HOW DOES AGE AND DIETARY METHIONINE AFFECT THE TRANSCRIPTOME OF BROILER CHICKENS REARED UNDER THERMONEUTRAL AND HEAT STRESS CONDITIONS?

by

JAMES COPLEY FOUTZ

(Under the Direction of Samuel E. Aggrey)

ABSTRACT

Heat is a major environmental stressor in the poultry industry and results in significant economic loss through reduced weight gain and high mortality. The growth of the *Pectoralis major* is of great interest in broiler production, and heat stress limits growth of the muscle. To help mitigate the detrimental effects of heat stress on broiler production, research has turned to methionine supplementation, as this essential amino acid plays a significant role in processes affected by heat stress. Additionally, environmental intervention such as thermal conditioning looks to aid broiler production and resistance to heat stress. The general objective of this work was to understand the effect heat stress has on the broiler breast muscle transcriptome and how dietary DL-2-hydroxy-(4-methylthio)butanoic acid (MHA) or DL-methionine (DLM) changes this response. In addition, we sought to understand if and how thermal conditioning affects broiler performance and breast muscle transcriptome. The results of this work show heat stress downregulated the expression of genes related to aerobic respiration and the cellular cycle. When broilers were in the finisher phase of production, heat stress additionally upregulated proteolysis,

whereas this catabolic process was unaffected by heat stress in younger broilers. Similarly, the actin cytoskeleton underwent reorganization when subjected to heat stress at a young age; however, older broilers negatively expressed genes related to the actin cytoskeleton, indicating the cellular structure was less resilient at an older age or when heat stress was prolonged. Though broiler performance under heat stress did not improve with increased dietary inclusion of methionine, the transcriptome revealed above-requirement methionine likely benefits cellular processes, particularly at a younger age. Understanding how a broiler regulates these processes under heat stress and how dietary methionine alters this regulation is crucial in furthering the field of poultry nutrition. Performance and conventional biomarkers may not be sufficient when examining poultry under heat stress, as the transcriptome varies depending on diet and age of the bird.

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CHAPTER 1

INTRODUCTION

Heat is a major environmental stressor for poultry. Heat stress is induced when the amount of energy within the bird's body is greater than the energy exiting the bird. The imbalance creates a pooling of energy within the bird in the form of heat (Lucas and Marcos 2013). To dissipate heat and ease the rising temperature in the body, blood flow is diverted from the viscera to the periphery to facilitate heat exchange with the environment. To increase the exchange of heat, a bird will increase its surface area allowing for heat exchange via conduction and convection (Li et al. 2015). Evaporative cooling is additionally used by birds, exchanging heat between the air sac and inhaled air (Etches et al. 2008; Mutaf et al. 2009). These methods of cooling are in direct conflict with bird growth, as heat-stressed birds spend less time standing or moving, reducing feed intake (Mack et al. 2013).

Impaired growth during heat stress can also be attributed to reduced nutrient digestibility. In broilers, metabolizable energy and nitrogen retention were significantly altered by heat stress, resulting in poor performance (Bonnet et al. 1997). Additionally, the uptake of glucose, lipids, and amino acids are sensitive to heat stress (Sun et al. 2015; Habashy et al. 2017). This all leads to altered metabolism and rationing of nutrients, leading to catabolism of stored nutrients. Proteolysis is increased under heat stress (Yoshihara et al. 2015), and the limited number of available nutrients reduces protein synthesis in broiler skeletal muscles (Temim et al. 2000). Proteolysis is also induced when proteins become damaged (Chondrogianni et al. 2014). In an oxidative environment, oxidized amino acids can prompt protein degradation (Zhang et al. 2013). This is primarily done by the ubiquitin proteasome pathway (UPP) and the 26S proteasome (Baumeister et al. 1998).

Damaged proteins in a variety of tissue are prone to degradation under oxidative stress, including skeletal muscle and intestinal tissue. The oxidation and degradation of proteins involved with tight junctions between enterocytes can lead to a compromised intestinal barrier and a leaky gut, reducing nutrient uptake and exposing the animal to bacterial invasion (Bruewer et al. 2005; Quinteiro-Filho et al. 2012).

The oxidation and damage of proteins result from attacks by reactive oxygen species (ROS). Under heat stress, the diversion of blood to the periphery deprives cells of oxygen, inducing hypoxia (Hall et al. 1999). Oxygen is the terminal acceptor of electrons in electron transport, and the lack of adequate oxygen can lead to electron leak (Donnelly et al. 2012). Under normal conditions, 1% to 4% of useful oxygen is converted to superoxide by electron leak (Davies 1995; Jastroch et al. 2010). Superoxide and other ROS are handled and neutralized by the antioxidant defense of the mitochondria and cells. Glutathione is one such antioxidant present in both the mitochondria and cytosol of the cell (Lu 2009). This thiol-containing amino acid is highly active during heat stress (Habashy et al. 2019), and increasing methionine supplementation in the diets of heat-stressed broilers improves the oxidative status and increases glutathione synthesis (Zeitz et al. 2020).

Methionine is the first limiting amino acid in conventional broiler diets. Under thermoneutral temperatures, NRC (1994) recommends broiler diets contain 0.50%, 0.38%, and 0.30% methionine for starter, grower, and finisher phases, respectively. Under stressful conditions, these amounts may not be sufficient to maintain homeostasis of a broiler and may need to be increased. Previous work has shown increasing only essential amino acids, including methionine, benefits broilers subjected to heat stress (Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005; Soares et al. 2020). Suganya et al. (2015) recommend a 1-2% reduction in crude protein with a 5-

10% increase of essential amino acids under heat stress temperatures. In chronically heat-stressed broilers, the doubling of methionine inclusion in the diet increased weight gain (Balnave et al. 1999). The increased need for methionine under heat stress may be due to methionine's participation in immune response (Wu et al. 2012; Wu et al. 2013), protein synthesis (Kozak 1992), cell growth (Zhou et al. 2016), and its role in glutathione production (Zeitz et al. 2020).

Modern poultry producers supplement methionine in the diet through the inclusion of synthetic methionine, including D-, L-, or DL-methionine (DLM), or a methionine analogue. Only L amino acids are biologically available, therefore the conversion of the D enantiomorph or methionine analogue must be done for proper utilization by the bird. The added step of converting D-methionine to L-methionine has led to researchers questioning the efficacy of D-methionine. Research has shown D-methionine to be just as effective as L-methionine for broiler performance (Katz and Baker 1975; Dilger and Baker 2007; Lee et al. 2021). However, some work has shown L-methionine is more utilized than D-methionine in broilers aged 1 to 21 days (Wickramasuriya et al. 2019). This is likely due to reduced D-amino acid oxidase activity in young birds (Shen et al. 2015).

DL-2-hydroxy-(4-methylthio)butanoic acid (MHA) is the hydroxy analogue of DLM and is additionally used as a methionine source for poultry (Martín-Venegas et al. 2006). Unlike methionine, MHA is primarily absorbed through diffusion (Knight and Dibner 1984; Martin-Venegas et al. 2007), and not by amino acid transporters. As with the discussion around the efficacy of D-methionine, MHA supplementation has shown similar bird performance to DLM (Swick and Pierson 1988; Ribeiro et al. 2001), while additional work demonstrates MHA to be less effective for broiler performance (Balnave and Oliva 1990).

Regardless of the source, methionine supplementation improves the oxidative status of heat stressed-broilers (Del Vesco et al. 2015; Gasparino et al. 2018). However, nutritional intervention is not the only method by which poultry producers can use to improve health and performance of heat-stressed broilers. Thermal conditioning is thought to raise the temperature threshold for a bird to experience heat stress, allowing for improved health and performance. Exposing a bird to high temperatures early in development significantly lowers body temperature later in life. It is thought that lower body temperature is a sign of lower metabolic rate, which was indicated by reduced T3 levels (Yahav and Plavnik 1999; De Basilio et al. 2001). Heart size is also influenced by thermal conditioning, and broilers subject to hot temperatures early in life have reduced heart size, possibly influencing energy metabolism (Yahav and Hurwitz 1996). Along with a lower metabolic rate, thermal conditioning influences the expression of heat shock proteins (HSPs). By exposing birds to heat stress prior to subsequent stress challenge, thermally conditioned birds show reduced HSP expression compared to non-conditioned birds (Al-Aqil and Zulkifli 2009; Hiroshi et al. 2014). It is thought during the conditioning phase, HSP expression is increased. Those HSPs remain in the cell during the post-conditioning phase and the subsequent challenge phase, enacting protection (Krebs and Loeschcke 1994; Vinoth et al. 2015). Raising the bird's threshold for hat stress has proven advantageous in some scenarios (Abdel-Fattah et al. 2018; Oke et al. 2020), while other work has shown birds lack performance increases (Yahav and Hurwitz 1996; Yahav and McMurtry 2001; Kang et al. 2019). Ncho et al. (2021) conducted a current meta-analysis of thermal conditioning in broilers. Their work found the performance of thermally conditioned birds was highly correlated with the conditioning time and length of challenge. When subjected to acute heat stress, thermal conditioning can improve body weight, feed intake, and body temperature. When chronic heat stress is induced, the positive effect of thermal conditioning is limited.

The objectives of this work will look deeper into the mechanisms behind heat stress and the effect methionine supplementation and thermal conditioning have on broilers. The first objective will use transcriptomics to investigate molecular pathways influenced by heat stress in the broiler's breast muscle. Through the supplementation of different synthetic methionine sources at different inclusion levels, this objective will additionally determine molecular differences the diet enacts during heat stress. The second objective seeks to determine if a broiler will respond differently to heat stress if exposed to elevated temperatures during different phases of production. Also, this objective will investigate if methionine source or inclusion level changes the transcriptome. Finally, the third objective will test thermal conditioning's effect on performance parameters and the transcriptome response of broilers. Different diets will additionally be tested to determine the combined effect of thermal conditioning and methionine supplementation on heat-stressed broilers.

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CHAPTER 2

LITERATURE REVIEW

AMINO ACIDS

Amino acids play a critical role in the health, growth, and development of an organism. These organic compounds contain an amino group, a carboxyl group, and a unique sidechain all attached to a central carbon atom. This sidechain, called an R group, differentiates one amino acid from another (Lehninger et al. 2005). There are over 500 naturally occurring amino acids (Wagner and Musso 1983), however, only a fraction are used in genetic code. Proteins are formed using the 20 canonical amino acids during the process of translation, where mRNA codes for a sequence of these amino acids, forming a protein (Wu 2013). Along with being the fundamental building block of proteins, amino acids regulate protein synthesis and degradation. Amino acids promote protein synthesis as well as inhibit proteolysis, the process of breaking down proteins (Tesseraud et al. 2006; Tesseraud et al. 2011).

The classification of the 20 canonical amino acids is well known. The general classification for these amino acid sidechains are negatively and positively charged sidechains, uncharged polar sidechains, and hydrophobic sidechains. Additionally, cysteine, glycine, and proline are grouped as "irregular" and do not belong to a particular conventional grouping (Creighton 1993). There are ways to otherwise classify amino acids, including the classification of sulfur-containing amino acids.

Sulfur-containing amino acids

Sulfur, one of the most abundant inorganic elements in organisms, is present in three forms in animals. It is incorporated into proteins, either in a thiomethyl group within methionine or

present in the sulfhydryl group of cysteine. Sulfur is also found in sulfates as a part of steroids, glycosaminoglycans, or other ester or amide metabolites (Varin et al. 1997a; Varin et al. 1997b; Bick and Leustek 1998).

There are 4 common sulfur-containing amino acids, two of which are incorporated in proteins. Methionine and cysteine are both found in proteins whereas taurine and homocysteine are both free amino acids (Atmaca 2004; Brosnan and Brosnan 2006). These amino acids are notable because of sulfur's lower electronegativity compared to oxygen (Colovic et al. 2018). By incorporating sulfur instead of oxygen, the sulfur-containing amino acids are much more hydrophobic. Additionally, sulfur easily makes linkages with other sulfur atoms, called disulfide bonds, due to the easy dissociation of the thiolate ion (Brosnan and Brosnan 2006). These linkages are common between two cysteines, forming cystine. Unlike cysteine, serine contains a terminal hydroxyl group in the placement of the thiol group and does not easily make dioxide linkages.

Gomez-Tamayo et al. (2016) sought to further investigate the interactions of both methionine and cysteine with other amino acids. Because of the inclusion of sulfur, Van der Waals interactions were thought to be strong in instances including the sulfur amino acids. The researchers found the sulfur-containing amino acids displayed stronger interactions than either aliphatic or aromatic amino acids, arriving at the possibility of cysteine and methionine contributing significant forces which determine protein structure.

Methionine

Perhaps the most prominent role of methionine is its function as the initiating amino acid of protein synthesis. The codon AUG, coding for methionine, is almost always the start codon in eukaryotes. However, other codons have been found to initiate synthesis, though the exceptions are few (Kozak 1992). Although methionine is needed for protein synthesis in almost all cases, the

amino acid is not present in many proteins. This is because methionine is often removed post-translationally (Giglione and Meinnel 2001).

Methionine was first discovered in casein by scientist J.H. Muller (Baernstein 1932). It is a hydrophobic amino acid, mainly found in the hydrophobic core of globular proteins and in the hydrophobic layer of membrane proteins (Gomez-Tamayo et al. 2016). The organic sulfur is present in a thiomethyl group within methionine's sidechain. This review will further explore the properties and role of methionine metabolism.

Cysteine

The semi-essential amino acid, cysteine, is another sulfur-containing canonical amino acid. The thiol-containing side chain gives cysteine unique properties, most notably its ability to form disulfide bonds either within a protein or between two peptide chains. The bond is formed without the use of an enzyme, but enzymes such as disulfide isomerase can move around a mismatched disulfide bond to assist in protein folding (Brosnan and Brosnan 2006). The dimerization of two cysteine residues either intra- or inter-protein is referred to as cystine (Miseta and Csutora 2000). Because of this disulfide bond, cysteine is critical for proper protein folding and stabilization. Proteins utilizing cystine are commonly found within the plasma membrane (Colovic et al. 2018). However, the formation of disulfide linkages are not easy, as physical restraints can prevent bonds from forming. Because of the inherent rigid nature, disulfide linkages do not form between neighboring cysteine residues. The known alignment of disulfide linkages follows the rule of the C-$(X)_2$-C motif, where X is any coded amino acid (Miseta and Csutora 2000).

The classification of cysteine as either hydrophobic or hydrophilic is controversial. Incorporated within proteins, cysteine is classified as a hydrophobic residue (Janin 1979). However, in its free form, cysteine is difficult to classify as either hydrophobic or hydrophilic

(Damodaran and Song 1986). Marino and Gladyshev (2010) found cysteine was the least exposed residue within a protein structure, revealing cysteine to be a highly hydrophobic amino acid. However, this result does not agree with the known polarity of cysteine's sidechain, which contains a polarized group. When the group used a quantum mechanics-based approach, they found cysteine was highly polarized when the secondary structure was accounted for. Additionally, the polarity of cysteine was similar to that of serine, a polar amino acid. Together, Marino and Gladyshev (2010) conclude cysteine is considerably more hydrophilic than hydrophobic.

Homocysteine

Though methionine and cysteine are often referred to as the two sulfur-containing amino acids, there are multiple non-proteinogenic sulfur-containing amino acids. Most similar to cysteine, homocysteine contains an additional methylene bridge in addition to the terminal thiol group in its sidechain. Like cystine, homocystine is the product of two homocysteine residues linked by a disulfide linkage. This occurs at physiological pH when reduced homocysteine's thiol group is extremely reactive and likely to undergo auto-oxidation. Additionally, homocysteine has the ability to form a mixed disulfide with cysteine utilizing disulfide linkages (Harrington 2019).

Homocysteine is utilized as an intermediate amino acid during the methionine cycle, thus it is not obtained from the diet (Selhub 1999; Harrington 2019). The fate of homocysteine is determined by the body's requirement at that time; either re-methylated to methionine or converted to cysteine or other products through trans-sulfuration, both to be examined in depth later.

Taurine

Taurine, the most abundant free amino acid, is derived from cysteine or absorbed from the diet. The chemical structure is unlike conventional amino acids, as taurine lacks a carboxyl group. Its designation as a sulfur amino acid comes from the terminal sulfonate group. Taurine is present

in high concentrations in proinflammatory cells (Atmaca 2004), which could be because of its cytoprotective properties such as functioning as an antioxidant.

Methionine cycle

The metabolism of methionine and subsequently homocysteine and cysteine occur through a few different pathways. Overall, the transmethylation pathway utilizes methionine and yields homocysteine. The remethylation pathway regenerates methionine from homocysteine, while the transsulfuration pathway consumes homocysteine and yields cysteine. All three pathways are influenced by methionine content and their interactions are indicative of methionine levels within the system.

Transmethylation pathway

The transmethylation pathway has two additional sulfur containing compounds, S-Adenosylmethionine (SAM) and S-Adenosylhomocysteine (SAH). SAM is made up of adenosine linked to methionine at the 5' carbon of adenosine. This reaction is catalyzed by methionine adenosyltransferase (MAT) and requires ATP. All three phosphates are cleaved from ATP and adenosine is linked to the sulfur in methionine. The three phosphates leave as a single inorganic phosphate and an inorganic pyrophosphate, the latter being catabolized to yield energy (-19.2 KJ/mole) (Atmaca 2004). MAT is activated by SAM, its product. SAM also activates cystathionine β-synthase (CBS) and suppresses methylenetetrahydrofolate reductase (MTHFR). Therefore, high SAM levels promote transsulfuration and inhibit remethylation (Finkelstein 1998).

SAH is similar to SAM structure, only the sulfur is not methylated, resulting in homocysteine linked to adenosine. When looking at transmethylation, the key reaction is the conversion of SAM to SAH by a methyl transferase. There are a variety of methyl transferases that transfer the methyl group of SAM onto nitrogen, oxygen, and occasionally carbon. These

methyltransferases utilize a SN2 reaction where a base deprotonates either the nitrogen, oxygen, or carbon on the methyl acceptor. That forces the electrons from the acceptor to attack the methyl group of SAM. The methyl group on SAM is a hot electrophile because of the positive charge on the adjacent sulfur, which sulfur does not like. The leaving group for this reaction is SAH, and the product is the new methylated compound. A common methyl acceptor in this reaction is DNA, which often methylates the fifth carbon of a cytosine ring. DNA methylation is vital to many cellular processes including embryonic development, X-chromosome inactivation, genomic imprinting, gene suppression, and chromosome stability. Abnormal DNA methylation is also linked to a number of human diseases (James et al. 2008).

Once the methyl group has been removed and SAH is formed, SAH hydrolase (SAHH) hydrates SAH, releasing adenosine and producing homocysteine. This hydrolysis reaction serves as a regulator for methylation reactions. SAH inhibits many methyltransferases that utilize SAM as a methyl donor. Thus, the SAM/SAH ratio indicates the transmethylation and the use of SAM as a methyl donor. A decrease in the ratio points to reduced methylation capacity. Under normal conditions, SAH is in equilibrium with homocysteine, however the hydrolysis of SAH is rapid, maintaining a flux towards hydrolysis. There are then 2 paths for homocysteine. It can be re-methylated to methionine or enter the transsulfuration pathway (Selhub and Miller 1992; Mato and Lu 2007; Lu 2009; Zhang et al. 2016).

Remethylation pathway

If the fate of homocysteine is not transsulfuration, it can be re-methylated to methionine through the enzyme methionine synthase (MS). This reaction takes homocysteine and adds a methyl group donated from N^5-methyltetrahydrofolate (5-methyl-THF). The methyl group on 5-

methyl-THF is first transferred to vitamin B_{12} that synthesizes methylcobalamin, transferring the methyl group to homocysteine, creating methionine (Finkelstein 1998).

The important methyl donor in remethylation is 5-methyl-THF, which is produced and recycled in the folate cycle. This cycle starts with the intake of folate. Folate is reduced to dihydrofolate through dihydrofolatesynthase (DHFR), which oxidizes NADPH in the process. DHFR further reduces dihydrofolate to tetrahydrofolate (THF) and oxidizes NADPH to $NADP^+$. THF is methylated by serine hydroxymethyl transferase (SHMT), which consumes serine and releases glycine, ultimately producing 5,10-methylene-THF. Methylenetetrahydrofolate reductase (MTHFR) oxidizes NADPH and reduces 5,10-methylene-THF, freeing the methyl group to be transferred to homocysteine and producing 5-methyl-THF (Finkelstein 1998).

