may lend insight into how NFE2L2 levels play an important role in predicting FE when a certain stressor is applied. It should be mentioned though that multiple studies have shown that SOD1 or SOD2 expression are not completely dependent on NFE2L2 activation as heat shock proteins and other cellular messengers play a vital role as well (Zelko et al., 2002). Recently, NFE2L2 has been viewed as something to be used to combat corticosterone effects in the body (Freitas et al., 2016). Corticosterone can negatively affect the morphology of astrocytes and microglia in various regions of the brain (Freitas et al., 2016), and it has long been known to suppress protein synthesis and degradation in skeletal muscle (Odedra et al., 1983). The results from the HS line could be a result of high corticosterone levels modulating the expression of NFE2L2 mRNA, thus affecting how FE is regulated in these birds. Like many of the genes in this study, much is still unknown about CUL3, especially in the avian species. In mammalian cells, the CUL family of proteins have shown they can undergo process called neddylation in which they will interact with small ubiquitin-like protein modifiers (Wu et al., 2005). To our knowledge, no detailed studies have been carried out in an avian cell line concerning the role of neddylation on CUL3 specifically and its targets. However, Kong et al. (2011) has shown that neddylation genes (e.g. Nedd8) were up regulated in muscle tissue of high FE broilers when compared to low FE broilers. Neddylation proteins are essential for cell viability (Osaka et al.,

2000), and could be signaling more efficient growth mechanisms in high FE birds compared to low FE birds.

In the mature quail that were individually phenotyped for FE, the expression of the downstream antioxidant enzymes (SOD1, SOD2, and GPx-1) showed higher mRNA expression in the high FE phenotype, even though NFE2L2 expression was not differentially expressed between the high and low FE phenotype (Figure 2.1, D). It should be pointed out both male and female birds were selected to be used in this comparison. These birds were highly selected for growth – similar to the pedigree broiler male. The results shed light on the complexity of the NFE2L2 pathway and its downstream targets. The fact that both male and female birds were used in this line, could have had an effect on the data as well. Current literature supports the notion that expression of various cytoprotective genes can differ based on sex, specifically genes regulated by NFE2L2 (Sheng et al., 2003). Definitive evidence that NFE2L2 is responsible for the difference in cytoprotective mRNA expression between sexes remains to be discovered, and some studies suggest that it is not responsible at all for the differences even though downstream targets are affected (Rohrer et al., 2014). The question remains, if NFE2L2 is not responsible for the differences between sexes, than what is?

As an organism ages SOD's and GPx's become increasingly important in muscle tissue to combat muscle oxidation and deterioration. Mutations in SOD's have been linked to diseases such as amyotophic lateral sclerosis (ALS) (Bozzo et al., 2016). NFE2L2 not being differentially expressed supports that a tremendous amount of cross talk between various pathways is occurring in the cell pertaining to the effects of oxidative stress. The high FE birds within this group showed much higher mRNA expression of NFE2L2's downstream targets,

despite no difference in NFE2L2 being observed. This lends insight into just how important these antioxidant genes are in relation to FE in older animals.

QM7 Cells

The effect of 4-HNE treated QM7 cells showed various differences across the six different genes of interest during the different time treatments (Figure 2.2). Cell viability determined by trypan blue exclusion method ranged from 95-99% throughout the study (data not shown). As expected, the QM7 cells treated with 20 µm 4-HNE at different time points showed significantly different mRNA expression when compared to the control cells. Concerning the downstream targets, GPx-1 was the only one that tended to mirror the expression trend that was seen in NFE2L2 (Figure 2.5). 4-HNE has important electrophilic properties and reacts with proteins, lipids and nucleotides to form 4-HNE adducts (Pillon et al., 2012). Given that the mitochondria is a major source of endogenous ROS, it makes it a target for HNE adduct formation (Echtay et al., 2003). This could in fact explain the upregulation that is seen between NFE2L2 and GPx-1 in the 120 min treatment, and the down regulation shown during the 30 min time course. Surprisingly, minimal differences were observed in the SOD1 and SOD2 mRNA expression levels between the different treatments. SOD2 showed no significant differences between the treatments while SOD1 did at the 30 min and 120 min time course. In a study done by Yoon et al. (2007), they noticed as a high expression of 4-HNE and SOD1 in the hippocampus of aged dogs (10-12 years of age) when compared to dogs that where only 2-3 years old. The data acquired here suggests that in QM7 cells, an increase of 4-HNE does not always correspond with a direct increase in SOD1 mRNA expression. The most notable relationship examined in the QM7 cell data is between NFE2L2 and GPx-1. At both 30 min and 120 min following 4-HNE treatment the mRNA levels of these two genes showed a similar trend