An important step in the methionine cycle involves the incorporation of choline. Choline is an essential nutrient that plays many roles in the cell, but the one played in the methionine cycle is the reduction of homocysteine. Choline’s structure is similar to homocysteine, the difference being the thiol group in homocysteine is an alcohol group in choline. Through the action of choline oxidase, betaine is formed from choline. Betaine will be the methyl donor and hand off its methyl group to homocysteine through betaine-homocysteine methyltransferase (BHMT). Betaine is a polar compound where the carboxyl oxygen has a negative charge and the nitrogen with 3 methyl groups has a positive charge. When a methyl group is transferred, the negatively charged oxygen picks up the hydrogen from homocysteine’s thiol group, enabling the methyl group to join and form methionine. This transfer creates dimethylglycine (DMG) which is released. DMG is oxidized and a methyl is released producing sarcosine. Sarcosine can be converted to glycine by a reversible reaction with the involvement of SAM and SAH and the transfer of a methyl group (Zeisel and da Costa 2009; Fu et al. 2016).

Transsulfuration pathway

Homocysteine can go through transsulfuration resulting in the production of cysteine. This is done by a couple of enzymes, the first being cystathionine-β-synthase (CBS) catalyzing a dehydration reaction. CBS produces cystathionine and water from homocysteine and the addition of serine, an alcohol containing amino acid. This step prepares for the formation of cysteine, which is produced in the following reaction catalyzed by cystathionine γ-lyase (CGL). The ammonia group is released from the former homocysteine, forming α-ketobutyrate, and the thiol group is linked to the former serine, which is now cysteine. α-ketobutyrate and cysteine are split through hydrolysis catalyzed by CGL. Regulation of this pathway is thought to be centered around CBS because of its sensitivity to oxidative properties. Antioxidants suppress the pathway where peroxidases increase the flux (Selhub and Miller 1992; Mato and Lu 2007; Aitken et al. 2011).

Cysteine now has 2 fates. It can either enter glutathione (GSH) synthesis or be converted to taurine. If it is converted to taurine, cysteine dioxygenase (COO) adds O_2 to produce cysteine sulfinic acid. Sulfinoalanine-decarboxylase (CSO) oxidizes cysteine sulfinic acid by removing CO_2 and producing hypotaurine, which then reduces NAD^+ to NADH by hypotaurine dehydrogenase and the addition of water to form taurine (James et al. 2008; Lu 2009).

Importance of the sulfur containing amino acids in poultry

Both methionine and cysteine have important roles in poultry immune health. Methionine is an essential building block of protein, which is a primary focus of broiler production. Additionally, methionine is a precursor for the strong antioxidant GSH. The essential sulfur amino acid is also critical for polyamine synthesis and DNA methylation. It is therefore important to monitor methionine supplementation in poultry as it plays a role in numerous pathways which influence bird health and production.

Methylation

The epigenetic modification of methylation is a widely explored topic in genetics. Briefly, methylation is a mechanism to modify the genome in response to the environment or other signals (Jaenisch and Bird 2003; Turner 2007). Through the addition of methyl groups onto CpG islands, genes can be silenced. Methionine is central in this epigenetic modification, as the amino acid gives rise to the methyl donor SAM, outlined above. By increasing methionine supplementation, hypermethylation can be induced (Tremolizzo et al. 2002; Dong et al. 2005), directly linking methionine intake to DNA methylation. Healthy levels of methylation assist in proper T cell proliferation (Lee et al. 2001) and proper inflammatory response (Hartnett and Egan 2012).

Antioxidant activity and immune system associations

The term reactive oxygen species (ROS) refers to free radicals containing oxygen such as superoxide, hydrogen peroxide, and hydroxyl radicals (Salami et al. 2015). An excess of free radicals can cause damage to biological molecules and create undue molecular stress (Surai et al. 2017). To combat these damaging ROS, antioxidants scavenge for ROS and interrupt oxidation (Halliwell and Gutteridge 1999). Some antioxidant compounds and amino acids which incorporate sulfur in their structure are methionine, cysteine, taurine, GSH, lipoic acid (LA), and N-acetylcysteine (NAC) (Atmaca 2004).

It is known low levels of methionine will induce remethylation in cells, reducing production of cysteine, GSH, and taurine. When examining K_m values for enzymes responsible for the recycling of homocysteine to methionine, values are two orders of magnitude lower than the K_m values for enzymes at the beginning of transsulfuration. This implies homocysteine will be re-methylated to methionine rather than be a product of transsulfuration when methionine levels are low (Grimble 2006). Production of GSH and taurine, both of which contribute greatly to proper

immune response, is then only feasible when proper dietary methionine levels are met, increasing flux of transsulfuration.

Muscle degradation allows proteogenic amino acids to be utilized for other tasks and systems (Aquilani et al. 2005), including being utilized by the immune system and to produce antioxidants when dietary methionine is reduced. During infections, loss of lean tissue is brought on by proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)–α (Watchorn et al. 2001; Schreiner et al. 2012). The degradation of lean muscle also increases urinary nitrogen and sulfur secretion. In humans, nitrogen secretion during heavy malaria infections is equivalent to the loss of 500 grams of protein per day. Sulfur secretion during times of infection are disproportionally lower compared to nitrogen secretion, suggesting catabolism of sulfur containing amino acids is reduced, perhaps due to the importance of these amino acids for immune function (Cuthbertson 1931). In rats, bacterial challenge has shown an increase in taurine and GSH production (Malmezat et al. 1998; Malmezat and Breuille 2000). Additionally, GSH function was artificially reduced by diethyl maleate injection. Compromised rats were then challenged with a sublethal dose of the proinflammatory cytokine TNF–α. The GSH-compromised rats experienced high mortality even at the sublethal dose, displaying the protective role of GSH (Zimmerman et al. 1989).

The production of ROS by the host's immune system activate nuclear transcription factor $\kappa\beta$ (NF-$\kappa\beta$) and activator protein 1 (AP1) (Newton and Dixit 2012). NF- $\kappa\beta$ exists in the cytosol of a cell in its inactive state, being bound to inhibitor of NF-κB protein (I $\kappa\beta$). It is a part of the rapid response primary transcription factors, meaning its activation is fast acting. Activation of NF-$\kappa\beta$ occurs through detection of proinflammatory cytokines, ROS, infection, or radiation. The dissociation from I $\kappa\beta$ when stimuli is detected activates NF-$\kappa\beta$, moving it to the nucleus where

it binds to the DNA at enhancer and promotor regions. The activation NF-$\kappa\beta$ and subsequent expression of genes leads to expression of inflammatory and immune responses which encode for cytokines and growth factors (Schreck et al. 1991). As mentioned previously, the increase in proinflammatory cytokines proves detrimental to lean muscle. Antioxidants scavenge for ROS, which reduces NF-$\kappa\beta$ activity, thus limiting proinflammatory cytokines. This can be seen in a study done by Pena et al. (1999), who saw an inverse relationship between GSH activity and the proinflammatory cytokines IL-1β, IL-6, TNF $-\alpha$ in human patients suffering from cirrhosis.

GSH does not only curtail proinflammatory response by reducing NF-$\kappa\beta$; it can also increase AP1 activity, thus increasing cellular proliferation. Similar to NF-$\kappa\beta$, AP1 is a family of fast acting primary transcription factors present in the cytosol in their inactive state and moving to the nucleus and activated when stimuli are detected. It has been shown in human immune cells that an increase in GSH brings about a rise in IL-2 production and lymphocyte proliferation while simultaneously reducing proinflammatory mediators (Wu et al. 1994).

Humeral and cellular immunity is greatly affected by methionine because of clonal proliferation of lymphocytes, effector molecule synthesis, and immunoglobulin affinity in the bursa; all of which need the sulfur containing amino acid to function properly. The process through which methionine contributes to immune response is not fully known, however it is thought that intracellular GSH and cysteine, both participants in methionine metabolism, contribute to immune cell proliferation (Wu et al. 2012; Wu et al. 2013; Rama Rao et al. 2021).

Kinscherf et al. (1994) looked at GSH concentrations of human T lymphocytes. In healthy individuals, $CD4^+$ and $CD8^+$ T cells had a positive relationship with intracellular GSH content. This relationship plateaued and eventually become negative between 30 and 50 nmol GSH per mg protein. When those individuals with optimal GSH levels began daily exercise, GSH

concentrations declined along with a decline in $CD4^+$ T cell numbers. Through the supplementation of N-acetyl cysteine (NAC), the reduction of $CD4^+$ T cells was curtailed. A study on immunosuppressed humans with HIV/AIDS observed the effects of NAC supplementation on the immune system. It was observed in a highly controlled study that supplementing 600 mg of NAC per day over a 7-month period had both an anti-inflammatory and an immunoenhancing effect in patients. Similar to previously mentioned works, the proinflammatory cytokine IL-6 concentrations were reduced while lymphocyte numbers increased (Breitkreutz et al. 2000).

Additional antioxidant properties of sulfur containing amino acids

All amino acids act, to some degree, as an antioxidant (Stadtman 1993). Thiols are especially strong antioxidants, so sulfur-containing amino acids are some of the strongest antioxidants. Methionine is susceptible to oxidation and is often vulnerable when exposed on the surface of a protein. The product of methionine oxidation is methionine sulfoxide (Levine et al. 1996). The oxidized forms of both cysteine and methionine can be restored by reductases. For methionine, methionine sulfoxide reductase reduces oxidized methionine back to methionine. However, even when oxidized, methionine retains its biological function, further proving its importance as an antioxidant and protection of protein structures (Fliss et al. 1983; Atmaca 2004; Zhang et al. 2013).

Cysteine is the limiting amino acid for taurine and GSH synthesis, proving critical for antioxidant function and regulation (Atmaca 2004). However, cysteine also functions as an antioxidant thanks to its sulfhydryl group. In the protein β-lactoglobulin, a cysteine residue within the structure was found to play a role in the antioxidant activity of β-lactoglobulin (Allen and Wrieden 1982). Recently, Kim et al. (2020) saw antioxidant effects of various sulfur amino acids, including cysteine, which protected food products from oxidative decay.

Numerous other amino acids and compounds function as antioxidants. Selenocysteine, an amino acid analogue of cysteine, contains selenol in the place of the thiol group (Johansson et al. 2005; Marino and Gladyshev 2010). Because of its lower reduction potential, selenocysteine is highly involved in antioxidant activity (Byun and Kang 2011). In response to increased ROS, human mesenchymal stem cells treated with selenocysteine increased the expression of selenoproteins while decreasing the expression of proinflammatory markers (Suh and Lee 2017).

Taurine is yet another example of an amino acid functioning as an antioxidant. To observe taurine's effect on oxidative status, Jong et al. (2012) inhibited the transport and utilization of taurine by the incorporation of β-alanine in cell culture medium. The researchers found reduced cellular taurine leads to impaired electron transport function in the mitochondria, resulting in superoxide production.

Dietary sources and conversions of methionine

Methionine cannot be synthesized by animals, but rather sourced from either plant products or from synthetic sources. Today, to supply a sufficient amount of methionine in broiler diets, synthetic methionine is the primary source for broilers. Synthetic methionine can be supplemented in various forms, including D-, L-, DL-methionine, or methionine analogue. Only L amino acids are biologically available, therefore the conversion of the D enantiomorph or methionine analogue must be done for proper utilization by the bird. It has been shown D-methionine has equal efficacy to L-methionine, however, when supplemented below requirement, bird weight gain may be delayed when supplemented with just D-methionine (Katz and Baker 1975). Consistent with those findings, Dilger and Baker (2007) found DL-methionine is equally efficacious as L-methionine when supplemented at required levels. However, some work has shown L-methionine is more

utilized than D-methionine in broilers aged 1 to 21 days (Wickramasuriya et al. 2019). This is due to reduced D-amino acid oxidase activity in young birds (Shen et al. 2015).

DL-2-hydroxy-(4-methylthio)butanoic acid (MHA) is the hydroxy analogue of DL-methionine and is often used as a methionine source for poultry (Martín-Venegas et al. 2006). MHA is absorbed primarily by passive diffusion, though H^+- and sodium-dependent transporters are also used (Knight and Dibner 1984; Martin-Venegas et al. 2007). Once absorbed, conversion from MHA to L-methionine begins in the intestine. Peroxisomal L-2-hydroxy acid oxidase and mitochondrial D-2-hydroxy acid dehydrogenase oxidize the alpha-carbon of L-2-hydroxy-(4-methylthio)butanoic acid and D-2-hydroxy-(4-methylthio)butanoic acid, respectively, to 2-keto-(4-methylthio) butanoic acid (KBM). D-2-hydroxy acid dehydrogenase is present in a large number of tissues including the intestine, liver, kidney, skeletal muscle, pancreas, spleen, and brain of birds while L-2-hydroxy acid oxidase is present in the liver and kidney. Transamination of KBM is carried out by a transaminase resulting in L-methionine (Dibner and Knight 1984; Dibner and Ivey 1992).

HEAT STRESS

A significant stressor in the poultry industry is heat (Surai et al. 2019). Heat stress is induced when the amount of energy entering the bird's body is greater than the energy exiting the bird. The imbalance creates a pooling of energy within the bird in the form of heat (Lucas and Marcos 2013). The two external factors determining the flow of energy in the form of heat are environmental temperature and humidity. If sensible heat loss is inhibited by higher temperatures, the bird's net heat loss through conduction, convection, and radiation are limited. Additionally, high ambient humidity limits latent heat loss of evaporation (Aggarwal and Upadhyay 2013). Upon experiencing a larger heat load, blood flow is diverted from the viscera to the periphery to facilitate

heat exchange, cooling the bird. This diversion of blood flow during heat stress leads to hypoxia and nutrient deficiency in various tissues including the liver, muscle, and intestines (Hall et al. 1999). This deficiency leads to disfunction because of energy depletion and increase in ROS, which compromises DNA, cellular structure, and gut integrity. Leaky gut results in an increased immune response, further compounding the inflammatory response.

Energy metabolism

Under heat stress, birds attempt to limit energy expenditure by remaining immobile and panting to facilitate heat loss (Mack et al. 2013). By reducing movement, the bird limits its energy expenditure and reduces ATP catabolism. Both the breakdown and synthesis of ATP are somewhat inefficient, and energy is lost in the form of heat during both anabolism and catabolism of ATP. The conversion of energy stored in glucose to energy stored in ATP is only 38% efficient under standard conditions. In the mitochondria, that efficiency is increased to about 50% (Beals et al. 1999). Endogenous heat production must be reduced in heat stressed birds; therefore, ATP production and consumption must be reduced. The reduction of ATP synthesis in the mitochondria is achieved by diffusing the proton gradient. Uncoupling facilitates the movement of protons across the inner membrane, bypassing ATP synthase (Mitsuru et al. 1997; Mujahid et al. 2005). Power et al. (2014) subjected intact rat hearts *ex vivo* to heat stress temperatures to observe the effects on energy production. It was shown when environmental temperatures were between 40°C and 43°C, there was loss of an adequate proton gradient in the mitochondria to maintain ATP synthesis. Both adenine nucleotide translocase and uncoupling protein 1 facilitate the movement of protons, reducing ATP synthesis (Jastroch et al. 2010). However, a sufficient amount of ATP must be produced to provide energy needs for cell survival. Wang et al. (2012) saw a decrease in Complex I and III activity in cells overexpressing heat shock protein 70, a highly conserved protein

greatly expressed during heat stress. When measuring ATP pools within those cells, overexpression of heat shock protein 70 had no effect on ATP levels, however. The authors attributed the net-zero loss to cellular ATP to increased glycolysis and anaerobic respiration.

During anaerobic respiration, pyruvate derived from glycolysis is instead converted to lactate by lactate dehydrogenase, regenerating NAD^+ used in glycolysis and ATP production. Ward and Peterson (1973) found circulating lactate dehydrogenase was higher in broilers subjected to acute heat stress than broilers kept at thermoneutral temperatures. Similarly, Melesse et al. (2011) saw higher levels of plasma lactate dehydrogenase in various breeds of layer hens subjected to high ambient temperatures. In humans, a deficient complex IV can cause numerous diseases, including as lactic acidosis; lactic acid buildup produced in anerobic respiration (Shoubridge 2001). Lactic acidosis has also been observed in humans with ATP synthase deficiency (Houštek et al. 1999). Ren et al. (2019) saw downregulation of genes related to oxidative phosphorylation and upregulation of genes encoding for phosphofructokinase, phosphoglycerate kinase, enolase, and pyruvate kinase in the tree species *Populus tomentosa* when subjected to heat stress. This all suggests glycolysis is more responsible for the generation of ATP during times of heat stress, and pyruvate is diverted away from the citric acid cycle towards anaerobic respiration.

Triiodothyronine (T_3) is a well-known regulator of metabolic rate in animals. Under heat stress, circulating T_3 is significantly reduced (Singh et al. 2015; Beckford et al. 2020), contributing to reduced ATP synthesis and altering energy metabolism. Sterling et al. (1980) found binding sites for T_3 on the mitochondria, and injection of the thyroid hormone greatly increased mitochondrial activity. Recent work has shown two T_3 binding proteins incorporated in the mitochondrial membrane greatly influence cellular events via the p43 pathway (Wrutniak-Cabello

et al. 2018). Additionally, hyperthyroidism and the increase of T_3 activity greatly increase cellular respiration and energy production (Venediktova et al. 2020).

Oxidative stress

High environmental temperatures are known to produce an oxidative environment within skeletal muscle tissue. By the diversion of blood to the periphery, oxygen and nutrient availably to more internalized tissue and cells are reduced, inducing hypoxia (Hall et al. 1999). Oxygen is the terminal acceptor of electron in electron transport, and the absence adequate oxygen can lead to electron leak (Donnelly et al. 2012). In poultry, skeletal and cardiac muscle contain the largest concentration of mitochondria (Reverter et al. 2017), so it can be expected that the breast muscle of modern broilers would be a highly oxidative environment. Under non-stressful conditions, 1% to 4% of useful oxygen is converted to superoxide by electron leakage through complexes I and III of the electron transport chain (Davies 1995; Jastroch et al. 2010). Additionally, the increase of mitochondrial derived ROS is positively corelated with mitochondrial generation of ATP (Green et al. 2004), meaning increased flux through the electron transport chain increases ROS generation. These amounts of ROS are controlled by the antioxidant defense of the cells (Pamplona and Costantini 2011), however heat stress can increase the generation of ROS to uncontrollable levels.

When produced at higher-than-normal levels, ROS cause damage and disruption to cellular structure, function, and metabolites including proteins, lipids, and nucleic acids (Fang et al. 2002). Under heat stress conditions, Feng et al. (2008) observed an increase in H_2O_2 production as well as an increase in lipid peroxidation in broiler muscle tissue, evident from malondialdehyde, a product of lipid peroxidation (Ismail et al. 2013). The oxidation of lipids by free radicals and other ROS leads to further lipid oxidation by means of newly formed lipid peroxyl radicals, creating a snowball effect detrimental to lipid metabolism and homeostasis (Benzie 1996). The oxidative

state of proteins can similarly be defined by the presence of protein carbonyl (Levine et al. 1994) which is common in muscle and liver tissue of heat stressed birds (Tan et al. 2010). Damage from oxidative stress can be seen in other tissues, as examined by Banan et al. (2001) who saw cellular damage to intestinal epithelial cells, reducing intestinal integrity. Damaged intestinal epithelium compromises the barrier between the bird and intestinal pathogens, as well as decreases bird performance by affecting nutrient absorption (Quinteiro-Filho et al. 2010).

GSH has long been known to be a powerful antioxidant for organisms (Hopkins and Dixon 1922). The majority of GSH is located in the cytosol of the cell, with about 15% additionally located in the mitochondria (Lu 2009). GSH peroxidase can act on a number of oxidants, further cementing its central role in antioxidant defense (Marí et al. 2009). In the mitochondria, GSH can neutralize superoxide generated directly from electron leak while also defending against H_2O_2. The nature of H_2O_2 allows the oxidant to traverse through the mitochondrial membrane when not neutralized by antioxidants in the mitochondria, causing damage to other organelles and components of the cell (Winterbourn 2013). The great presence of GSH in the cytosol again acts on traveled oxidants, including lipid radicals. Malondialdehyde is a product of lipid oxidation, and the formation of this enol is elevated during heat stress. During both acute and chronic heat stress, broilers display an increase in malondialdehyde concentrations (Wang et al. 2009; Azad et al. 2010). This also coincides with GSH activity in both skeletal muscle and the liver (Habashy et al. 2019), indicating the need for GSH during oxidative stress.

Immune system

It is important to note how oxidative stress is an initiator for an inflammatory reaction. Ca^{2+} signaling is well known as a secondary messenger for many processes within the cell, including immune regulation (Feske et al. 2001). Oxidative stress is known to increase intracellular Ca^{2+}

(Roveri et al. 1992), resulting in the activation of lymphocytes through the activation of transcription factors such as NF- $\kappa\beta$ and AP-1. The expression of IL-1 also initiates NF- $\kappa\beta$ translocation, furthering inflammatory immune response (Oeckinghaus and Ghosh 2009). IL-1 is the primary cytokine and is highly influential in the cellular response to stressors (Croston et al. 1995). In addition to initiating NF- $\kappa\beta$ translocation, IL-1 activates of the inflammatory cytokines interferon gamma (IFNγ), IL-12, and IL-18 (Zhang and An 2007; Borge et al. 2009; Turner et al. 2014). In response to inflammatory cytokines, anti-inflammatory cytokines act to control the expression of inflammatory cytokines, preventing further damage to host cells. This is achieved primarily by the inhibition of inflammatory cytokine receptors by the major anti-inflammatory cytokines IL-4, IL-6, IL-10, IL-11, and IL-13 (Opal and DePalo 2000).

Damage done by inflammatory cytokine activation can cause more harm to the cell or organism than the stressor which caused the primary activation. This is evident in the gut, where inflammatory cytokines influence gut integrity (Al-Sadi et al. 2009). Two of the major proteins which make up tight junctions, occludin and claudin-1, undergo macropinocytosis when IFNγ is highly expressed, leading to a compromised barrier (Bruewer et al. 2005). Increased oxidative damage to the enterocyte caused by ROS also leads to leaky gut (Quinteiro-Filho et al. 2012). Tight junctions form a barrier between epithelial cells, protecting against pathogens and pathogenetic molecules. These tight junctions are formed around the circumference of each cell near the apical membrane and are comprised of numerus proteins which anchor each cell to another. During heat stress, the dysfunction of electron transport in the mitochondria produces ROS, which can damage proteins forming tight junctions. Numerous studies have identified the interaction between ROS and tight junction proteins (Kevil et al. 2000; Rao et al. 2002; Basuroy

et al. 2003; Sheth et al. 2003), and it has become clear ROS play a direct role in leaky gut, resulting in immune activation.

Lambert et al. (2002) investigated intestinal damage by heat stress in rats using FITC-dextran. They saw an increase in intestinal permeability when rats were stressed with a core body temperature of 41.5^{o}C or above compared to 37°C. Histology further displayed epithelial damage in heat stressed rats. Other alterations to the intestine during heat stress include increased pathogenic bacteria presence. When housed at 30°C for 24 hours, market weight broiler chickens had an increased load and attachment of *Salmonella* Enterditis in the ileum, as well as an altered gut microbiome (Burkholder et al. 2008).

As a result to compromised intestinal integrity, lipopolysaccharides derived from bacteria cross the epithelium and trigger an immune response (Andreasen et al. 2008). Lipopolysaccharides have been shown to increase TNF–α and IL-6 expression (Lin et al. 2000), indicating an inflammatory reaction and immune activation. The activation of immune cells from increased Ca^{2+} signaling and detection of lipopolysaccharides increases the expression of nitric oxide (Ikeda et al. 1996). Nitric oxide is a reactive nitrogen species (RNS) which is secreted by activated macrophages (Shapira et al. 2000) and acts as a signal transducer for immune regulation (Ignarro 1991). Work in mice has shown angiopoietin-like 4 (ANGPTL4) to be a potent regulator of nitric oxide and inflammation (Chong et al. 2014). Both Lan et al. (2016) and Coble et al. (2014) identified the importance of *ANGPTL4* during heat stress. Their research used transcriptomics to identify integral pathways in the liver of heat stressed birds, leading to the identification of *ANGPTL4* as an important marker for heat stress and resistance in poultry. In addition, Coble et al. (2014) linked *ANGPTL4* to endocrine system development and function through gene ontology, along with the genes *CCK*, *TRPC5*, *DIO2*, and *DIO3*. This agrees with Uribe et al. (1999), who

saw that increased secretion of nitric oxide stimulated the hypothalamic-pituitary-adrenal (HPA) axis.

The HPA axis controls an organism's stress response by regulating biological systems including digestion, immune activity, and energy metabolism (Tsigos and Chrousos 2002; Smith and Vale 2006). When experiencing stress, corticotropin-releasing hormone and arginine vasopressin are secreted by the hypothalamus. The release of these hormones stimulates the pituitary gland to release adrenocorticotropin hormone, which in turn stimulates the release of glucocorticoids from the adrenal cortex (Turrin and Rivest 2006). In poultry and humans, corticosterone and cortisol are the major glucocorticoids, respectively. Adrenocorticotropin hormone secretion additionally activates the locus caeruleas/norepinephrine system, which controls the fight or flight response of the organism (Jansen et al. 1995). The role of glucocorticoids is to prioritize short-term processes which facilitate immediate wellbeing and survival. Under acute glucocorticoid secretion, an organism may better deal with metabolic and immune changes, however chronic exposure to glucocorticoids can result in dysfunction and disease (Bronstein 2011).

To assist in the fight or flight mechanism, glucocorticoids inhibit insulin signaling to prevent glucose storage and facilitate energy production. Along with this, cortisol acts as an anti-inflammatory agent, suppressing the overall inflammatory response while prioritizing local inflammatory responses (Heim et al. 2000; Tsigos and Chrousos 2002). The anti-inflammatory effect of glucocorticoids are so powerful in fact, corticosteroids are often supplemented to patients with chronic inflammatory diseases (Barnes 2010). During long term exposure, glucocorticoids inhibit growth and reproduction, negatively impacting immune tissue and cell proliferation. The suppression of insulin signaling also leads to increased insulin levels, increasing fat deposition

during times of prolonged stress. The increase in fat deposition initiated by prolonged glucocorticoid secretion increases oxidative stress and expression of inflammatory cytokines (Palmieri et al. 2006).

It has been shown that heat stress reduces white blood cell counts (Mashaly et al. 2004), decreases the percentage of peripheral blood lymphocytes (Borges et al. 2004), reduces spleen weight (Trout and Mashaly 1994), decreases macrophage activity (Bartlett and Smith 2003), and reduces CD4+ and CD8+ lymphocytes (Khajavi et al. 2003). When constant stress was replicated by the continuous administration of adrenocorticotropin in broilers, there was an increase in heterophile:lymphocyte ratio as well as a reduction in lymphoid organ size (Puvadolpirod and Thaxton 2000). Lower spleen, thymus, and bursa of Fabricius weights in heat stressed broilers have been linked to the high levels of circulating corticosterone (Sapolsky et al. 2000; Quinteiro-Filho et al. 2010; Jahanian and Rasouli 2015). As discussed, the release of cortisol and the effect on the immune system is time-dependent. Acute stress can stimulate immune activation and enhance innate and adaptive immunity, while chronic stress suppresses the immune system by reducing immune cell proliferation and function (Dhabhar 2009). Lan et al. (2016) saw a similar effect in the liver of heat stressed birds. Through transcriptomics, the researchers determined acute heat stress activated immune function while prolonged stress reduced immune response.

Cytoskeleton and focal adhesion

The cytoskeleton of a cell is comprised of microtubules, intermediate filaments, and microfilaments. Actin is the primary protein of microfilaments and is present in all eukaryotic cells in various forms. Although there are many isoforms of actin, each has a distinct structure and role in the shape, motility, anchorage, and signaling of the cell. During myogenesis, actin plays a crucial role in cell fusion. Various actin-binding proteins interact with actin filaments to regulate actin

growth, organization, and signaling which contributing to the growth and structure of a cell. The Rho family of small guanosine triphosphateases (GTPases) interact with actin to control focal adhesion and actin stress fibers. Small GTPases also activate mitogen-activated protein kinases (MAPK), a part of a signaling cascade. HSPs also bind to actin when under stress, attempting to mitigate damage to the cellular structure. sHSPs are primarily responsible for this action, responding to stress, cell growth, apoptosis, and signal transduction. ROS commonly attack cysteine and methionine residues in actin monomers. Three methionine residues and one cysteine residue are fully exposed to the cytoplasm while 4 other methionine residues are additionally vulnerable to oxidation (Dalle-Donne et al. 2002; Dalle-Donne et al. 2003). When challenged with heat stress or oxidative stress, cells overexpressing HSP27 showed higher stability of actin and more rapid recovery (Lavoie et al. 1993a; Lavoie et al. 1993b; Huot et al. 1996). Additionally, HSP25 expression is elevated during the first phases of myogenesis (Dubińska-Magiera et al. 2014; Thakur et al. 2019), indicating protection of actin filament assembly and organization during the development of muscular tissue. In addition to ROS disrupting actin structure, proteases such as calpains target skeletal muscle for degradation. Oxidative stress increases the activity of calpains (McClung et al. 2009; Dargelos et al. 2010), which cleave cytoskeletal proteins such as actin (Goll et al. 2003). Just as actin is a part of initial formation of cells, the actin cytoskeleton is one of the last structures to be degraded during the final stages of apoptosis (Coleman and Olson 2002). Nakashima et al. (2004) used chicken thigh muscles *in vitro* to study the effects of heat stress on myofibrillar proteolysis. They found 6 hours of heat stress lowered protein content, and 24 hours of heat stress lowered the content even further. Additionally, proteasome activity was elevated at the two time points, and calpain activity was significantly higher after 24 hours of heat stress. Cruzen et al. (2017) discovered mechanisms that prevent actin disassembly expressed in the

muscle after 4 hours of heat stress. Overall actin abundance did not decrease as time progressed to 6 hours, however, there was significantly less actin present after 12 hours of heat stress.

Focal adhesion complexes are a part of the mechanical and signaling mechanisms of a cell with the extracellular matrix (ECM) (Zaidel-Bar et al. 2004). These focal adhesions are comprised of integrin protein structures which link the cytoskeleton to the ECM. In skeletal muscle, focal adhesions function as force transducers, transferring mechanical forces from one cellular structure to the next. Focal adhesion kinase (FAK) is involved with these cell-to-cell and cell-to-ECM interactions and plays a role in focal adhesion complex formation. During FAK inhibition, apoptosis is induced (Hungerford et al. 1996; Wei and Vander Heide 2008), while FAK activation protects against apoptosis (Frisch et al. 1996). It has been shown FAK is a target of oxidative stress, resulting in apoptosis in epithelial cells (Mian et al. 2008). When presented with ROS, cellular stress fibers and focal adhesions were disrupted, coinciding with the reduction of FAK activity (Schneider et al. 1998). When apoptosis was induced by compromising microtubules, cells became more rounded and more migratory, a sign of reduced focal adhesions (Kook et al. 2000). In poultry, birds exposed to heat stress significantly regulated genes associated with focal adhesion in the breast (Park et al. 2019) and liver (Emami et al. 2020).

Apoptosis and proteolysis

Damage to the cellular structure and adhesion complexes are not the only way heat stress and ROS arrest cellular development and proliferation. It is known that oxidative environments induce DNA damage (Kantidze et al. 2016). Ricci et al. (2008) investigated the occurrence of apoptosis when mitochondrial DNA was damaged. The researchers found apoptosis occurred in compromised mitochondria, along with impairments in cellular respiration and mitochondrial

protein expression. Mitochondrial dysfunction and apoptosis subsided upon DNA repair, indicating DNA damage plays a significant role in apoptosis.

In addition to compromised mitochondrial DNA, genomic damage causes problems in the cellular cycle, leading to cell damage and death. Under heat shock, the checkpoints at G1/S and G2/M transitions are compromised (Elledge 1996). Cell-dependent kinases (Cdks) drive the cell cycle, and their binding to different cyclins determine their activation during a certain stage of the cell cycle (Noble et al. 1997). Cdk1 and cyclin A are active in the late S phase to early M phase while Cdk1 and cyclin B are active from early G2 phase to mid M phase. Cdk2 and cyclin E are active from mid G1 to mid S phases while Cdk2 and cyclin A are active from early S to mid G2 phases. Cdk4 and Cdk6 bind to cyclin D and are active during the G1 phase (Morgan 1997). Inactivation of the G1 phase has been contributed to inhibition of the MAPK signaling pathway (Nebreda 1994). However, short term heat stress has been shown to increase MAPK signaling, inducing cellular growth (Dubois and Bensaude 1993). Liu et al. (2015) concluded MAPK activation is a downstream event to ROS accumulation, leading to anti-apoptotic events. Additional research revealed that the expression of receptor-interacting protein 3 (RIP3) is elevated in cells subjected to heat stress, leading to apoptosis through MAPK regulation (Huang et al. 2020).

Apoptosis is also correlated with proteolysis. The ubiquitin proteasome pathway (UPP) is a major proteolytic pathway and involves the linking of ubiquitin onto intracellular proteins, marking them for degradation. The protease complex 26S proteasome then degrades the targeted protein into small peptides (Baumeister et al. 1998). The enzymes responsible for marking the intracellular proteins are ubiquitin-activating enzyme (E1), ubiquitin-carrier proteins (E2), and ubiquitin-protein ligase (E3) (Glickman and Ciechanover 2002). Ubiquitin is a 76 amino acid

protein which does not exist freely, rather as polyubiquitin chains or attached to ribosomal subunits. Deubiquitinating enzymes free ubiquitin, enabling its utilization for proteolysis. E3 serves the main function of UPP, and there are over 1,000 E3s (Jackson et al. 2000), each associated with specific substrates.

It is well known that biological components such as amino acids are subject to oxidation. This oxidation and subsequent damage induce proteolytic processes (Zhang et al. 2013). It has been shown that an increase of ROS stimulates the expression of E2 and E3 in myocytes (Li et al. 2003). The means by which stress stimulates the UPP is not fully characterized. When exposed to alcohol-related stress, atrophied cells greatly upregulated the expression of various cytokines and oxidative markers, including antrogin-1. Antrogin-1 is believed to be responsible for the activation of the UPP through its relationship with FoxO1 signaling (Furukawa et al. 2016; Pomiès et al. 2016). In broilers, the reduction of atrogin-1 expression in the muscle reduced the occurrence of proteolysis (Li et al. 2019). Zuo et al. (2015) additionally found protein degradation was increased in the thigh muscle of heat stressed broilers. Glucocorticoids have long been known to induce proteolysis (Schakman et al. 2008) and inhibit protein synthesis (Kostyo and Redmond 1966; Shah et al. 2000). Inhibition of protein synthesis is likely the result of mTOR inhibition by glucocorticoids (Wang et al. 2006; Southgate et al. 2007). Additionally, glucocorticoids inhibit proper growth factor function (Gayan-Ramirez et al. 1999), attenuating growth.

Physiological response

When a bird is unable to dissipate their internal heat into the environment and their body temperature begins to rise, the molecular mechanisms outlined above react to allow for the best response to combat oxidative stress and other deleterious effects. In addition, a bird will alter its physiological response to further dissipate internal heat. As mentioned, blood flow will move

towards the periphery of the bird to exchange heat from the blood to the environment. Birds often spread their extremities when experiencing heat stress, which increases the bird's surface area allowing for heat exchange via conduction and convection (Li et al. 2015). Additionally, poultry increase heat exchange via evaporative cooling (Etches et al. 2008; Mutaf et al. 2009). Hillerman and Wilson (1955) observed laying hens acclimated to 60°F showed a significant spike in body temperature when the environmental temperature rapidly changed to 95°F. In addition, respiration rate increased from about 40 to 70 breathes per minute during the rapid temperature change. Panting enables heat within the body of the bird to be released through gas exchange. Air sacs within the bird are extremely thin, which enable gas exchange needed for respiration. The thin air sacs are also ideal for heat exchange, allowing the bird to cool, which is further increased by panting (Fedde 1998).

It is thought broilers can dissipate over 80% of their heat production through evaporative cooling (Esmail 2012). This requires moisture loss from the bird, and it has been shown water loss is increased upon heat stress (Abdelqader and Al-Fataftah 2014). Water consumption is therefore increased to replenish moisture lost from evaporative cooling when heat stressed (May and Lott 1992; Aswathi et al. 2019).

Performance

The preference for a bird to rest during heat stress limits feed intake (Mack et al. 2013). Mello et al. (2015) saw 19% lower body weight in heat stressed broilers at 35 days of age. Feed conversion over that period was increased 15% and feed intake was numerically decreased. Corticosterone regulates feed intake and the satisfaction of feeding, and when feed intake is reduced, weight gain is subsequently affected (Costa-Pinto and Palermo-Neto 2010). When administering exogenous corticosterone to simulate stress in broilers, bird performance was

significantly reduced (Post et al. 2003). However, decreased weight gain is not fully contributed to lower feed intake. When heat stressed broilers were pair-fed with their thermoneutral counterparts, birds under stress had reduced weight gain even with similar feed intake (Dale and Fuller 1980; Geraert et al. 1996). These results suggest mechanisms other than feed intake contribute to decreased performance, such as reduced protein accretion, altered gut microbiome, and decreased nutrient digestibility.

Reduced broiler performance is partly attributed to impaired digestibility of nutrients. Bonnet et al. (1997) subjected broilers to a constant ambient temperature of 32°C from 4 to 6 weeks of age. They found heat stressed birds had lower digestibility of a conventional corn and soybean diet compared to unstressed birds. Metabolizable energy and nitrogen retention was also lower in heat stressed birds. There is conflicting evidence in the literature as to the effect of heat stress on metabolizable energy, as some studies suggest no change in metabolizable energy (Husseiny and Creger 1980; Keshavarz and Fuller 1980; Geraert et al. 1992) while others do see a decrease (Yamazaki and Zi-Yi 1982; Bonnet et al. 1997). Sun et al. (2015) investigated nutrient transport in the jejunum of broilers subjected to heat stress. They found glucose and lipid transporters were more greatly affected by heat stress than amino acid transporters. Coincidently, body weight was significantly affected. This agrees with Habashy et al. (2017) who saw increased expression of glucose transporters in the ileum of heat stressed broilers. Additionally, PepT1, a protein transporter, was upregulated in the ileum, suggesting fasting of heat stressed broilers increases the need for protein uptake.

Protein synthesis, as well as degradation, has been shown to be greatly influenced by ambient temperature. Temim et al. (2000a) observed significantly reduced protein synthesis in chronically heat stressed broilers. To counteract reduced muscle growth, the researchers fed an

increased crude protein diet to heat stressed birds. The attempt failed as heat stress lowers the capacity for protein synthesis, preventing high protein diets from affecting muscle protein content. Cheng et al. (1997) saw similar results, as broilers fed either low or high protein diets had no change in body weight when both groups were subjected to heat stress. Protein utilization was markedly decreased in heat stressed birds, regardless of crude protein content in the diet. It has been shown that protein deposition during heat stress varies from tissue to tissue. In the breast muscle, protein synthesis is reduced while in the leg muscle, protein degradation is increased (Zuo et al. 2015). Further, Yoshihara et al. (2015) determined heat stress regulated proteolysis differently dependent on the muscle group and usage in rat skeletal muscle.

Protein degradation, limited nutrient uptake, reduced feed intake, impaired immunity, and infection through leaky gut all lead to decreased bird performance and meat quality during heat stress (Hurwitz et al. 1980; Niu et al. 2009; Attia et al. 2011; Imik et al. 2012; Sohail et al. 2012; Habashy et al. 2017; Awad et al. 2018; Goo et al. 2019; Lin Law et al. 2019). Because of these losses in production, it is estimated that the poultry industry endures an economic loss of $128 to $165 million annually due to heat stress (St-Pierre et al. 2003). It is therefore vital to mitigate the detrimental effects of heat stress to improve the bird's wellbeing and performance.

Current methods of mitigating heat stress

Birds are capable of releasing heat through conduction, convection, and radiation by increasing respiration, increasing surface area, and decreasing activities which produce body heat. However, a bird can only dissipate the amount of heat an environment will allow. Saeed et al. (2019) provides a current review of methods for reducing the stress of heat on poultry. Current methods include altering the environment through housing management such as insulation and ventilation, the reduction of other stressors, genetics, and nutrient supplementation.

Ambient temperature, relative humidity and air velocity are three environmental factors that greatly affect a bird's ability for thermoregulation. When housed in a thermoneutral environment, poultry maintain a deep body temperature of 41.2^oC to 42.2^oC. When broiler breeders were subjected to ambient temperatures ranging from 31 to 33^oC for 4 hours, deep body temperature jumped from about 41.5^oC to almost 43.5^oC during the 4 hours of raised ambient temperature. However, when birds experienced tunnel ventilation (air velocity 3.6 m/s) during the 4 hours of raised ambient temperature, deep body temperature was only elevated to just over 42.5^oC (Hamrita and Conway 2017). Yahav et al. (2004) found when broilers were housed at 35^oC from 5 to 7 weeks of age, air velocity had a negative correlation with performance with a peak in negative performance at 2 m/s. Performance did improve, however, when air velocity was increased to 3 m/s. This result is puzzling, as it was thought increased air movement would alleviate the effects of heat stress and improve broiler performance. It was concluded increased air velocity has deeper deleterious effects, such as contribution to surface water loss, which ultimately harm the bird more than heat stress to a certain extent. It is therefore important to balance ambient temperature, relative humidity, and ventilation for optimal performance.