in expression. The 30 min treatment of 4-HNE at 20 µM brought about a significant decrease in mRNA levels GPx-1 when compared to the control group, while NFE2L2 showed the same trend ($P < .07$). The 120 min treatment of 4-HNE brought about a significant increase in mRNA expression of NFE2L2 and GPx-1 in the 20 µM treated group when compared to the control cells that received no 4-HNE. The results regarding SOD2 mRNA expression are somewhat baffling, given the known relationship that exists between NFE2L2 and phase II detoxifying enzymes. Even when NFE2L2 showed differential mRNA expression throughout the different treatments, SOD2 had no significant differences in expression. This data supports the notion that NFE2L2 is more involved with mitochondria function that perhaps initially thought years ago. In both the in *vivo* and in *vitro* studies, a clear correlation is seen between the trend of NFE2L2 mRNA expression and GPx-1 mRNA expression.

Cleary, more studies need to be conducted exploring NFE2L2's role as a possible biomarker for FE in avian species. This study aimed to examine what mRNA expression would like for NFE2L2 when comparing different lines of birds that have been phenotyped for high and low FE. The QM7 cell studies proved to show that 4-HNE at sublethal levels can bring about changes in oxidative stress related genes, but the amount of time in which they are treated proves crucial as to what kind of response is observed.

CHAPTER 2 – LITERATURE CITED

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TABLES AND FIGURES

Table 2.1: FI, Gain, and FE for the Different Avian Lines Used.

Avian Line (n)	FI Average ± SEM (g)	Gain Average ± SEM (g)	FE Average ± SEM
Pedigree Broilers	970.4 ± 36.9	*628.0 ± 25.1	*0.647 ± 0.006
High FE (5)	1021.6 ± 24.4	470.4 ± 10.7	0.461 ± 0.006
Low FE (5)			
LS Quail	197.6 ± 7.1	*38.33 ± 1.2	*0.195 ± 0.005
High FE (6)	218.8 ± 10.8	28.33 ± 1.8	0.129 ± 0.003
Low FE (6)			
HS Quail	189.9 ± 8.1	*42.5 ± 2.5	*0.224 ± 0.012
High FE (6)	217.2 ± 8.8	25.2 ± 1.7	0.115 ± 0.004
Low FE (6)			
Mature Quail	272.5 ± 13.5	*135.8 ± 5.9	*0.500 ± 0.012
High FE (6)	309.2 ± 14.0	108.5 ± 4.1	0.354 ± 0.012
Low FE (6)			

*Indicates High FE > Low FE (P< .05)

Table 2.2: Primer Sequences

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
18S	TCCCCTCCCGTTACTTGGAT	GCGCTCGTCGGCATGTA
NFE2L2	CCACCCTAAAGCTCCATTCA	GGATCTGCAGAGCTTTTGCT
KEAP1	TACAACCCCATGACCAACCG	CGGCGTAGATCATCCCATCG
KEAP1 (Cells)	CGCCATCGACTGTTACAACC	GCGTAGATCATCCCGTCGAT
CUL3	AGCAGAGGTAACTCAGCAGC	CTTTGCGATCCTCAGGTGTT
SOD1	TGGCTTCCATGTGCATGAAT	AGCACCTGCGCTGGTACAC
SOD2	GCTGGAGCCCCACATCAGT	GGTGGCGTGGTGTTTGCT
GPx-1	TCCCCTGCAACCAATTCG	AGCGCAGGATCTCCTCGTT