THERMAL CONDITIONING

Adaptation occurs in three levels: genetic, phenotype, and epigenetic (Nichelmann and Tzschentke 2002). Genetic adaptation is an encapsulation of all morphological, physiological and biochemical traits of a genetically fixed organism to its environment. Phenotypic adaptation occurs during the lifetime of an organism, altering the control system and partially influencing morphological characteristics. Epigenetic adaptation occurs through gene expression, causing an organism's adaptation to an environment.

In humans, it has been shown stress inducing environments or events enact epigenetic changes. During the winter of 1944 to 1945, Germany imposed a food embargo for parts of Europe, resulting in famine. Because health records remain intact, Bastiaan et al. (2008) were able to trace individuals during this time to study the effects of prenatal stress. Their investigation focused on the methylation of the gene insulin-like growth factor II (IGF2). Based on their previous work, methylation of IGF2 under normal conditions is consistent up to middle aged humans. They found methylation of IGF2 was significantly decreased in middle-aged people conceived during the time and site of the famine compared to their siblings not conceived during the famine. It is believed the reduced methylation was caused by a reduction in methyl donors normally present within the methionine cycle. The study shows environmental conditions can enact epigenetic changes still present well after development and maturity in humans.

Thermal conditioning exposes pre- and postnatal animals to stressful environmental temperatures in the hope of developing adaptations to better suit the stressful environment later in life. It is believed thermal conditioning lowers the threshold at which an animal will be stressed (Yahav and McMurtry 2001). Work has demonstrated exposing a bird to heat stress temperatures during the first days post hatch significantly lowers body temperature later in life. It is thought the lower body temperature is a sign of lower metabolic rate, which is indicated by decreased T_3 levels (Yahav and Plavnik 1999; De Basilio et al. 2001). Work done in humans has shown the benefits of heat acclamation are gained during the first 7 days of initial heat exposure, with the majority of acclimation occurring during days two and three (Eichna et al. 1945). In plants, environmental temperature plays a role in epigenetics. Vernalization causes early flowering in plants which were exposed to unusually low temperatures. The Flowering Locus C gene is a primary gene behind this response and is controlled via DNA methylation. It has been shown that there is a quantitative

relationship between cold temperature exposure and downregulation of the Flowering Locus C gene (Candice et al. 2000).

Varying age and length of thermal conditioning seems to alter biological response to heat stress. When fruit flies were exposed to high temperatures at a young age for either one, two, or three regimens of thermal conditioning, those exposed to three regimens performed the best. However, the increase in bouts of thermal conditioning did not attain a linear increase in performance. The best performance jump was from no thermal conditioning to one bout of thermal conditioning. The performance jump from one to two bouts was less than none to one, while the jump from two to three bouts of thermal condition showed an even lower increase in performance. The plateau in performance gain is likely due to post-transcriptional regulation. When exposed to stress, mRNA is quickly transcribed, and a protein is produced. Upon removal of the stressor, transcription is quickly halted. However, the removal of a stressor does not immediately trigger the degradation of the protein. Because heat shock proteins still enact protective characteristics post-stress, another bout of stress (i.e. subsequent rounds of thermal conditioning) does not provoke the similar strong response as the first time encountering a stressor (Krebs and Loeschcke 1994).

When thermally conditioned during embryogenesis, broilers show downregulation of genes for various heat shock proteins (HSP 90 α, HSP 90 β, HSP 70, HSP 60, and HSP 27) in the brain compared to broilers newly exposed to heat stress at 42 days of age (Vinoth et al. 2018). Vinoth et al. (2015) found embryonic thermal conditioning enacts numerically higher protein expression of HSP 90 (alpha and beta) and significantly higher protein expression of HSP 70, HSP 60, and ubiquitin in the liver of non-stressed broilers at 42 days of age. These results are in accordance to the hypothesis put forth by Krebs and Loeschcke (1994) who assert proteins

manipulated by stress are long-lived, so proteins expressed during thermal conditioning are still present some time later, enacting protection against additional heat stress.

Stress conditioning can additionally be applied to help broilers cope with the stress of transport prior to slaughter. Al-Aqil and Zulkifli (2009) has shown subjecting broilers to cyclical heat stress or restricting feed intake increased the expression of HSP70. Those subjected to stress prior to transport also showed reduced increase in circulating corticosterone than broilers experiencing stress for the first-time during transport. In thermally conditioned chicks, plasma corticosterone levels were lower than newly heat stressed birds when both were subjected to 40°C environmental temperature at 10-days of age. Those thermally conditioned birds also showed lower gene expression of brain-derived neurotrophic factor, thyrotropin-releasing hormone, IL-6, and lipopolysaccharide-induced tumor necrosis factor (Hiroshi et al. 2014). Organ size is also a contributing factor to thermal conditioning's positive effect during heat stress. Heart size is decreased when broilers are exposed to hot temperatures for 24 hours at 5 days of age (Yahav and Hurwitz 1996). The reduced heart size is thought to reduce the metabolic load, helping thermally conditioned birds cope with heat stress conditions later in life. As mentioned, the cause behind lymphoid organ involution is thought to be related to HPA activation (Sapolsky et al. 2000; Jahanian and Rasouli 2015), an action which happens under heat stress (Quinteiro-Filho et al. 2010). Thus, thermal conditioning may reduce the size of metabolically demanding organs, reducing the metabolic load of the animal which better suites them for additional stress.

Along with the reduction of organ size, thermal conditioning effects metabolic rate, endogenous heat production, and oxidative status by altering the ETC. After acute heat stress, broilers previously subjected to heat stress temperatures showed lower expression of uncoupling protein and carnitine palmitoyltransferase 1 (Ouchi et al. 2021). Uncoupling proteins play a role

in endogenous heat production (Mujahid et al. 2007), so the reduced expression seen by Ouchi et al. (2021) could explain lower rectal temperatures in thermally conditioned broilers. Panting and wing drooping were not altered by thermal conditioning, thus the authors concluded the lower body temperature may be a result of reduced heat production. Additionally, body weight was improved in thermally conditioned broilers, which could be associated with the higher expression of myosin and actin related genes in thermally conditioned broilers compared to newly stressed broilers.

METHIONINE AND HEAT STRESS

Methionine supplementation provides a potential for growth through protein synthesis (Del Vesco et al. 2013). Additionally, the immune benefits and antioxidant properties of methionine and other sulfur containing amino acids are well noted (Atmaca 2004; Wu et al. 2012; Wu et al. 2013). Because heat stress-inducing temperatures are known to increase proteolysis and oxidative stress through the production of ROS (Del Vesco et al. 2015a), methionine is more critical under the stressful condition. Under thermoneutral temperatures, the NRC (1994) recommendations of methionine for broilers are 0.50, 0.38, and 0.30% for starter, grower, and finisher phases, respectively. However, under suboptimal conditions, particularly heat stress, these recommendations may not be adequate enough to fulfill the bird's requirement for methionine to protect from performance loss and impaired immunity.

Studies have shown increasing the dietary concentrations of amino acids has no beneficial effects to heat stressed birds (Han and Baker 1993; Mendes et al. 1997; Temim et al. 2000b; Zarate et al. 2003). Cheng et al. (1997) looked at the relationship between environmental temperature and dietary crude protein. When environmental temperatures were below 25.3 ^{o}C, weight gain was

impacted by dietary crude protein up to 22.4% inclusion. At higher temperatures, between 26.7 and 32.2 °C, crude protein above 20% inclusion depressed weight gain.

It is known providing excess crude protein in a diet further increases the heat load of the bird, particularly in a hot environment (Musharaf and Latshaw 1999; Bell et al. 2002). It is thought the increased heat load experienced by feeding high crude protein diets enhances the detrimental effects of heat stress, rather than help the bird overcome the stress. It would appear advantageous to supplement only the essential amino acids, reducing non-essential amino acids in the diet. This benefit was seen by Leeson et al. (2000) when the reduction of crude protein with at-requirement essential amino acids led to similar performance of birds fed a conventional diet. However, MacLeod (1997) observed increased heat production upon increasing only lysine's inclusion in the diet. It was concluded excess crude protein was not the primary culprit behind heat production, rather essential amino acids. These findings are in contradiction to various other studies which have shown benefits of feeding above-required amounts of essential amino acids (Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005). Ojano-Dirain and Waldroup (2002) have shown that under moderately hot temperatures (26.7ºC), methionine and lysine requirements for broilers are more than the requirements suggested by NRC (1994). Suganya et al. (2015) recommends a 1-2% reduction in crude protein with a 5-10% increase of critical amino acids under heat stress temperatures. In chronically heat stressed broilers, the doubling of 2-hydroxy-4-(methylthio) butanoic acid (MHA) inclusion in the diet increased weight gain (Balnave et al. 1999). Soares et al. (2020) reared broilers under cyclical heat stress while feeding diets differing in protein content during 22-42 days of age. They found weight gain and FCR were improved in heat stressed broilers supplemented with 220 g/kg protein compared to those supplemented with 160 g/kg protein.

Improved performance and overall health was seen by Del Vesco et al. (2015b) who increased methionine supplementation in heat stressed broilers. As discussed, methionine plays many metabolic roles, including the precursor for various other amino acids. When methionine demand is higher than supply, homocysteine will be re-methylated to methionine rather than go down transsulfuration (Grimble 2006). This decreased flux through transsulfuration can lead to taurine deficiency. During taurine restriction, electron transport activity in the mitochondria is decreased. Upon supplementation of taurine, normal ETC activity is resorted and the production of ROS is reduced (Jong et al. 2012). In agreement, Sahebi Ala et al. (2019) found increasing methionine content in a broiler's diet increased complex I and III activity under heat stress. The increased activity of complexes along the ETC could indicate reduced mitochondrial-derived ROS with increased methionine supplementation. GSH is also a product of transsulfuration, thus methionine absorption must be adequate to produce GSH in sufficient amounts. Under stress, Campbell et al. (2016) saw an increase in flux through PPP in cells supplemented with methionine. The PPP gives rise to NADPH which can be utilized by GSSG to reform GSH for further protection against oxidants. Increasing methionine supplementation to above-requirement levels increased antioxidant status in heat stressed broilers (Zeitz et al. 2020), improving health and performance. It is generally thought the increase of methionine in the diet positively affects the oxidative status and health of heat stressed animals.

DL-methionine versus DL-2-hydroxy-(4-methylthio) butanoic acid

Though methionine inclusion in the diet is generally agreed upon, methionine source has been a point of controversy for decades in the poultry industry. As mentioned, synthetic methionine is mostly supplemented as DL-methionine or as a hydroxy analogue. Results vary regarding broiler performance in relation to methionine source. Some research shows under heat

stress conditions, broilers supplemented with MHA have better performance and lower mortality than broilers supplemented with DL-methionine (Swick and Pierson 1988). Studies have additionally shown DL-methionine improves broiler performance while MHA does not (Balnave and Oliva 1990). However, it has been shown methionine supplement source has no effect on broiler performance under both thermoneutral conditions or heat stress conditions (Ribeiro et al. 2001).

In vitro work has shown promising protective effects of MHA, where its supplementation induced higher levels of taurine and GSH compared to DL-methionine supplementation when subjected to stress through H_2O_2 exposure (Martín-Venegas et al. 2013). By stimulating the transsulfuration pathway, it was concluded by the researchers that MHA shows protection of the epithelial barrier in stressed animals. *In vivo* work has additionally shown the beneficial effects of MHA over DL-methionine. Chronically stressed broilers receiving MHA had improved performance over those given DL-methionine, as well as reduced oxidative damage (Willemsen et al. 2011). When Guo et al. (2018) investigated the protective benefits of different methionine sources on Peking ducks, they found MHA supplementation allowed for overall higher intestinal and liver HSP70 expression than DL-methionine. However, the results varied depending on the age of the bird and segment of intestine, indicating age as well as tissue dictate which methionine source is preferred. In broilers, Wang et al. (2019) found DL-methionine supplementation increased the synthesis of GSH, whereas MHA stimulated the expression of thioredoxin. Additionally, MHA supplementation coincided with increased SAM levels compared to DL-methionine, suggesting MHA stimulates other metabolic pathways to produce SAM.

Recent work has examined the effects of either D- or L-methionine supplementation in heat stressed broilers. Lee et al. (2021) determined methionine source in a broiler's diet does enact

a different transcriptome response under heat stress. Their work resulted in similar performance of heat stressed broilers fed either L-methionine or D-methionine. However, the transcriptome of both dietary treatments did differ. It appears nutrient metabolism is altered when heat stressed birds are fed either D- or L-methionine. Similar to results seen in other work, L-methionine supplementation resulted in fewer differentially expressed genes related to metabolic and cellular processes in heat stressed broilers. This is likely due to the L isomer of methionine being biologically active and requiring less metabolic processing than D-methionine. Because of this, broilers fed D-methionine upregulated more genes associated with oxidative stress, possibly due to the reduced efficacy of D-methionine.

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CHAPTER 3

UNDERSTANDING THE BROILER CHICKEN *PECTORALIS MAJOR* UNDER HEAT STRESS THROUGH RNA-SEQUENCING

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ABSTRACT

The importance of methionine to broilers succumb to heat stress is well noted, however efficacy of methionine or its hydroxy analogue is less clear. By using transcriptome analysis, this study aimed to identify the transcriptome response of heat stressed broilers who were fed different sources and dietary inclusion level of methionine. This study used a 2x2x2 design consisting of environmental temperature (25°C or 35°C), dietary methionine source [D,L-methionine (DLM) or D,L-2-hydroxy-(4-methylthio) butanoic acid (MHA)], and dietary methionine inclusion level (at- or above-requirement). Broilers were fed their respective diet and placed in their respective environment at-hatch and reared until 35 days post-hatch. Tissue from the *Pectoralis major* was collected from broilers in all 8 treatment groups at days 10, 21, and 35 post-hatch. RNA-sequencing was performed using the Illumina Hi-seq platform. Heat stressed broilers gained significantly less weight during the starter, grower, and finisher phases. Neither methionine source nor inclusion level improved performance of heat stressed broilers. The transcriptome does indicate, however, regulation of central pathways is influenced by the diet. At a younger age, regulation of energy metabolism is most effected by heat stress. Both MHA supplementation and increased methionine in the diet of heat stressed broilers resulted in fewer differentially expressed genes associated with oxidative phosphorylation, suggesting methionine source does influence mitochondrial function. By increasing methionine to above-requirement levels, regulation of proteolysis and actin reorganization was reduced. This suggests positive effects of raising methionine inclusion in the diets of heat stressed broilers, possibly reducing the effect of oxidative damage to proteins in the breast muscle.

INTRODUCTION

A significant stressor in the poultry industry is heat. Heat stress is induced when the amount of energy generated by the bird is greater than the energy exiting the bird. The imbalance creates a pooling of energy within the bird in the form of heat (Lucas and Marcos 2013). Upon experiencing a larger heat load, blood flow is diverted from the viscera to the periphery to facilitate heat exchange, cooling the bird. This diversion of blood flow during heat stress leads to hypoxia and nutrient deficiency in various tissues including the liver, muscle, and intestines (Hall et al. 1999). Numerous studies have determined heat stress is detrimental to broiler performance (Hurwitz et al. 1980; Attia et al. 2011; Sohail et al. 2012; Awad et al. 2018; Goo et al. 2019; Lin Law et al. 2019). Additionally, heat stress inhibits proper immune function (Trout and Mashaly 1994; Bartlett and Smith 2003; Borges et al. 2004; Mashaly et al. 2004). The deleterious effects of heat stress on the bird's immune system as well as the depression in broiler performance cause significant economic loss to poultry producers (St-Pierre et al. 2003). It is in the interest of poultry producers to therefore minimize the deleterious effects of heat stress through various practices, such as addressing nutrition.

Methionine is often referred to as the first limiting amino acid in modern poultry production. Under thermoneutral temperatures, NRC (1994) recommends broiler diets contain 0.50%, 0.38%, and 0.30% methionine for starter, grower, and finisher phases, respectively. However, under suboptimal conditions, particularly when heat stressed, these recommendations may not be adequate to fulfill the bird's requirement for methionine to mitigate performance loss and impaired immunity. Previous research has shown increasing only essential amino acids, including methionine, benefit broilers subjected to heat stress (Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005). Ojano-Dirain and Waldroup (2002) have shown under moderately hot

temperatures (26.7ºC) methionine and lysine requirements for broilers are more than the requirements suggested by NRC (1994). Suganya et al. (2015) recommends a 1-2% reduction in crude protein with a 5-10% increase of critical amino acids under heat stress temperatures. When chronically exposed to high temperatures, the doubling of methionine inclusion in the diet increased broiler weight gain (Balnave et al. 1999). Soares et al. (2020) reared broilers under cyclical high temperatures while feeding diets differing in protein content during 22-42 days of age. They found weight gain and FCR were improved in heat-stressed broilers supplemented with 220 g/kg protein compared to those supplemented with 160 g/kg protein.

Today, synthetic methionine is the primary source of methionine in broiler diets. Synthetic methionine can be supplemented in various forms, including D-, L-, or DL-methionine (DLM), or as a methionine analogue. Only L amino acids are biologically available, therefore the conversion of the D enantiomorph or methionine analogue must occur for proper utilization (Dibner and Knight 1984). Results vary regarding broiler performance in relation to methionine source. Some research shows under heat stress conditions, broilers supplemented with DL-2-hydroxy-(4-methylthio)butanoic acid (MHA) have better performance and lower mortality than broilers supplemented with DLM (Swick and Pierson 1988). Studies have additionally shown DLM improves broiler performance while MHA does not (Balnave and Oliva 1990). However, it has also been shown supplemental methionine source has no effect on broiler performance under both thermoneutral conditions or heat stress conditions (Ribeiro et al. 2001).

Recently, Lee et al. (2021) studied the transcriptome of heat-stressed broilers fed either D-methionine or L-methionine. Their work found gene expression in both the liver and ileum differed between broilers fed D-methionine and L-methionine, though performance was not different between those same birds. Transcriptomics can be a powerful tool in studying molecular changes

in birds subjected to high temperatures and have been used in several recent studies (Coble et al. 2014; Lan et al. 2016; Jun-Mo et al. 2017; Hubbard et al. 2019). This study sought to expound upon nutritional intervention during heat stress in broiler chickens, specifically between DLM and MHA supplementation, as well as above-requirement methionine supplementation. The transcriptomic response in the breast muscle will offer insight as to heat stress's effect on fast growing broilers and to what extent methionine supplementation mitigates those deleterious effects.

MATERIALS AND METHODS

A 2x2x2 factorial design consisting of environmental temperature [heat stress (HS) or thermoneutral (TN)], dietary methionine source (DLM or MHA), and dietary methionine inclusion (100% or 140% of requirement). At hatch, chicks were placed in pine shaving-covered floor pens (0.072 m^2/bird) with ambient temperature at 32°C. Each of the 8 unique groups consisted of 6 replicate pens with 20 birds per replicate. Ambient temperature was gradually reduced over the next 7 days in accordance with industry standards. At the beginning of day 8, HS was initiated, and ambient temperature was set to 35°C. Broilers subjected to TN temperatures were kept at 25°C.

Feed and water were provided *ad libitum*. Broiler body weight and feed weight were taken on days 0, 10, 21, and 35. The 4 unique diets were formulated for broiler starter (day 0-10), grower (day 11-21), and finisher (22-35) phases from a common basal diet (Table 3.1). For each phase, diets containing 100% and 140% of Cobb500 methionine requirement were formulated using DLM. Then, diets containing MHA were formulated to remain equimolar to 100% DLM and 140% DLM, respectively. Starter diets contained 22% crude protein (CP) and 12.4 MJ apparent metabolizable energy (AME). The 100% DLM and 140% DLM starter diets contained 0.653% and 0.792% methionine and 0.997% and 1.131% total sulfur amino acids (TSAA), respectively.

Grower diets for 100% DLM and 140% DLM contained 20% CP and 12.8 MJ AME with 100% DLM and 140% DLM diets containing 0.573 and 0.669% methionine, and 0.880 and 0.975% TSAA, respectively. Finisher diets 100% DLM and 140% DLM contained 18% CP and 12.8 MJ AME. The 100% DLM and 140% DLM finisher diets contained 0.511 and 0.630% methionine, and 0.880 and 0.918% TSAA, respectively (Table 3.2).

Tissue from the *Pectoralis major* was taken at days 10, 21, and 35 (outlined in Figure 3.). Five birds per group were randomly sampled for tissue collection. Samples were snapped frozen in liquid nitrogen and stored at -80°C. Sampled *Pectoralis major* tissue was coarsely ground in liquid nitrogen and further broken down via lysing matrix tubes (MP Biomedicals, Santa Ana, CA). Total RNA was extracted using TRIzol reagents (Invitrogen, Carlsbad, CA), then cleaned with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA was treated with RNase-Free DNase (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. Purified RNA was resuspended in RNase-free water. RNA concentration and purity were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm an $OD_{260/280}$ ratio greater than 1.9. cDNA libraries were prepared with 4 µg total RNA using TruSeq RNA sample Preparation Kit to obtain cDNA fragments of 229 bp average size, or 355 bp including adapter sequences. All cDNA libraries were sequenced with 150 bp paired-end read chemistry using the Illumina HiSeq 2000 system.

FastQC was used to identify the quality of raw reads. Low-quality bases and adapter sequences were trimmed using Trimmomatic v.0.36 (Bolger et al. 2014) and Flexbar v.2.4 (Dodt et al. 2012). Reads were aligned to the chicken reference genome (*Gallus gallus* 4.0, version 81, Ensembl) using STAR aligner v.2.5.2b (Dobin et al. 2013). Hit counts were counted using featureCounts v.1.5.2 (Liao et al. 2014), using only unique reads moving forward. DESeq2

(Anders and Huber 2010; Love et al. 2014) was used for differential gene expression analysis. For pairwise analysis, four comparisons were devised for each sampling day: broilers kept at TN and fed 100% DLM versus broilers kept at HS and fed 100% DLM (100DLM), broilers kept at TN and fed 100% MHA versus broilers kept at HS and fed 100% MHA (100MHA), broilers kept at TN and fed 140% DLM versus broilers kept at HS and fed 140% DLM (140DLM), and broilers kept at TN and fed 140% MHA versus broilers kept at HS and fed 140% MHA (140MHA). Counts were normalized using the transcripts per million method. An adjusted p-value for false discovery rate (FDR) was generated using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). Genes were considered differentially expressed when the FDR ≤ 0.05 and fold change ≥ 1.2 or ≤ -1.2.

The ClueGO Plugin v2.5.4 (Bindea et al. 2009) was used for gene ontology (GO) within Cytoscape v3.7.2. Only genes considered to be differentially expressed were used for analysis. The majority of analysis was done using Kyoto Encyclopedia of Genes and Genomes (KEGG) terms, however molecular and biological GO pathways/networks were used, when stated.

Aliquots from all RNA samples used for RNA-sequencing were retained and used for RT-qPCR validation of selected genes. Nine highly differentially expressed genes identified by RNA-sequencing were used for validation. These included *CHRNG*, *FBLN5*, *GSN*, *PVALB*, *GLO1*, *CDK1*, *SLC7A11*, *ATP5G1*, and *PEAK1* (Table 3.3). cDNA was synthesized from two micrograms of total RNA using high-capacity cDNA Reverse Transcription Kit in accordance with the manufacture's protocol (Applied Biosystems, Foster City, CA) and using a thermocycler (Eppendorf, Hauppage, NY) with the following settings: 10 min at 25°C, 120 min at 37°C, five min at 85°C and final cycle at 4°C. Newly synthesized cDNA was stored at -20°C until further use. cDNA was diluted so two ng would be used for each reaction during RT-qPCR, additionally

using 0.3 μL of forward primer (10 μM), 0.3 μL of reverse primer (10 μM), 8.4 μL of RNase-DNase-free water, and 10 μL of SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA). Each biological sample was run in triplicate for each gene. Conditions for RT-qPCR were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds using a StepOnePlus (Applied Biosystems, Carlsbad, CA). Chicken β-actin was used as an endogenous control and differential expression was measured using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001), with relative expression comparisons identical to the comparisons used for RNA-sequencing.

Statistical analysis for body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) results for each day were determined using the GLMMIX procedure in SAS (SAS Institute, Cary, North Carolina). All tests were performed with a 5% level of significance. Environment, methionine source, and methionine level were considered as main effects. Least square means was used to compare effects using PDIFF in SAS when found to be significant. Probability values were generated using analysis of variance for main and interaction effects. For validation of differentially expressed genes (DEGs), linear regression using PROC REG (SAS Institute, Cary, North Carolina) tested the strength of RNA-sequencing $\log_2$ fold change predicting RT-qPCR $\log_2$ fold change.

RESULTS

Broiler performance

During the starter phase, elevated temperature had no impact on broiler BWG, feed intake, or FCR (Table 3.4). Additionally, neither methionine source nor inclusion level had an effect on these performance parameters during the first 10 days. During days 11 to 21, however, heat stress did reduce broiler feed intake and BWG, regardless of methionine source or inclusion level (Table

3.5). FCR during this period was numerically lowest in TN broilers, while FCR of heat stressed broilers fed at-requirement MHA and above-requirement DLM did not significantly differ from any non-stressed broiler. From days 22 to 35, heat stressed broilers again showed reduced BWG compared to TN broilers (Table 3.6). Feed intake during this period was significantly lower in all heat stressed groups compared to TN groups. The FCR of heat-stressed broilers fed at-requirement DLM or MHA and above-requirement DLM were also significantly higher than all TN broilers. Only heat stressed broilers fed above-requirement MHA had statistically similar FCR to non-stressed broilers fed above-requirement MHA. The total BWG was numerically highest in non-stressed broilers fed above-requirement DLM. All TN broilers gained significantly more weight than heat stressed broilers. Feeding above-requirement MHA resulted in numerically higher BWG in heat stressed broilers, though the total BWG of heat stressed broilers did not significantly differ between dietary treatments.

Alignment, mapping, and RT-qPCR validation

A total of 4.01 billion 150 base pair paired-end reads were produced using Illumina HiSeq platform. On day 10, an average of 34.2 million reads per sample were obtained and an average of 88.8% of reads were mapped to the *Gallus gallus* reference genome. Of those, 84.1% of reads were uniquely mapped. On day 21, an average of 34.5 million reads were obtained; 88.5% being mapped. An average of 28.8 million reads per sample were uniquely mapped to the reference genome on day 21. On day 35, an average of 31.5 million reads per sample were obtained. Of those, 84.0% were uniquely mapped to the reference genome. A total of 43 data-points were selected for validation via RT-qPCR based on significance, comparison, and sampling time. Regression analysis revealed strong correlation ($p < 0.0001$, $R^2 = 0.8492$) between the $\log_2$ fold change of significantly DEGs determined by RNA-sequencing and RT-qPCR (Figure 3.2).

Effects of heat stress and methionine on breast muscle transcriptome at day 10

After heat stress from days 8 to 10, broilers in 100DLM differentially regulated the most genes (Figure 3.3a). The most upregulated genes in that comparison were *ADPRHL1*, *PDK4*, *CHAC1*, *CEBPD*, and *DDIT4* (Table 3.7). Those broilers also downregulated *KCNU1*, *CHADL*, *PEAK1*, *FAM126B*, and *UBE2C* the most (Table 3.8). Six genes related to the electron transport chain (ETC) were additionally downregulated in 100DLM at day 10, including *ATP5G1*, *ATP6V1C1*, *COX6A1*, *COX7A2*, *ND1*, and *NDUFV2*. The gene *UCP3*, coding for a mitochondrial uncoupling protein, was upregulated in these stressed broilers. Cysteine and methionine metabolism were additionally affected by heat stress in 100DLM, with the genes *BCAT1* and *MAT1A* being downregulated.

Increasing DLM inclusion in the starter diet of heat stressed broilers did reduced the number of DEGs. The gene *TCIM* was the only DEG in 140DLM and was additionally upregulated in 100DLM.

Heat stressed broilers in 100MHA differentially expressed only 9 genes on day 10: *RPL3L*, *GRIN3B*, *MINDY4B*, and *BRSK2* were upregulated and *FABP1*, *CELSR1*, *CPOX*, *SDF2*, and *PEAK1* were all downregulated. When dietary MHA inclusion was raised, heat stressed broilers differentially regulated 71 genes at day 10 (Figure 3.3a). The genes *FGA*, *FGG*, *ALB*, *APOA4*, and *MAL2* were most upregulated in 140MHA (Table 3.9) while *SDR42E1*, *PPP1R3C*, *SLC38A4*, *LBH*, and *CHRNG* were most downregulated (Table 3.10). The upregulated genes *ARHGAP35*, *GIT1*, and *SLC9A1* regulated the actin cytoskeleton according to KEGG pathway analysis. No other comparison differentially regulated genes related to the actin cytoskeleton on day 10.

Effects of heat stress and methionine on breast muscle transcriptome at day 21

At 21 days of age, 100DLM upregulated 161 genes and downregulated 139 genes. In 140DLM, broilers upregulated 251 genes and downregulated 365 genes. Broilers in 100MHA upregulated 151 genes and downregulated 84 genes. When MHA was increased, 140MHA upregulated 117 genes and downregulated 140 genes (Figure 3.3b). Figure 3.4b shows 7 genes were similarly differentially expressed among all four comparisons; *ND6*, *LANCL2*, and *NAP1L4* were downregulated in all four comparisons while *FBLN5*, *TBC1D22B*, *FBXW4*, and *SCN2B* were upregulated in all four comparisons.

The top upregulated genes observed in 100DLM were *FGA*, *GC*, *PIT54*, *ALB*, and *FGG* (Table 3.11). The genes *FOS*, *HBAD*, *NR4A2*, *GJD2*, and *HBAA* were the top downregulated genes for the same comparison (Table 3.12). When stressed during the starter and grower phases, broilers in 100DLM upregulated 9 genes associated with the actin cytoskeleton. Similarly, a majority of genes related to focal adhesion were also upregulated. KEGG pathway analysis also shows genes related to oxidative phosphorylation and the cell cycle were downregulated in 100DLM (Table 3.13).

Increasing DLM inclusion in the diet of heat stressed broilers revealed *PVALB*, *HS3ST2*, *GPM6A*, *BBOF1*, and *SRD5A2* to be most upregulated (Table 3.14) while *CCK*, *GJD2*, *SLC7A11*, *ELFN1*, and *ND6* were the most downregulated genes (Table 3.15). Oxidative phosphorylation was the top KEGG term associated with negatively expressed genes in 140DLM, along with the TCA cycle and pyruvate metabolism. Wnt signaling, FoxO signaling, and p53 signaling pathways were all associated with upregulated genes (Table 3.16).

The genes *FOSB*, *PVALB*, *FOS*, *CEBPD*, and *SYPL1* were most upregulated in heat stressed broilers in 100MHA at day 21 (Table 3.17). Similar to 140DLM, *CCK* was most downregulated, along with *SCUBE2*, *NR5A2*, *CPEB2*, and *UBE3D* (Table 3.18). Table 3.19 shows

MAPK signaling, Wnt signaling, and Jak-STAT signaling pathways were a part of the top pathways associated with positively expressed genes in 100MHA. The calcium signaling pathway, phagosome, and peroxisome were associated with negatively expressed genes in heat stressed broilers fed at-requirement MHA.

Unlike 140DLM and 100MHA, heat stressed broilers in 140MHA upregulated the gene *CCK*. Those broilers upregulated the genes *MINDY4B*, *PVALB*, *PI15*, and *SYPL1* as well (Table 3.20). *CSMD1*, *STAC*, *GSTZ1*, *RGR*, and *GATM* were the most downregulated in 140MHA (Table 3.21). GO reveals the peroxisome and Wnt signaling pathway were the KEGG terms most associated with positively expressed genes while oxidative phosphorylation, pyrimidine metabolism, and the cell cycle were associated with negatively expressed genes (Table 3.22).

GO analysis on day 21 reveals oxidative phosphorylation to have the most downregulated genes associated than any other KEGG term. Conversely, the Wnt signaling pathway has the most positively expressed genes associated, however 100DLM did not differentially regulate any genes related to this pathway. Figure 3.5 highlights expression differences of KEGG terms in all 4 comparisons. It is shown broilers in 100MHA did not differentially express any genes related to glycolysis or the TCA cycle, while all other comparisons downregulated a majority of genes related to these pathways. Additionally, feeding at-requirement MHA resulted in the upregulation of genes related to the cell cycle, while feeding at-requirement DLM resulted in more downregulated genes related to the cell cycle when broilers were stressed. Regulation of the actin cytoskeleton also saw dissimilar regulation when heat stressed broilers were fed different diets. Those in 100DLM more positively expressed genes related to actin while increased DLM inclusion resulted in primarily downregulated genes related to the regulation of actin in 140DLM.

Effects of heat stress and methionine on breast muscle transcriptome at day 35

The number of DEGs increased approximately 10-fold from day 21 to day 35 for most comparisons. In 100DLM, there were 1,974 upregulated and 1,821 downregulated genes. When dietary DLM was increased, heat stressed broilers in 140DLM upregulated 1,158 genes and downregulated 1,008 genes. In 100MHA, there were 1,520 upregulated and 1,681 downregulated genes. Heat stressed broilers in 140MHA upregulated 1,276 and downregulated 1,039 genes (Figure 3.3c). In all, there were 930 genes shared across all 4 comparisons, 354 being commonly downregulated and 576 commonly upregulated (Figure 3.4c).

After experiencing high temperatures during all three phases of production, broilers in 100DLM upregulated the genes *SYPL1*, *PVALB*, *NXNL1*, *FKBP5*, and *ZBTB16* the most (Table 3.23). Additionally, *CCK*, *TGM4*, *PHGDH*, *CSMD1*, and *GAP43* were most downregulated in 100DLM at day 35 (Table 3.24). Protein processing in endoplasmic reticulum, RNA transport, and ubiquitin mediated proteolysis were most associated with positively expressed genes while the KEGG terms focal adhesion, regulation of actin cytoskeleton, cell cycle, phagosome, and oxidative phosphorylation were related to negatively expressed genes (Table 3.25).

When dietary DLM was increased to above-requirement levels, heat stressed broilers in 140DLM upregulated *PVALB*, *PDK4*, *ZBTB16*, *FKBP5*, and CXCR5 most (Table 3.26). The genes *ZP1*, *CSMD1*, *SPIRE2*, *WNT16*, and *OASL* were most negatively expressed in these broilers (Table 3.27). Similar to 100DLM, heat stress upregulated genes associated with RNA transport and ubiquitin mediated proteolysis in 140DLM, along with autophagy, the ribosome, and the spliceosome. Heat stress additionally downregulated genes related to the regulation of the actin cytoskeleton, focal adhesion, and the cell cycle (Table 3.28).

In 100MHA, *PVALB*, *BRSK2*, *ZBTB16*, *MINDY4B*, and *SYPL1* were most upregulated (Table 3.29), while *MLANA*, *CALB2*, *MMP7*, *CCL19*, and *CTHRC1* were most downregulated (Table 3.30). GO shows ubiquitin mediated proteolysis and RNA transport to be associated with upregulated genes in heat stressed broilers. The KEGG terms endocytosis, regulation of actin cytoskeleton, focal adhesion, and the cell cycle were most associated with downregulated genes (Table 3.31).

Increasing MHA inclusion in the diet of heat stressed broilers still resulted in the upregulation of *PVALB*, *ZBTB16*, and *SYPL1* (Table 3.32). The genes *CCK*, *TRPC3*, *CSMD1*, *SBK2*, and *GIPC3* were additionally downregulated in 140MHA at day 35 (Table 3.33). Like all other comparisons at day 35, ubiquitin mediated proteolysis was associated with a large number of positively expressed genes in heat stressed broilers, along with the KEGG terms ribosome, RNA transport, and the spliceosome. Regulation of actin cytoskeleton, oxidative phosphorylation, and the cell cycle were also highly associated with negatively expressed genes in 140MHA (Table 3.34).

After being subjected to hot environmental temperatures from days 8 to 35, broilers in all comparisons downregulated a majority of genes related to oxidative phosphorylation, glycolysis, the TCA cycle, the cell cycle, and regulation of the actin cytoskeleton (Figure 3.6). Genes regulating ubiquitin mediated proteolysis were primarily upregulated in all comparisons, however supplementing above-requirement methionine resulted in fewer DEGs related to this proteolytic pathway.

DISCUSSION

Heat stress is induced when the amount of energy generated by the bird is greater than the energy exiting the bird. This imbalance creates a pooling of energy within the bird in the form of

heat (Lucas and Marcos 2013). To offload this heat, a bird will utilize conductive, convective, and evaporative cooling. Blood carries heat throughout the body and by increasing blood flow to the periphery and air sac, heat can be exchanged with the environment. To increase the surface area to the environment, birds lay down and spread their extremities (Li et al. 2015), increasing contact with the circulating air and surfaces in the environment and enabling cooling by conduction and convection. The preference for a bird to rest during heat stress limits feed intake (Mack et al. 2013). Mello et al. (2015) saw 19% lower body weight in heat stressed broilers at 35 days of age. Feed conversion over that period was increased 15% and feed intake was numerically decreased. In the current study, heat-stressed broilers reduced their feed intake significantly from days 11 to 35, regardless of methionine source or inclusion level. BWG was also significantly reduced during this period. However, hot environmental temperatures from days 8 to 10 post-hatch does not reduce broiler BWG or affect feed intake. Younger broilers have a higher heat tolerance (Chowdhury et al. 2012), attenuating the effects of a hot environment (Andretta et al. 2021), enabling more optimal performance.

Increasing methionine inclusion in broiler diets has been shown to benefit performance (Balnave et al. 1999; Ojano-Dirain and Waldroup 2002; Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005; Soares et al. 2020), however this study did not see such an effect on BWG. During the grower phase, increased DLM inclusion did lower FCR of broilers similar to TN broilers, though this was not the case during the finisher phase. Interestingly, feeding at-requirement MHA additionally improved the FCR of broilers when stressed during the grower phase, though this too did not carry into the next phase of production.

Oxidative stress

Under heat stress, the diversion of blood flow to the periphery gives rise to a hypoxic environment (Hall et al. 1999). Under normal conditions, electron leak in the mitochondria's electron transport chain (ETC) generates superoxide (Turrens 2003). In a hypoxic environment, this generation of superoxide and other ROS reach uncontrollable levels (Pamplona and Costantini 2011). Additionally, increasing mitochondrial membrane potential and ATP production increases the rate of ROS generation (Korshunov et al. 1997; Starkov and Fiskum 2003). By reducing ATP production and decreasing the flux of electrons through the ETC, ROS generation may be reduced. Uncoupling proteins are a mechanism to reduce ATP synthesis by diffusing the proton gradient and reducing membrane potential (Caldeira da Silva et al. 2008). In the current study, 100DLM showed upregulation of the gene *UCP3* on day 10. Poultry with higher expression of avian uncoupling proteins are more adapt to heat stress and produce less ROS (Mujahid et al. 2007). This, along with the downregulation of genes coding for complexes along the ETC, may indicate heat-stressed broilers in 100DLM are reducing membrane potential, limiting ROS production.

These results can be interpreted in another way, however. Heat stress is known to induce mitochondrial dysfunction (Downs and Heckathorn 1998; Monti et al. 2001; Zhao et al. 2006), and the downregulation of genes coding for certain subunits of complexes along the ETC could result in altered function. Complex I is the main source of mitochondria-derived ROS (Murphy 2008) resulting from electron leak. The genes *ND1* and *NDUFV2* code for two of the 43 subunits of complex I, and their downregulation possibly contributes to dysfunction of complex I and the generation of ROS. Similarly, *COX6A1* and *COX7A2* code for two of the 13 subunits of complex IV of the ETC. Complex IV catalyzes the transfer of electrons to oxygen, and the downregulation of two genes coding for subunits may inhibit proper function. Zhang et al. (2019) saw the

downregulation of *COX6A1* in heat stressed broilers, which may have contributed to the production of ROS. Additionally, the dysfunction of ATP synthase may produce ROS and further contribute to oxidative damage. *In vitro*, the genetic knockout of *ATP5G1* reduces ATP synthesis and increases the generation of ROS (Comelli et al. 2003; Seth et al. 2009). At day 10, 100DLM additionally downregulated the gene *ATP5G1*, possibly contributing to ROS generation.