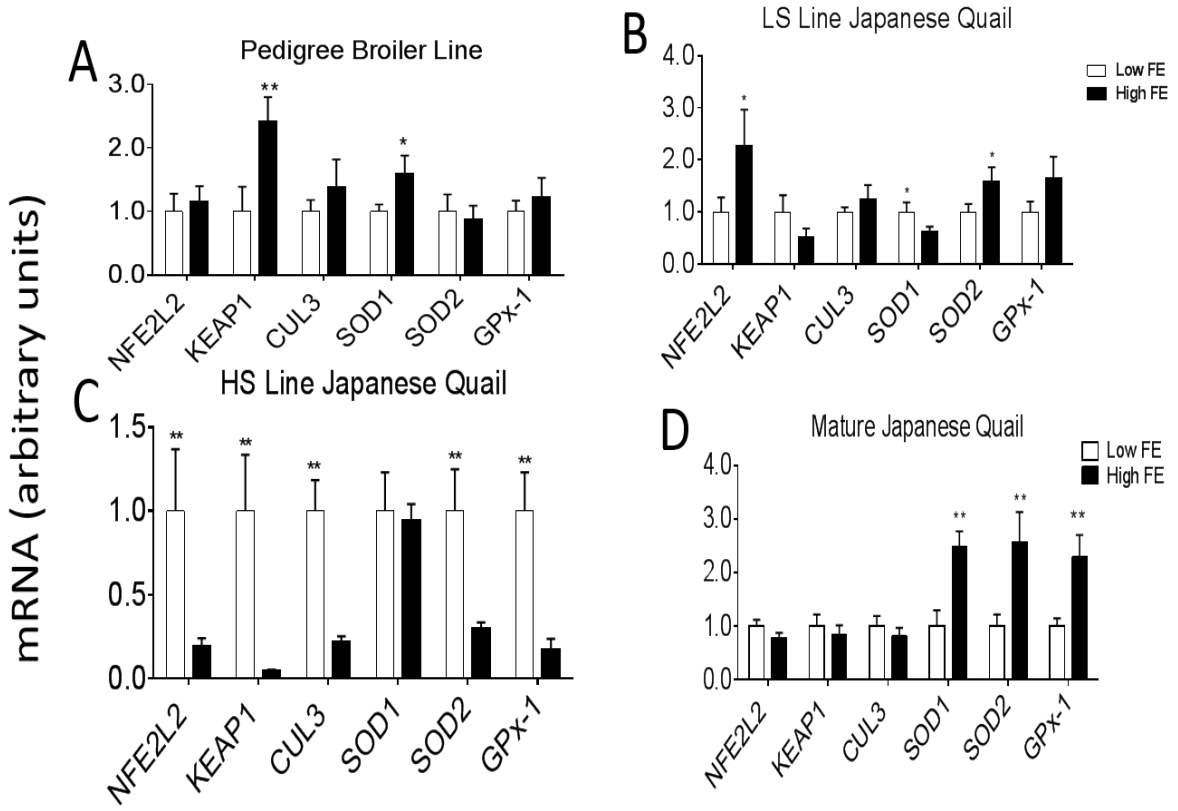

Figure 2.1: Avian Lines mRNA Expression: The expression of mRNA (arbitrary units) in breast muscle in A) Pedigree Broiler Males B) Low Stress (LS) Japanese Quail C) High Stress (HS) Japanese Quail D) Mature Japanese Quail exhibiting high or low feed efficiency (FE) for nuclear factor (erythroid 2)-like 2 (NFE2L2), Kelch-like ECH-associated protein 1 (Keap1), Cullin3 (CUL3), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and glutathione peroxidase 1 (GPx-1).
Bars represent the mean $\pm$ SE (n=4-6).
* Values are different ($P < 0.05$)
** Values are different ($P < 0.01$)