If the ETC is compromised, the antioxidant system of the bird should respond to the increase of ROS. However, no genes coding for any major antioxidant system were differentially expressed in any comparison at day 10. Younger animals have higher capacity for oxidative stress (Sandercock et al. 2001; Del Vesco et al. 2017), which could explain the lack of DEGs related to antioxidant response. This was additionally seen on day 21 in 100DLM, however there was upregulation of genes within the pentose phosphate pathway (PPP). The PPP performs many functions within a cell, including the generation of substrates for glycolysis, the citric acid cycle, and nucleic acid synthesis, as well as the generation NADPH (Stine et al. 2014; Litwack 2018). NADPH is utilized by glutathione reductase to reduce glutathione disulfide (GSSG) to glutathione (GSH), enabling for additional neutralization of ROS (Harvey 2008). The upregulation of *ALDOB* and *PGLS* suggest heat stressed broilers in 100DLM increased flux through the PPP, increasing products such as NADPH to maintain a proper GSH:GSSG ratio. These same broilers downregulated 10 genes associated with the ETC in 100DLM at day 21, including *COX6A1*, indicating possible dysfunction in the mitochondria and increased ROS generation during heat stress.

In 140DLM, 33 genes associated with oxidative phosphorylation were downregulated in heat stressed broilers on day 21. This would seem to indicate dysfunction in the ETC, resulting in an increase of ROS. In response to possibly increased amount of ROS, the gene coding for catalase,

CAT, was upregulated in heat stressed broilers in 140DLM. Catalase is responsible for the neutralization of hydrogen peroxide in the mitochondria, a job also done by glutathione peroxidase (GPX). The lack of genes associated with GSH metabolism indicates catalase may be more active in protecting skeletal muscle cells from oxidative stress than GSH at this age. Habashy et al. (2019) saw catalase and GPX were not equally active when broilers were subjected to heat stress. GPX showed significantly higher expression in broilers heat stressed for 12 days whereas catalase activity was unaffected by heat stress. The authors concluded catalase, GPX, and GSH are coordinated differently depending on the tissue and tolerance to oxidative stress.

No genes associated with methionine, GSH, or any other antioxidant system were differentially expressed in 100MHA or 140MHA at day 21. Heat stressed broilers in 100MHA downregulated only two genes in association with oxidative phosphorylation, *ATP6V0A1* and *ND6*, while those in 140MHA downregulated 9 genes. Just as downregulation of genes associated with the ETC may indicate increased ROS generation (Zhang et al. 2019), the lower number of DEGs may indicate more normal function of the mitochondria. Dietary methionine plays a role in the oxidative status of animals (Swennen et al. 2011; Pan et al. 2017). Willemsen et al. (2011) saw MHA supplementation improved oxidative status and performance of heat stressed broilers. The current study agrees that DLM and MHA induce a different molecular response to heat stress, and MHA supplementation may improve the oxidative status of broilers when heat stressed from days 8 to 21.

Unlike on day 21, heat stressed broilers in all comparisons differentially expressed genes associated with the metabolism of methionine and GSH on day 35. This coincides with an overall increase in the number of DEGs associated with oxidative phosphorylation, although regulation is not uniform (Figure 3.7). Heat stressed broilers in 100DLM and 100MHA downregulated 36 and

15 genes related to oxidative phosphorylation, respectively, while heat stressed broilers in 140DLM and 140MHA downregulated 20 and 22 genes, respectively. Older broilers are less apt at coping with a hot environment (Sandercock et al. 2001), leading to oxidative stress resulting in elevated antioxidant activity (Del Vesco et al. 2017). Antioxidant activity is correlated with dietary methionine inclusion (Waschulewski and Sunde 1988; Tchantchou et al. 2008; Del Vesco et al. 2014; Zeitz et al. 2020), and increasing dietary DLM appears to reduce oxidative stress in broilers. No genes related to GSH metabolism were differentially expressed in 140DLM on day 35, while *HPGDS*, *MGST3*, and *PGD* were downregulated in 100DLM. As mentioned, the PPP works with GSH metabolism to combat ROS, so the additional regulation of 8 genes related to the PPP in 100DLM could be in response to reduced GSH activity. Similarly, both 100MHA and 140MHA negatively expressed genes related to both GSH metabolism and the PPP. All heat stressed broilers reduced their feed intake during the finisher phase of this study, which could explain reduced GSH activity (Deneke et al. 1985). In general, dietary restriction does not influence GSH activity (Walsh et al. 2014), although methionine restriction does reduce GSH levels in some tissue (Maddineni et al. 2013). Indeed, methionine metabolism was downregulated in all heat stressed broilers. The genes *BCAT1* and *MAT1A* were downregulated in all 4 comparisons, however 140DLM only differentially regulated these two genes related to methionine metabolism. Heat stressed broilers in 140MHA differentially regulated an additional 7 genes while those in 100MHA differentially regulated another 6 genes and those in 100DLM another 9 genes. These results seem to indicate increasing DLM inclusion in the diet of heat stressed broilers affects methionine metabolism, as it resulted in fewer differentially regulated genes.

Energy metabolism

Under heat stress, birds attempt to limit energy expenditure by remaining immobile and panting to facilitate heat loss (Mack et al. 2013). By reducing movement, the bird limits its energy expenditure and reduces ATP hydrolysis. Both the breakdown and synthesis of ATP are somewhat inefficient, and some energy is lost as heat during both anabolism and catabolism of ATP. The conversion of energy stored as glucose to energy stored as ATP is only 38% efficient under standard conditions. In the mitochondria, that efficiency is increased to about 50% (Beals et al. 1999). Endogenous heat production must be reduced in heat stressed birds; therefore, ATP production and consumption must be reduced. Wang et al. (2012) saw a decrease in Complex I and III activity in cells overexpressing heat shock protein 70, a highly conserved protein greatly expressed during heat stress. When measuring ATP pools within those cells, overexpression of heat shock protein 70 had no effect on ATP levels, however. The zero-net loss to cellular ATP was contributed to the increased expression of enzymes along the glycolytic pathway, which produces some ATP, while ATP synthesis from the electron transport system was decreased. A similar scenario may be happening in 100DLM at day 10. Genes associated with the ETC (*ATP5G1*, *ATP6V1C1*, *COX6A1*, and *COX7A2*, *ND1*, and *NDUFV2*) were all downregulated. Conversely, no genes coding for enzymes along glycolysis, the citric acid cycle, or anaerobic respiration were differentially expressed. There appears to be a disconnect in energy production, as the oxidation of glucose and reduction of electron donors appears unaltered while oxidative phosphorylation appears to be downregulated. This was only seen in 100DLM, and it seems heat stress does not affect energy metabolism of broilers fed MHA or above-requirement DLM after experiencing a hot environment from days 7 to 10.

At day 21, 100DLM downregulated 10 genes associated with oxidative phosphorylation. *COX6A1* was the only gene commonly downregulated between days 10 and 21, however the remaining downregulated genes do code for complexes I and IV, as well as ATP synthase, similar to day 10. Perhaps more interesting is the effect of above-requirement DLM supplementation on oxidative phosphorylation during heat stress. At day 21, 33 downregulated genes were associated with oxidative phosphorylation in 140DLM. Of these, 13 genes code for subunits of complex I, four genes code for subunits of complex III, 5 genes code for subunits of complex IV, including *COX6A1* and *COX7A2*, 10 genes coding for subunits of ATP synthase, and one gene, *PPA1*, which codes for the enzyme responsible for the conversion of pyrophosphate to two phosphate ions. It is also important to note genes coding for components of the pyruvate dehydrogenase complex (*DLAT, DLD,* and *PDHB*) where downregulated along with genes associated with the citric acid cycle (*ACO2*, *CS*, *DLST*, *IDH3A*, *IDH3B*, *MDH1*, *MDH2*, *PDHB*, *SUCLA2*, *SUCLG1*). During anaerobic respiration, pyruvate derived from glycolysis is diverted from aerobic respiration and instead converted to lactate by lactate dehydrogenase, regenerating NAD^+ used in glycolysis and ATP production. Ward and Peterson (1973) found circulating lactate dehydrogenase was higher in broilers subjected to acute heat stress than broilers kept at thermoneutral temperatures. Similarly, Melesse et al. (2011) saw higher levels of plasma lactate dehydrogenase in various breeds of layer hens subjected to high ambient temperatures. In humans, complex IV deficiency leads to lactic acidosis; lactic acid buildup produced from anerobic respiration (Shoubridge 2001). Lactic acidosis has also been observed in humans with ATP synthase deficiency (Houštek et al. 1999). Wang et al. (2012) hypothesized the glycolytic pathway may be utilized to maintain an adequate ATP pool under mitochondrial dysfunction, which would increase the production of lactic acid. In agreement with this hypothesis, Azad et al. (2010) saw reduced expression of 3-hydroxyacyl CoA

dehydrogenase and citrate synthase in the skeletal muscle of heat stressed broilers, suggesting reduced aerobic respiration of broilers subjected to heat stress. In the current study, the downregulation of genes responsible for the citric acid cycle and oxidative phosphorylation seems to indicate heat stressed broilers fed above-requirement DLM do not favor aerobic respiration and ATP synthesis through the ETC. Instead, ATP may have been generated by anaerobic respiration. This regulation of energy metabolism was not seen in any other dietary treatment at day 21.

Both D- and L-methionine and MHA elicit different responses in the metabolism of energy in production animals (Jeon et al. 2018). Additionally, increasing dietary methionine increases glycolytic potential and further alters energy metabolism (Zhai et al. 2012; Yuan et al. 2020; Gondret et al. 2021). On day 21, no genes related to glycolysis or the TCA cycle were differentially regulated by heat in broilers fed at-requirement MHA. In addition, only the genes *ATP6V0A1* and *ND6* were differentially regulated in relation to oxidative phosphorylation. Conversely, 100DLM and 140DLM downregulated genes related to glycolysis, the TCA cycle, and oxidative phosphorylation on day 21. Broilers in 140MHA similarly downregulated *DLAT*, *PDHB*, *DLST*, *IDH3A*, *SUCLA2*, *ATP5C1*, *ATP5G3*, *ATP6*, *COX3*, *ND5*, *ND6*, *NDUFV2*, *UQCRC1*, and *UQCRFS1*, all related to aerobic respiration. Beta oxidation was also downregulated by heat stress in 140MHA, 140DLM, and 100DLM. Both fatty acid oxidation as well as glucose oxidation are reduced under heat stress (Zhao et al. 2018), so the lack of DEGs related to beta oxidation, glycolysis, and the TCA cycle in 100MHA indicate these pathways are less affected by heat stress when broilers are fed at-requirement MHA.

When reared at 35°C from days 8 to 35 post-hatch, broilers similarly regulated oxidative phosphorylation, regardless of diet. Growth rate influences mitochondrial activity in poultry skeletal muscle (Toyomizu et al. 2011; Hu et al. 2019), which could explain why heat stress at an

older age greatly influences regulation of the ETC. Older broilers are less able to adapt to warm environments (Sandercock et al. 2001), additionally requiring greater regulation of ATP production. All 4 comparisons negatively regulated genes related to glycolysis, the TCA cycle, and oxidative phosphorylation on day 35, although broilers in 100DLM downregulated more genes than any other comparison. Increasing DLM inclusion almost halved the number of DEGs related to these processes. Increasing methionine supplementation increases the flux through the ETC and aids in mitochondria function (Lozoya et al. 2018; Tripodi et al. 2018). However, this same affect was not seen when broilers were fed MHA. Heat stressed broilers in 140MHA downregulated 19 genes associated with complexes I, III, and IV, along with 7 genes associated with the citric acid cycle and three genes associated with glycolysis. In 140MHA, 14 genes coding for complexes I and IV and ATP synthase were downregulated. These same broilers downregulated 8 and 7 genes, respectively, related to glycolysis and the TCA cycle. In fish, MHA supplementation improves cell survival through the regulation of the mitochondria (Pan et al. 2016). Mitochondrial D-2-hydroxy acid dehydrogenase is responsible for the oxidation of MHA (Dupuis et al. 1990), possibly contributing to improved function of the mitochondria (Tang et al. 2011). This could explain why heat stress had less influence on the regulation of energy metabolism in broilers fed MHA in the current study.

The actin cytoskeleton and focal adhesions

The cytoskeleton of a cell is comprised of microtubules, intermediate filaments, and microfilaments. Actin is the primary protein of microfilaments and makes up a large portion of protein in skeletal muscles (Anderson 1976; Murakami and Uchida 1985). Under stress, heat shock proteins (Hsps) bind to actin in an attempt to mitigate damage to the cellular structure. Small Hsps (sHsps) are primarily responsible for this action, responding to stressors, cell growth, apoptosis,

and signal transduction (Mounier and Arrigo 2002). Under oxidative stress, ROS commonly attack cysteine and methionine residues in actin monomers. Three methionine residues and one cysteine residue are fully exposed to the cytoplasm, while 4 other methionine residues are additionally vulnerable to oxidation (Dalle-Donne et al. 2002; Dalle-Donne et al. 2003). When challenged with heat stress or oxidative stress, cells overexpressing Hsp27 showed higher stability of actin and more rapid recovery (Lavoie et al. 1993a; Lavoie et al. 1993b; Huot et al. 1996). Additionally, Hsp25 expression is elevated during the first phases of myogenesis (Dubińska-Magiera et al. 2014; Thakur et al. 2019), indicating protection of actin filament assembly and organization during the development of muscular tissue. In addition to ROS disrupting actin structure, proteases such as calpains target skeletal muscle for degradation. Oxidative stress increases the activity of calpains (McClung et al. 2009; Dargelos et al. 2010), which cleave cytoskeletal proteins such as actin (Goll et al. 2003). Just as actin is a part of the initial formation of cells, the actin cytoskeleton is one of the last structures to be degraded during the final stages of apoptosis (Coleman and Olson 2002). Nakashima et al. (2004) used chicken thigh muscles *in vitro* to study the effects of heat stress on myofibrillar proteolysis. They found 6 hours of heat stress lowered protein content, and 24 hours of heat stress lowered the content even further. Additionally, proteasome activity was elevated at the two time points, and calpain activity was significantly higher after 24 hours of heat stress.

On day 10, only 140MHA differentially expressed genes associated with regulation of the actin cytoskeleton. Of the 4 upregulated genes associated with regulation of the actin cytoskeleton, *ARHGAP35* and *GIT1* are related to guanosine triphosphateases (GTPase) activity. Under heat stress, the activity of GTPases are increased, expressing actin stress fibers (Ridley and Hall 1992). Under oxidative stress, the cytoskeleton is reorganized, and focal adhesion complexes are formed using actin filaments (Huot et al. 1998). The upregulation of the gene coding for GTPase-activating

protein suggests increased actin polymerization in the breast of heat stress broilers fed above-requirement MHA.

After experiencing heat stress from days 8 to 21, broilers in all dietary treatments upregulated genes associated with regulation of the actin cytoskeleton when heat stressed. Heat stressed broilers fed MHA upregulated the genes *FGF1* and *GSN* only, while those supplemented with DLM upregulated *ARHGAP35*, along with numerous other associated genes. *GSN* codes for gelsolin, an actin-binding protein which regulates actin assembly and is shown to inhibit apoptosis (Koya et al. 2000), indicating some induced protection of actin under heat stress. The regulation of focal adhesions was also positively regulated in heat stressed broilers on day 21. Focal adhesion complexes are a part of the mechanical and signaling mechanisms of a cell with the extracellular matrix (ECM) (Zaidel-Bar et al. 2004). These focal adhesions are comprised of integrin protein structures which link the cytoskeleton to the ECM. In skeletal muscle, focal adhesions function as force transducers, transferring mechanical forces from one cellular structure to the next (Hanks et al. 2003; Graham et al. 2015).

Heat stressed broilers in 100DLM upregulated the most genes associated with focal adhesions on day 21, including *ARHGAP35*, *PAK3*, *PIK3CA*, *PTEN*, *SOS1*, *SOS2*, and *VTN*. Increased expression of genes related to focal adhesions may be to strengthen the anchoring and structure of cells under heat stress. sHsps are highly expressed under heat stress. The sHsp αB-crystallin plays a role in cell adhesion, and its knockdown causes cells to be more migratory with reduced adhesion (Miho et al. 2016). In chickens, the genes *CRYAB* and *HSPB1* code for αB-crystallin and Hsp27, respectively. It has been shown an increase in amino acids and antioxidants stimulate Hsp expression. Methionine and taurine stimulate Hsp70 in rabbit myocardial tissue (Setyarani et al. 2014), vitamin C induces the protective effects of αB-crystallin and Hsp70 in

poultry (Yin et al. 2020), and increased methionine in the diet of Peking ducks upregulated Hsp70 expression in the small intestine and liver (Guo et al. 2018). Increasing DLM inclusion resulted in fewer DEGs associated with focal adhesion and the actin cytoskeleton. It may be increasing DLM inclusion under heat stress benefits cellular structure, possibly through protection by sHsps (Mounier and Arrigo 2002).

The amount of time a bird is subjected to heat stress influences the cytoskeleton's response. Acute stress strengthens myocyte's structure, while chronic stress greatly damages the cytoskeleton (Yao et al. 2015). After heat stress from days 8 to 35, broilers in all comparisons downregulated significantly more genes than were upregulated related to cellular structure (Figure 3.8). The positive expression of *FGF1, RRAS2*, *GSN*, and *ARHGAP35* in 100DLM indicate actin reorganization and polymerization, however the downregulation of genes related to alpha and gamma actin, integrin, lamellipodium, and epidermal growth factor suggest the cytoskeleton is severely compromised. In addition, 100DLM downregulated 44 genes associated with focal adhesion, including *PTK2*, which codes for focal adhesion kinase (FAK). FAK is involved with these cell-to-cell and cell-to-ECM interactions and plays a role in focal adhesion complex formation. During FAK inhibition, apoptosis is induced (Hungerford et al. 1996; Wei and Vander Heide 2008), while FAK activation protects against apoptosis (Frisch et al. 1996). It has been shown FAK is a target of oxidative stress, resulting in apoptosis in epithelial cells (Mian et al. 2008). When exposed to ROS, cellular stress fibers and focal adhesions were disrupted, coinciding with the reduction of FAK activity (Schneider et al. 1998). When apoptosis was induced by compromising microtubules, cells became more rounded and more migratory, a sign of reduced focal adhesions (Kook et al. 2000). In poultry, birds exposed to heat stress significantly regulated genes associated with focal adhesion in the breast (Park et al. 2019) and liver (Emami et al. 2020).

Fujigaki et al. (2010) found expression of sHsps are positively associated with FAK expression when cells are subjected to stress. This is in agreement with Lee et al. (2008) who saw overexpression of Hsp27 enhanced cell adhesion through regulation of FAK in fibroblasts. The current study came to a different conclusion, as 28 days of continuous heat stress showed increased expression of *CRYAB* with reduced expression of *PTK2* in 100DLM. This was additionally seen in 140DLM and 140MHA, where heat stressed broilers downregulated *PTK2* expression while upregulating the expression of *CRYAB*.

Raising dietary methionine reduced the number of DEGs related to the actin cytoskeleton and focal adhesions in heat stressed broilers on day 35. Increasing methionine supplementation reduces proteolysis (Del Vesco et al. 2015), and S-adenosyl-methionine enacts protection of the cytoskeleton from xenobiotics (Ramírez et al. 2003). Heat shock disrupts microfilaments, altering the cytoskeleton (Van Dongen et al. 1985). Additionally, protein turnover is more prevalent in older birds (Tesseraud et al. 1996; Northcutt et al. 1998). Because of this, it is unsurprising 35-day-old broilers showed a downregulation of actin proliferation, and increased dietary methionine led to fewer DEGs related to the regulation of actin.

Cell cycle

Oxidative environments induce DNA damage, leading to cell damage and death. On day 21, 100DLM downregulated the genes *CCNA2*, *CCNB2*, *CDC25A*, *CDC45*, and *E2F1*, all of which are associated with the cell cycle. Under heat shock, the checkpoints at G1/S and G2/M transitions are compromised (Elledge 1996). Cell-dependent kinases (Cdks) drive the cell cycle, and their binding to different cyclins determine their activation during a certain stage of the cell cycle (Noble et al. 1997). The genes *CCNA2* and *CCNB2* code for cyclin A2 and cyclin B2, respectively. The progression from G1 to S phase involves cyclin A2 and transcription factor E2F1, coded by *E2F1*.

Additionally, the genes *CCNB2* and *CDC25A* are involved with the regulation of G2/M transition (Morgan 1997). Methionine restriction leads to cellular arrest and apoptosis (Lu and Epner 2000; Su et al. 2005; Shiraki et al. 2014). Heat stressed broilers fed above-requirement DLM did not differentially express any genes related to the cell cycle on day 21. However, increased MHA inclusion resulted in the downregulation of *BUB1*, *CDC20*, *E2F1*, *MCM2*, *MCM3*, *MCM5*, and *PLK1* in 140MHA. More interestingly, broilers in 100MHA upregulated *CCND3*, *CREBBP*, and *E2F4* on day 21, indicating the cell cycle does not undergo arrest, similar to 140DLM. Both 140DLM and 100MHA upregulated genes related to MAPK signaling during this period, while 100DLM and 140MHA did not significantly upregulate this pathway. Cellular arrest is associated with the inhibition of the MAPK signaling pathway (Nebreda 1994), and increased MAPK signaling encourages cellular growth (Dubois and Bensaude 1993). However, more recent work shows MAPK signaling responds to stressors, inducing apoptosis (Rahman et al. 2014; Srikanth et al. 2017; Takac et al. 2018).

On day 35, heat stress prompted negative expression of genes related to the cell cycle in all comparisons (Figure 3.9). Additionally, MAPK signaling was differentially regulated in all 4 comparisons. Of the DEGs related to MAPK signaling, 12 genes were commonly upregulated in all 4 comparisons, while only *NF1*, *MAP3K1*, *PTPN7*, *RAC2*, and *PRKCB* were commonly downregulated. In addition, *ANAPC2*, *CCNE1*, *CREBBP*, *E2F4*, *MAD2L2*, *STAG2*, *ZBTB17* were all upregulated, while *ANAPC13*, *CCNB2*, *CDC20*, *CDC45*, *CDK1*, *PLK1*, *RAD21* were commonly downregulated in 100DLM, 100MHA, 140DLM, and 140 MHA. Zhou et al. (2019) concluded methionine and MHA elicit similar healing in mice succumb to intestinal injury. The similar regulation of these genes related to the cell cycle and MAPK signaling agree that these pathways are not influenced by methionine source when broilers are stressed from days 8 to 35.

Ubiquitin mediated proteolysis

The ubiquitin proteasome pathway (UPP) is a process in which enzymes link ubiquitin onto intracellular proteins, marking them for degradation. Under oxidative stress, protein misfolding and damage occurs more frequently, resulting in the activation of the UPP (Wang and Kaufman 2012). The enzymes responsible for marking the intracellular proteins are ubiquitin-activating enzyme (E1), ubiquitin-carrier proteins (E2), and ubiquitin-protein ligase (E3) (Glickman and Ciechanover 2002). Ubiquitin is a 76 amino acid protein which does not exist freely, rather as polyubiquitin chains or attached to ribosomal subunits. Deubiquitinating enzymes free ubiquitin, enabling its utilization for proteolysis. E3 serves the main function in the UPP, and there are over 1,000 E3s, each associated with specific substrates (Jackson et al. 2000). After protein tagging, protease complex 26S proteasome degrades the targeted protein into small peptides (Baumeister et al. 1998).

During the first 21 days, heat stress only influenced genes associated with the UPP in 140MHA. In those broilers, the genes *CDC20*, *CUL2*, *HERC3*, and *UBE2C* were downregulated while *PIAS1* was upregulated on day 21. The genes *CUL2* and *HERC3* are associated with E3 while *UBE2C* is associated with E2. Their downregulation may indicate proteolysis is slightly reduced during heat stress when broilers are fed above-requirement MHA. This result was not seen in any other dietary treatment at day 21 and is unexpected. In previous research, heat stress did induce protein degradation, however mRNA analysis did not reflect this, as proteasomal and ubiquitination genes were not differentially regulated (Wang et al. 1998; Luo et al. 2000). It may be that gene expression seen at day 21 may not reflect enzyme and protein activity. The performance results of the current study seem to agree that gene expression of the UPP cannot be completely associated with protein degradation and lower weight gain.

After 28 days of continuous heat stress, broilers in 100DLM, 140DLM 100MHA, and 140MHA upregulated significantly more genes associated with UPP. The genes *UBE4B*, *PIAS1*, *UBE2D2*, *UBE2O*, *UBE3B*, *STUB1*, and *ANAPC2* were all commonly upregulated between the 4 comparisons. The genes *STUB1*, *UBE4B*, *PIAS1*, and *UBE3B* are associated with E3, while *UBE2O* and *UBE2D2* are associated with E2. ROS stimulates the expression of E2 and E3 in myocytes (Li et al. 2003). Additionally, work has shown protein degradation by the 26s proteasome is increased under oxidative stress (Medicherla and Goldberg 2008; Lee et al. 2010; Manohar et al. 2019). It can be reasoned that these similarly regulated genes related to E2 and E3 are critical in the degradation of proteins in the breast muscle when broilers are subjected to prolonged heat stress.

Increasing methionine inclusion in the diet reduced the number of DEGs associated with proteolysis (Figure 3.10). Del Vesco et al. (2015) investigated methionine supplementation in heat stressed broilers. Their work found expression of genes related to protein degradation was reduced when broilers were fed excess amounts of methionine. Additionally, protein deposition was increased in those birds. Increasing methionine content in the diet of heat stressed poultry is not a new concept, and research has shown promising performance results (Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005; Suganya et al. 2015). It is thought that methionine inhibits proteolysis through the inhibition of atrogin-1 (Tesseraud et al. 2007). The gene *FBXO32* codes for atrogin-1, and expression has been associated with increased UPP activity (Furukawa et al. 2016). In the current study, *FBXO32* was significantly upregulated in 140DLM and 140MHA at day 35, however, *FBXO32* was not differentially regulated in 100DLM or 100MHA. Genes associated with the UPP were more differentially regulated in 100DLM and 100MHA, however. This result is in slight disagreement with Furukawa et al. (2015) and Furukawa et al. (2016), who saw

definitive increased expression of antrogin-1 and increased proteolysis in chickens exposed to heat stress.

FoxO signaling is crucial for the activation of antrogin-1 (Nakashima et al. 2006). Oxidative stress is known to induce the translocation of FoxO transcription factors, affecting proteolysis and apoptosis (Furukawa-Hibi et al. 2002). Both 140DLM and 140MHA upregulated 16 and 20 genes, respectively, related to FoxO signaling. Additionally, 140DLM and 140MHA downregulated only 7 and 9 genes, respectively, to FoxO signaling pathway. The upregulation of genes along this pathway is in agreement with the upregulation of *FBXO32* in 140DLM and 140MHA, indicating atrogin-1 activation. Also in agreement, heat stressed broiler in 100DLM and 100MHA did not upregulate more genes than they downregulated in association with FoxO signaling, indicating the signaling pathway was not impacted by heat stress when broilers were fed at-requirement methionine. These results suggest the regulation of *FBXO32* does not reflect the activity of the UPP after 28 days of heat stress.

CONCLUSION

Heat stress greatly affects regulation of energy metabolism, oxidative state, cellular structure, cellular signaling, and proteolysis in the breast muscle of broilers. At a young age, hot environmental temperatures from day 7 to 10 post-hatch appear to alter regulation of oxidative phosphorylation only in broilers fed at-requirement DLM. After 14 days of heat stress, however, the effects of heat stress on energy metabolism are seen in all broilers. It appears ATP production through aerobic respiration is reduced, possibly to decrease endogenous heat production or to reduce mitochondrial-derived ROS. Interestingly, increasing methionine in the diet did not alleviate the effects of heat stress on the regulation of ATP production. In fact, there were more DEGs associated with glycolysis, the citric acid cycle, and oxidative phosphorylation in broilers

fed above-requirement methionine after 14 days of heat stress. These results suggest methionine source and inclusion level in the diet does effect energy metabolism and possible mitochondrial-derived ROS production. Cellular structure and focal adhesions respond to heat stress differently dependent on methionine source and inclusion. Under heat stress, MHA supplementation resulted in fewer DEGs associated with the actin cytoskeleton and focal adhesion in comparison to DLM supplementation in broiler diets. Conversely, feeding at-requirement DLM appeared to upregulate actin proliferation and focal adhesion complexes, suggesting repair or tolerance to hot temperatures after 14 days of heat stress. After 28 days of continuous elevated temperatures, however, any tolerance or resilience to heat stress appears to diminish, as proteolysis seems to be greatly increased. Energy production through aerobic respiration appears to be reduced at day 35, and the response to ROS was more prevalent in broilers fed at-requirement methionine. Additionally, broilers fed above-requirement methionine differentially regulated fewer genes associated with the UPP, the actin cytoskeleton, and focal adhesion when heat stressed compared to broilers fed at-requirement levels of methionine. Together, these results suggest DLM and MHA supplementation elicit different transcriptome response to heat stress in the breast of broilers. Performance results do not tell the entire story, as no differences between DLM and MHA supplementation were observed. Methionine source and inclusion in the diet did greatly alter the transcriptional response to heat stress, and this should be considered when further researching and discussing heat stress and amino acid nutrition of broilers.

Table 3. 1. Ingredients and composition for diets[1].

Ingredient	Starter	Grower	Finisher
Corn	57.92	64.53	67.80
Soybean meal, 48.5% CP	33.23	27.43	24.04
Soybean oil	3.32	3.20	3.80
Monocalcium phosphate	1.78	1.57	1.28
Limestone ($CaCO_3$)	1.58	1.33	1.12
Biolys®	0.52	0.44	0.38
Premix[2]	0.50	0.50	0.50
Salt (NaCl)	0.31	0.31	0.35
Val AMINO (L-valine)	0.13	0.08	0.06
ThreAMINO®	0.15	0.11	0.10
Sodium biocarbonate	0.08	0.09	0.00
L-Isoleucine	0.05	0.04	0.04
Titanium oxide	0.00	0.00	0.20
Coccidiostat	0.05	0.05	0.05

[1] Starter diet: 100%DL: 0.37% MetAmino®; 140%DLM: 0.52% MetAmino®; 100% MHA: 0.86% DL-MHA-Sipernat®; 140% MHA: 1.20% DL-MHA-Sipernat®.
Grower diet: 100%DL: 0.31% MetAmino®; 140%DLM: 0.43% MetAmino®; 100% MHA: 0.72% DL-MHA-Sipernat®; 140% MHA: 1.02% DL-MHA-Sipernat®.
Finisher diet: 100%DL: 0.28% MetAmino®; 140%DLM: 0.39% MetAmino®; 100% MHA: 0.65% DL-MHA-Sipernat®; 140% MHA: 0.91% DL-MHA-Sipernat®.

[2]Premix provided the following (per kg of feed): Vitamin A (retinyl acetate, 10,000 IU; Vitamin D_3 (cholecalciferol), 2,500 IU; Vitamin E (dl-α-tocopherol), 50 mg; Vitamin K3 (menodione), 1.5 mg; Vitamin B_1 (thiamin), 2.0 mg; Vitamin B_2 (riboflavin), 7.5 mg; Vitamin B_6 (pyridoxine-Hcl), 3.5 mg; Vitamin B_{12} (cyanocobalamin), 20 μg; Niacin, 35 mg; D-pantothenic acid, 12 mg; Choline chloride, 460 mg; Folic acid, 1.0 mg; Biotin, 0.2 mg; Iron ($FeSO_4.H_2O$), 80 mg; Copper ($CuSO_4.5H_2O$), 12 mg; Manganese (MnO), 85 mg; Zinc ($ZnSO_4.H_2O$), 60 mg; Iodate, 0.8 mg; Selenium (Na_2SeO_3), 0.15 mg.

Table 3. 2. Analyzed crude protein, metabolizable energy (AME), sulfur amino acids (methionine and cysteine) and DL-2-hydroxy-(4-methylthio) butanoic acid composition of diets[1].

	100% DLM	140% DLM	100% MHA	100% MHA
Starter (Day 0-10)				
Crude Protein, %	22.36	22.44	21.77	22.11
Methionine,%	0.653	0.792	0.306	0.303
Cysteine, %	0.344	0.339	0.341	0.343
Methionine-Cysteine, %	0.997	1.131	0.647	0.646
MHA	0.000	0.000	0.533	0.738
AME (Kcal/kg)	2966	2959	2982	2958
AME (MJ/kg)	12.42	12.39	12.49	12.39
Grower (Day 11-21)				
Crude Protein, %	19.71	19.77	19.76	19.18
Methionine,%	0.573	0.669	0.272	0.274
Cysteine, %	0.307	0.306	0.306	0.305
Methionine-Cysteine, %	0.880	0.975	0.578	0.579
MHA	0.000	0.000	0.460	0.652
AME (Kcal/kg)	3012	3014	3043	3036
AME (MJ/kg)	12.61	12.62	12.74	12.71
Finisher (Day 22-35)				
Crude Protein, %	19.92	18.28	18.01	17.62
Methionine,%	0.511	0.630	0.255	0.254
Cysteine, %	0.284	0.288	0.287	0.286
Methionine-Cysteine, %	0.795	0.918	0.542	0.540
MHA	0.000	0.000	0.412	0.584
AME (Kcal/kg)	3071	3066	3062	3062
AME (MJ/kg)	12.86	12.84	12.82	12.82

[1]DLM = DL-methionine; MHA = DL-2-hydroxy-(4-methylthio) butanoic acid; 100 = 100% of methionine requirement; 140 = 140% of methionine requirement

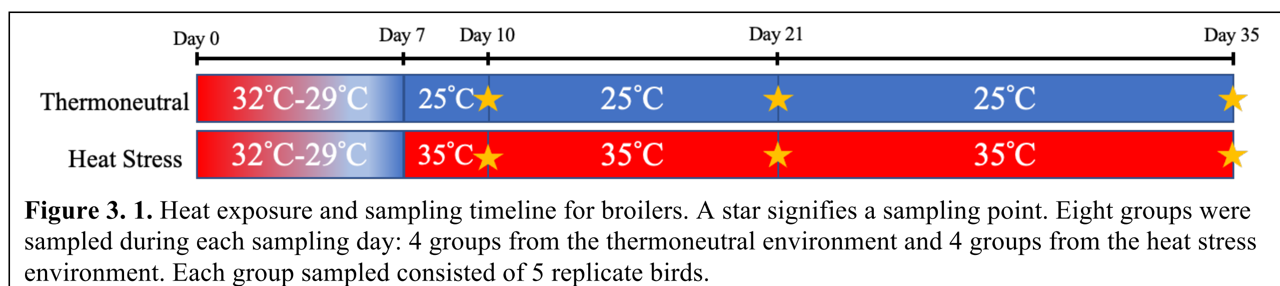

Figure 3. 1. Heat exposure and sampling timeline for broilers. A star signifies a sampling point. Eight groups were sampled during each sampling day: 4 groups from the thermoneutral environment and 4 groups from the heat stress environment. Each group sampled consisted of 5 replicate birds.

Table 3. 3. Forward and reverse primers used for RT-qPCR validation of RNA-sequencing.

Gene	Forward Primer	Reverse Primer
CHRNG	5′ GCTGGAGAAGATTGGACGTG 3’	5′ AAGAGCGAGGCCATGATGAA 3’
FBLN5	5′ AAGATCGTGCCAAGACGTGA 3’	5′ AATAGGCTCCAGGGTAGCGA 3’
ATP5G1	5′ GGACACGGCAAGTAATAGG 3’	5′ CATCAAACAGAAGAGACCCA 3’
GSN	5′ GAGGAGGGAGAAGCCCATTTG 3’	5′ CACCACTGATAGATGTTACTGCC 3’
PVALB	5′ TGGGAGCCTTTTCAGCAGC 3’	5′ CGTCCTTATCTCCAGCAGCC 3’
SLC7A11	5′ AACTGCTGGTTATTCGCCCT 3’	5′ GGGTAGTCCACACTTCCCAT 3’
PEAK1	5′ TTCAGCGAGGCGAAATCAGA 3’	5′ ATCGGGCACTTGACTGGGG 3’
GLO1	5′ CCGAGGATTTGGGCACATT 3’	5′ ATGCTCCTTGCTGAAGCTCAT 3’
CDK1	5′ TACCCACAGCCATGGAGGAT 3’	5′ GGGTGTCCCTAAAGCTCTGA 3’
β-actin	5′ AGACATCAGGGTGTGATGGTTGGT 3’	5′ TCCCAGTTGGTGACAATACCGTGT 3’

Table 3. 4. Effect of heat stress, dietary methionine, and methionine level on broiler chicken body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) during the starter phase.

Temperature[1]	Source[2]	Level[3]	BWG	FI	FCR
TN	DLM	100	172.0	137.9	0.80
TN	DLM	140	168.4	133.8	0.80
TN	MHA	100	157.3	120.6	0.77
TN	MHA	140	163.7	127.1	0.78
HS	DLM	100	160.5	122.6	0.76
HS	DLM	140	164.9	122.9	0.75
HS	MHA	100	166.8	128.6	0.77
HS	MHA	140	158.3	118.7	0.75
SEM			3.7	4.8	0.02
Main effect of temperature					
TN			165.4	129.8	0.79
HS			162.6	123.2	0.78
SEM			1.85	2.4	0.01
Main effect of methionine source					
DLM			166.4	129.33	0.78
MHA			161.5	123.7	0.77
SEM			1.85	2.4	0.01
Main effect of methionine level					
100			164.1	127.4	0.78
140			163.8	125.6	0.77
SEM			1.85	2.4	0.01
Probability					
Model			0.0919	0.0945	0.7054
Temperature			0.2965	0.0585	0.1110
Source			0.0661	0.1118	0.5219
Level			0.9067	0.6052	0.6631
Temperature x Source			0.0739	0.0670	0.3566
Temperature x Level			0.5073	0.3927	0.5209
Source x Level			0.7830	0.9798	0.8872
Temperature x Source x Level			0.0338	0.1358	0.8581

[1]TN=25 ^{o}C; HS=35 ^{o}C
[2]DLM=DL-methionine; MHA= DL-2-hydroxy-(4-methylthio) butanoic acid
[3]100=100% of methionine requirement; 140=140% of methionine requirement
Mean values within a column with no common superscript differ significantly (p<0.05)

Table 3. 5. Effect of heat stress, dietary methionine, and methionine level on broiler chicken body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) during the grower phase.

Temperature[1]	Source[2]	Level[3]	BWG	FI	FCR
TN	DLM	100	600.6^{a}	907.9^{a}	1.51^{bc}
TN	DLM	140	614.7^{a}	911.4^{a}	1.48b^{c}
TN	MHA	100	609.5^{a}	873.3^{a}	1.43^{c}
TN	MHA	140	632.9^{a}	919.4^{a}	1.45^{c}
HS	DLM	100	429.5^{b}	749.8^{b}	1.75^{ab}
HS	DLM	140	417.7^{b}	708.7^{b}	1.70^{abc}
HS	MHA	100	430.6^{b}	708.3^{b}	1.64^{abc}
HS	MHA	140	410.9^{b}	740.3^{b}	1.80^{b}
SEM			19.3	14.7	0.06
Main effect of temperature					
TN			614.4^{a}	903.0^{a}	1.48^{b}
HS			422.2^{b}	726.8^{b}	1.72^{a}
SEM			9.7	7.4	0.03
Main effect of methionine source					
DLM			515.6	819.5	1.62
MHA			520.9	810.3	1.59
SEM			9.7	7.4	0.03
Main effect of methionine level					
100			517.5	809.8	1.59
140			519.1	820.0	1.62
SEM			9.7	7.4	0.03
Probability					
Model			<0.0001	<0.0001	<0.0001
Temperature			<0.0001	<0.0001	<0.0001
Source			0.7008	0.3853	0.5498
Level			0.9116	0.3372	0.6081
Temperature x Source			0.5521	0.6899	0.4045
Temperature x Level			0.2146	0.1665	0.4846
Source x Level			0.9800	0.0083	0.1944
Temperature x Source x Level			0.7556	0.4660	0.3145

[1]TN=25 ^{o}C; HS=35 ^{o}C
[2]DLM=DL-methionine; MHA= DL-2-hydroxy-(4-methylthio) butanoic acid
[3]100=100% of methionine requirement; 140=140% of methionine requirement
Mean values within a column with no common superscript differ significantly ($p<0.05$)

Table 3. 6. Effect of heat stress, dietary methionine, and methionine level on broiler chicken body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) during the finisher phase.