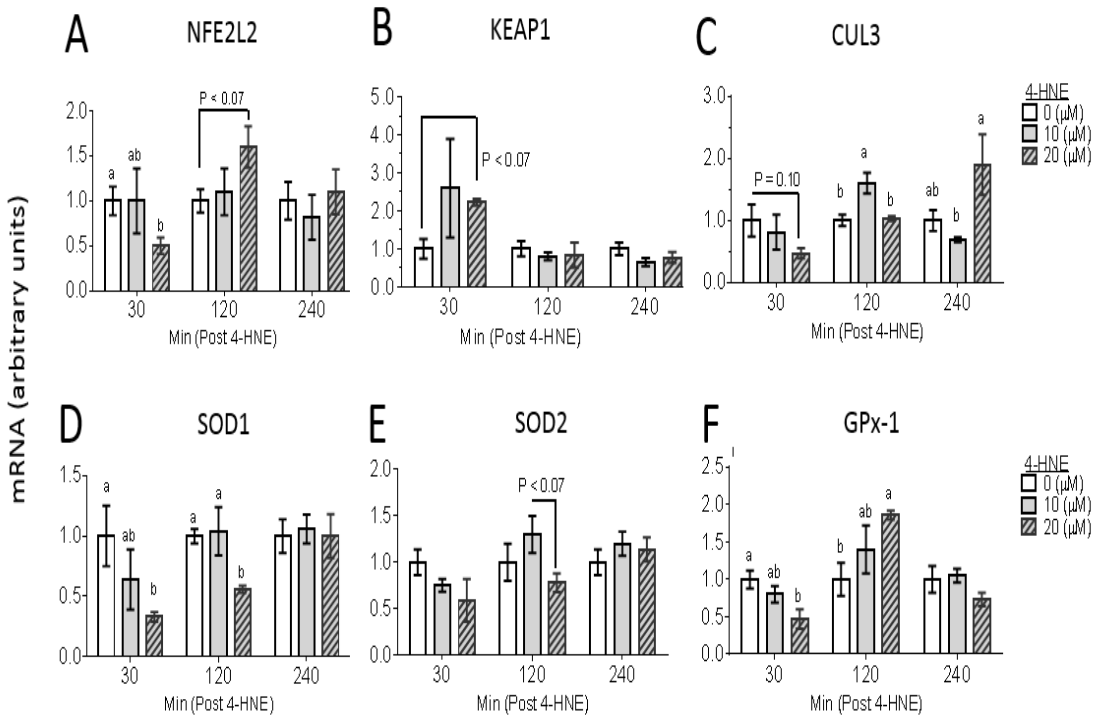

Figure 2.2: QM7 Cells mRNA Expression: The effect of 30 min, 120 min, and 240 min treatment of 4-hydroxy 2-nonenal (4-HNE) on mRNA expression (in arbitrary units) in QM7 cells of: A) nuclear factor (erythroid 2)-like 2 (NFE2L2), B) Kelch-like ECH-associated protein 1 (Keap1), C) Cullin3 (CUL3), D) superoxide dismutase 1 (SOD1), E) superoxide dismutase 2 (SOD2), and F) glutathione peroxidase 1 (GPx-1).
Bars represent the mean $\pm$ SE (n=4).
ab Values with different letters are significantly different ($P < 0.05$).

CHAPTER 3

I. Overall Conclusion

Although this study shed light on NFE2L2 mRNA expression in avian muscle individually phenotyped for FE, more studies are needed to understand the full complexity of the role NFE2L2 may play in the feed efficiency phenotype. Data acquired from the QM7 cells studies suggest intricate cross talk occurs within the oxidative stress pathways that modulates NFE2L2 expression as well as its regulators and downstream targets.

APPENDIX

Table A1: Abbreviations and Definitions

NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
bZIP	Basic-region leucine zipper
Neh	NFE2L2-homologous domains
CDDO-le CDDO-Me	Imidazole and methyl ester derivative of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
Maf Protein	Small protein that heterodimerizes with NFE2L2 upon activation in the nucleus
ARE	Antioxidant response element
KEAP1	Kelch-like ECH-associated protein 1
Bric-a-Brac	Region of the KEAP1 protein
CUL3	Cullin-3
SQSTM1 (p62)	Sequestosome 1
H_20_2	Hydrogen peroxide
SOD1	Superoxide dismutase 1 [Cu-Zn]
DJ-1 (Parkin7)	Parkinson disease protein 7
SOD2	Superoxide dismutase 2 [Mn]
GPx-1	Glutathione peroxidase 1
GSH	Glutathione
L-FABP	Fatty acid-binding protein 1
4-HNE	4-hydroxy-2-nonenal
FE	Feed efficiency
LS	Low stress
HS	High Stress
RFI	Residual feed intake
ALS	Amyotrophic lateral sclerosis
KLH40	Kelch-like-protein 40

Table A2: Pedigree Male Broilers Phenotype Data

Bird ID	Gender	Phenotype (FE)	FE	FI (g)	Gain (g)
G1	M	High	.630	920	580
G4	M	High	.652	1080	704
G5	M	High	.664	1012	672
G10	M	High	.659	844	556
G16	M	High	.631	996	628
B2	M	Low	.483	960	464
B6	M	Low	.455	1032	470
B7	M	Low	.444	974	432
B11	M	Low	.453	1112	504
B13	M	Low	.468	1030	482

Table A3: LS Japanese Quail Phenotype Data

Bird ID	Gender	Line (Stress)	Phenotype (FE)	FE	FI (g)	Gain (g)
20	M	L	H	.182	214.5	39
42	M	L	H	.220	168	37
33	M	L	H	.185	194.5	36
8	M	L	H	.194	185	36
40	M	L	H	.186	204.5	38
28	M	L	H	.201	219	44
32	M	L	L	.130	247	32
29	M	L	L	.133	225.5	30
21	M	L	L	.138	188	26
23	M	L	L	.123	195.5	24
37	M	L	L	.136	257.5	35
16	M	L	L	.116	199	23

Table A4: HS Japanese Quail Phenotype Data

Bird ID	Gender	Line (Stress)	Phenotype (FE)	FE	FI (g)	Gain (g)
24	M	H	H	.215	195.5	42
10	M	H	H	.181	209.5	38
26	M	H	H	.237	181.5	43
15	M	H	H	.280	189	53
31	M	H	H	.217	211.5	46
30	M	H	H	.216	152.5	33
2	M	H	L	.126	230	29
36	M	H	L	.098	214.5	21
11	M	H	L	.121	206	25
6	M	H	L	.107	177.5	19
45	M	H	L	.113	230.5	26
41	M	H	L	.127	244.5	31

Table A5: Mature Japanese Quail Phenotype Data

Wing Band	Gender	Phenotype (FE)	FE	FI (g)	Gain (g)
9009	M	High	.537	218	117
9058	M	High	.504	252	127
9192	M	High	.539	282	152
9124	F	High	.483	327	158
9433	F	High	.454	284	129
9300	F	High	.485	272	132
17296	F	Low	.344	311	107
9663	F	Low	.344	329	113
9458	M	Low	.386	236	91
9589	M	Low	.371	324	120
17262	M	Low	.377	313	118
9685	F	Low	.299	342	102

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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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.

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cc: Animal Welfare Veterinarian

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