Temperature[1]	Source[2]	Level[3]	BWG	FI	FCR
TN	DLM	100	1,412.1[a]	2,423.1[a]	1.72[c]
TN	DLM	140	1,417.1[a]	2,439.2[a]	1.72[c]
TN	MHA	100	1,407.7[a]	2,415.3[a]	1.72[c]
TN	MHA	140	1,257.8[a]	2,295.8[a]	1.83[bc]
HS	DLM	100	632.3[b]	1,353.5[b]	2.14[a]
HS	DLM	140	654.0[b]	1,374.7[b]	2.10[a]
HS	MHA	100	624.2[b]	1,367.3[b]	2.19[a]
HS	MHA	140	739.5[b]	1,470.5[b]	1.99[ab]
SEM			42.4	35.9	0.06
Main effect of temperature					
TN			1,373.6[a]	2,393.3[a]	1.75[b]
HS			662.5[b]	1,391.5[b]	2.12[a]
SEM			21.2	18.0	0.03
Main effect of methionine source					
DLM			1,028.9	1,897.6	1.93
MHA			1,007.3	1,887.2	1.95
SEM			21.2	18.0	0.03
Main effect of methionine level					
100			1,019.1	1,889.8	1.95
140			1,017.1	1,895.1	1.92
SEM			21.2	18.0	0.03
Probability					
Model			<0.0001	<0.0001	<0.0001
Temperature			<0.0001	<0.0001	<0.0001
Source			0.4760	0.6857	0.6664
Level			0.9484	0.8370	0.4321
Temperature x Source			0.0515	0.0142	0.2809
Temperature x Level			0.2390	0.0306	0.0311
Source x Level			0.6128	0.6018	0.7668
Temperature x Source x Level			0.0449	0.0384	0.1143

[1]TN=25 °C; HS=35 °C
[2]DLM=DL-methionine; MHA= DL-2-hydroxy-(4-methylthio) butanoic acid
[3]100=100% of methionine requirement; 140=140% of methionine requirement
Mean values within a column with no common superscript differ significantly ($p<0.05$)

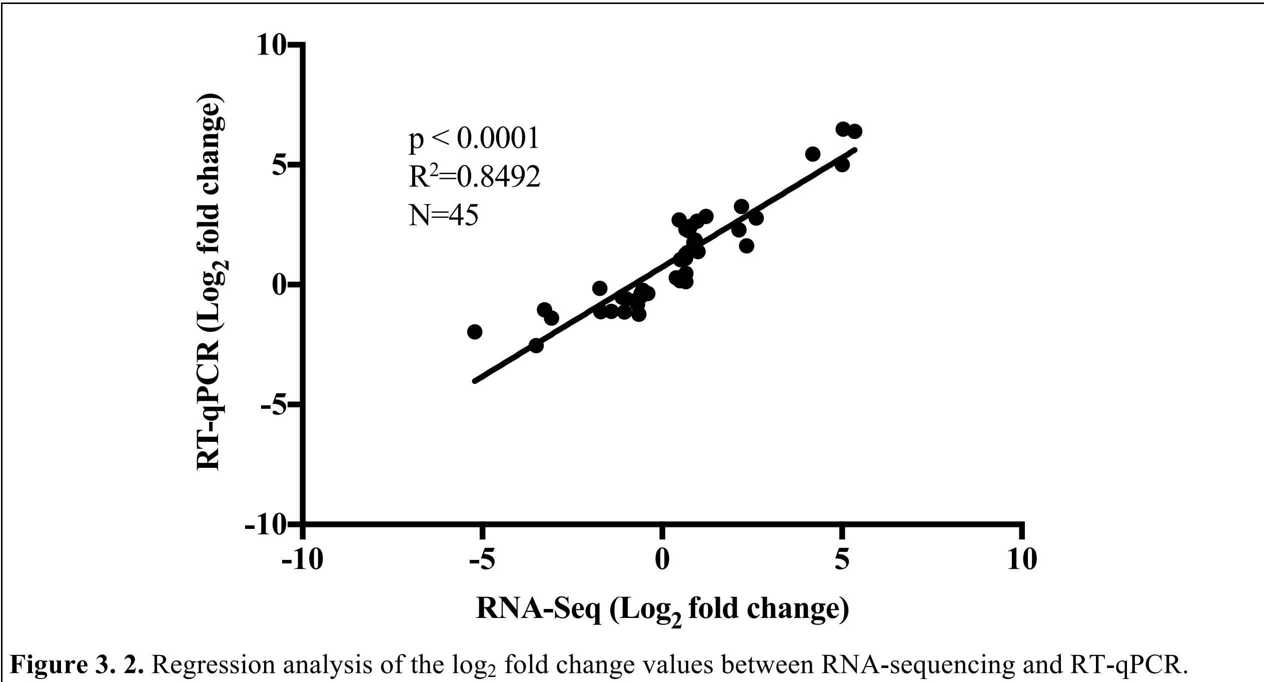

Figure 3. 2. Regression analysis of the $\log_2$ fold change values between RNA-sequencing and RT-qPCR.

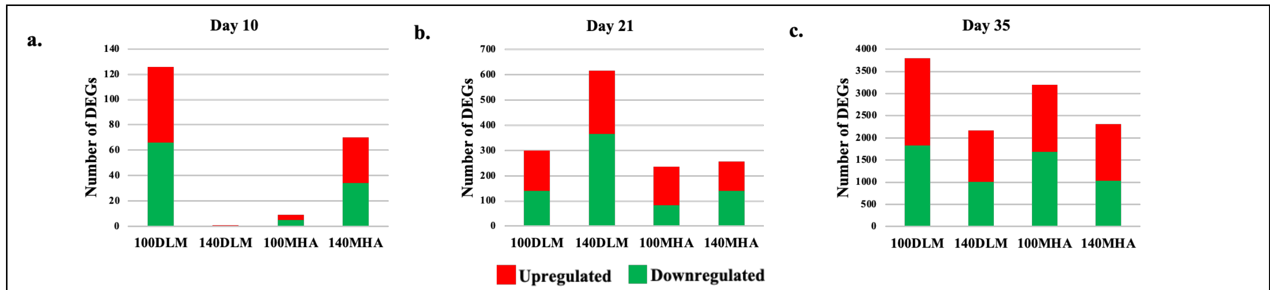

Figure 3. 3. Number of differentially expressed genes (DEGs) in the *Pectoralis major* of broiler chickens reared at 35°C on days 10 (**a**), 21 (**b**), and 35 (**c**). The X-axis represents the comparison for heat-stressed broilers in comparison to thermoneutral broilers fed at-requirement D,L-methionine (100DLM), above-requirement D,L-methionine (140DLM), at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), or above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA).

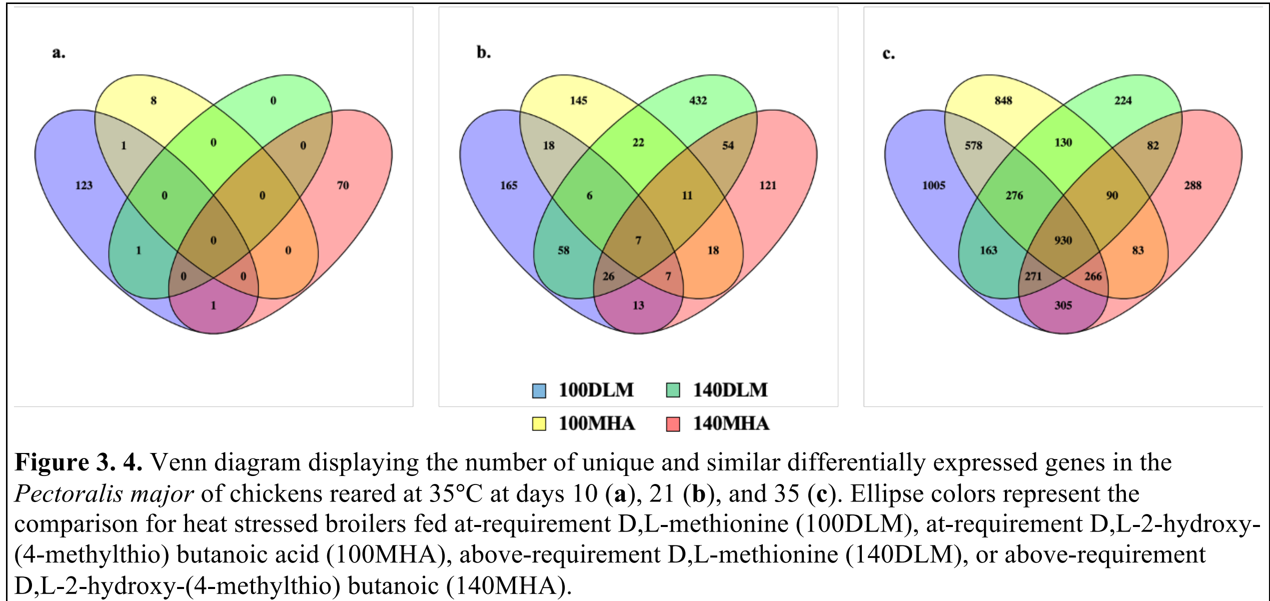

Figure 3. 4. Venn diagram displaying the number of unique and similar differentially expressed genes in the *Pectoralis major* of chickens reared at 35°C at days 10 (**a**), 21 (**b**), and 35 (**c**). Ellipse colors represent the comparison for heat stressed broilers fed at-requirement D,L-methionine (100DLM), at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), above-requirement D,L-methionine (140DLM), or above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA).

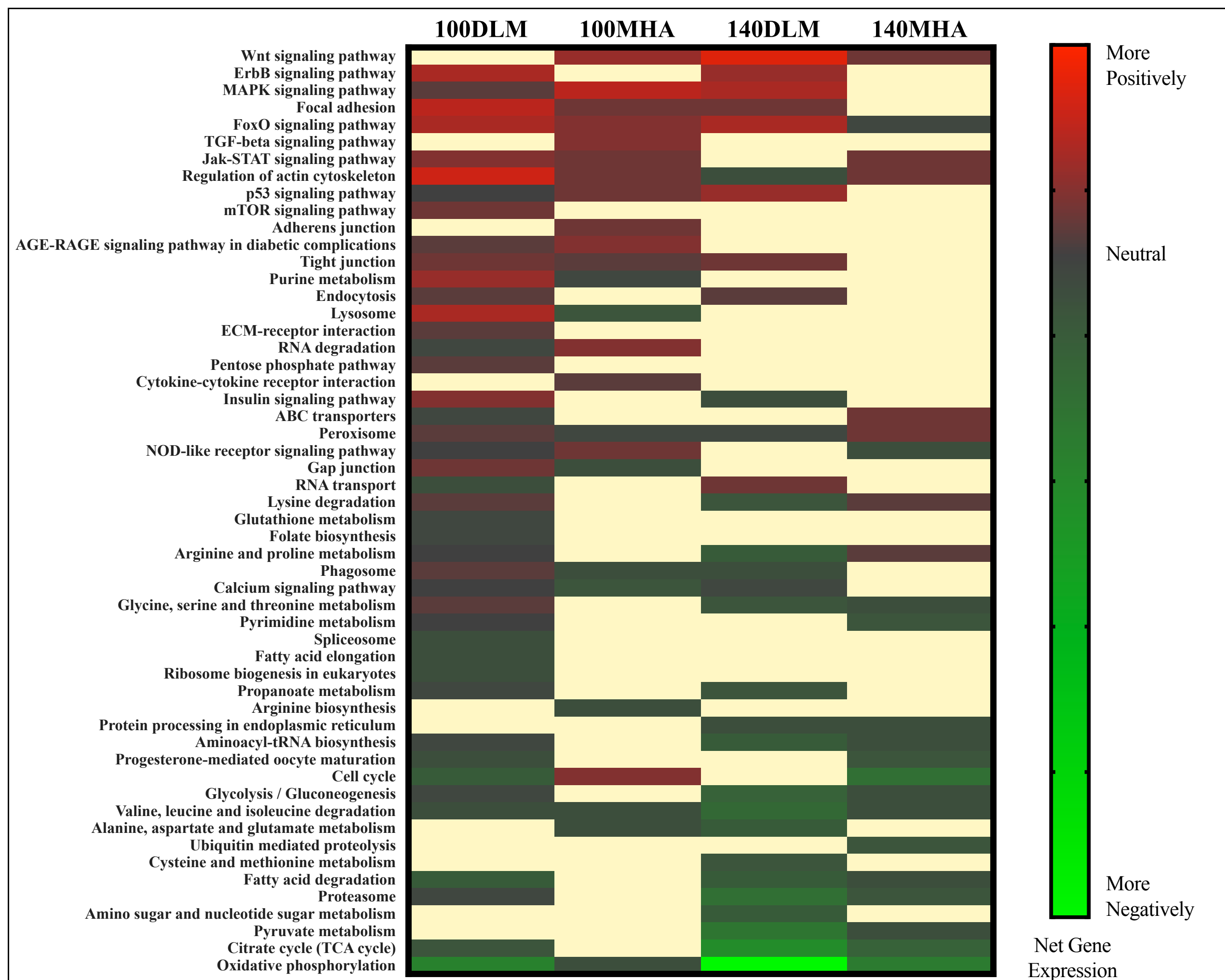

Figure 3. 5. Net gene expression of KEGG terms in the *Pectoralis major* of broiler chickens reared at 35°C on day 21. For each individual KEGG term, the number of positively (+1) and negatively (-1) differentially expressed genes were added to obtain net expression for heat stressed broilers fed at-requirement D,L-methionine (100DLM), at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), above-requirement D,L-methionine (140DLM), or above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA). Yellow fill indicates non-enriched pathway.

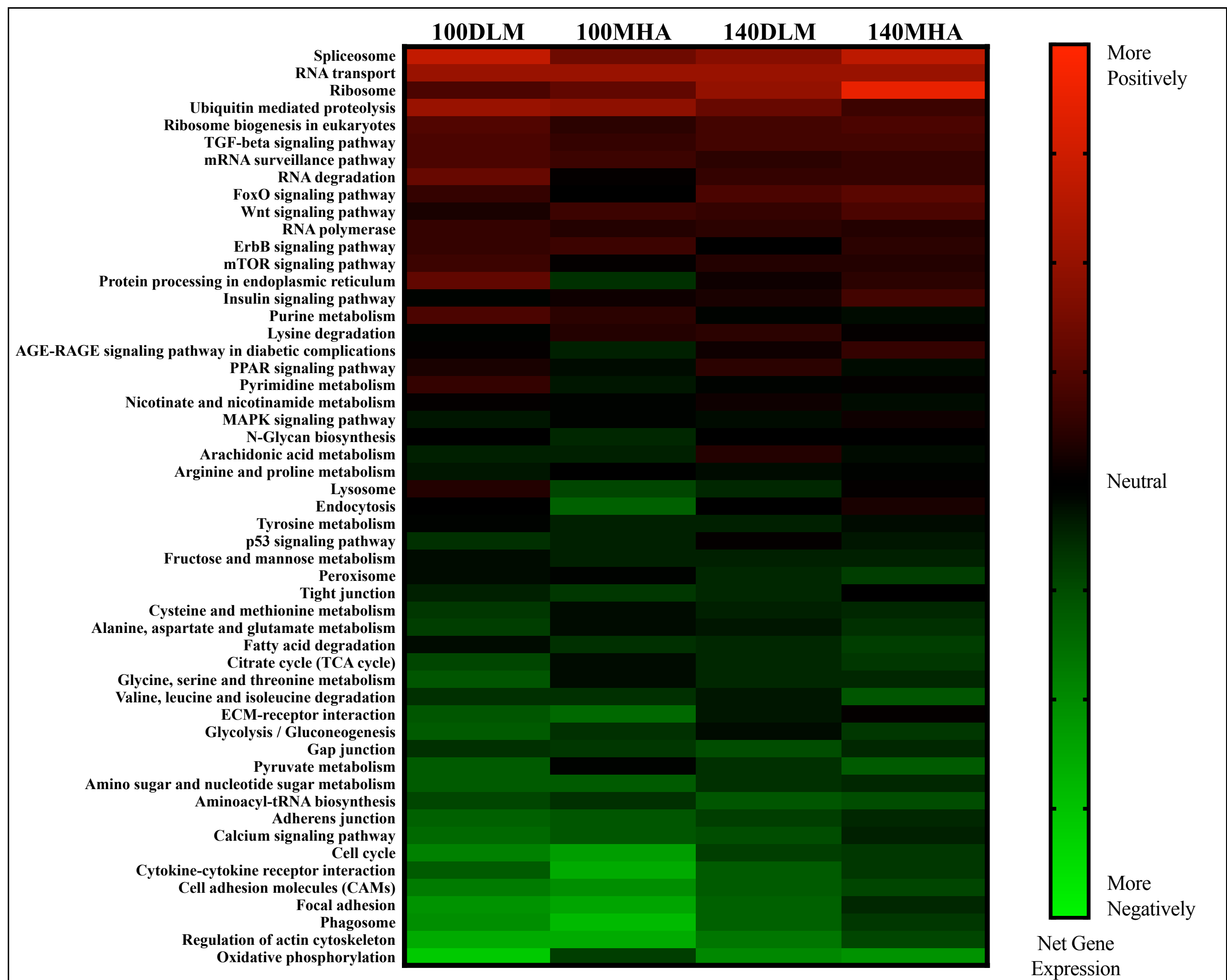

Figure 3. 6. Net gene expression of KEGG terms in the *Pectoralis major* of broiler chickens reared at 35°C on day 35. For each individual KEGG term, the number of positively (+1) and negatively (-1) differentially expressed genes were added to obtain net expression for heat stressed broilers fed at-requirement D,L-methionine (100DLM), at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), above-requirement D,L-methionine (140DLM), or above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA).

Table 3. 7. Top 50 upregulated genes in the *Pectoralis major* of 10-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement DLM (100DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
ADPRHL1	3.8797911	0.04497614	*GLUL*	0.80031719	5.57E-06
PDK4	3.31166488	0.00098905	*ACKR3*	0.79117938	0.00098905
CHAC1	2.84418651	0.0285082	*RAP1GAP2*	0.78804856	0.02704437
CEBPD	1.67740002	0.04425893	*OASL*	0.77800913	0.02631827
DDIT4	1.56957319	0.02070429	*MOV10*	0.72614059	0.01480909
PFKFB3	1.3732097	0.01480909	*PLXDC2*	0.72238885	0.01068511
UCP3	1.26877029	0.00031213	*WNK4*	0.70651086	0.02372718
C15orf48	1.25884961	0.02064247	*NRP2*	0.65946212	0.01910991
BLB1	1.18137202	0.00189836	*CEBPB*	0.6456388	0.00276762
KLF15	1.15987187	0.01457484	*SCARA5*	0.63689902	0.03737089
KCNN1	1.10796411	0.00151751	*SORD*	0.62964415	0.04709544
RGCC	1.02660976	3.03E-06	*HAGHL*	0.61655182	0.01910991
SESN1	1.02522415	0.01480909	*FAM20A*	0.59799142	0.01480909
RAMP1	1.00064344	0.0115224	*UCK1*	0.58250148	0.02064247
CHRNG	0.9954942	0.02889147	*TSPAN15*	0.57645249	0.02064247
MAPK12	0.97280472	0.04574474	*ATP13A2*	0.56511404	0.04319361
HPGD	0.96403993	0.01340838	*STAT2*	0.56131583	0.01490933
WBP1L	0.96144092	0.02274444	*FZD1*	0.55743889	0.04859013
NTHL1	0.95712338	0.04984122	*ZFP36L2*	0.55607618	0.02992318
INSRR	0.94448675	0.0488771	*CDK5*	0.55550573	0.04984122
DDO	0.92807163	0.02722143	*ANGPTL2*	0.5271664	0.01340838
TCIM	0.89851153	0.01910991	*MKS1*	0.52201533	0.02671711
NFKBIA	0.88790622	4.81E-06	*RNF168*	0.49345606	0.01910991
EPSTI1	0.87153805	0.04098329	*FBXL20*	0.48243239	0.01713928
SLC4A1	0.85122389	0.02070429	*LRP1*	0.45735918	0.04984122

Table 3. 8. Top 50 downregulated genes in the *Pectoralis major* of 10-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement DLM (100DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
KCNU1	-1.6288197	0.01910991	*MAPK9*	-0.6237443	0.02274444
CHADL	-1.4363533	0.01480909	*SMG1*	-0.6202327	0.01480909
PEAK1	-1.0712806	0.00151751	*CELF1*	-0.6177177	0.02111774
FAM126B	-1.0417825	0.01480909	*DHX29*	-0.5998132	0.02889147
UBE2C	-0.9519076	0.01652299	*ATP6V1C1*	-0.5738815	0.03785743
LRRC14B	-0.8621858	0.00031213	*CMAS*	-0.5526303	0.02064247
AQP9	-0.8607574	0.00660077	*RAB22A*	-0.5440139	0.02048893
GLS	-0.8519881	0.00031213	*NABP1*	-0.541113	0.01480909
KIFC1	-0.8014367	0.03785743	*DES*	-0.5362728	0.00729162
GATM	-0.7918436	0.04319361	*ZBTB25*	-0.5216293	0.04859541
METTL6	-0.7777988	0.03737089	*COX6A1*	-0.5109624	0.04319361
CIT	-0.7668352	0.0393124	*COX7A2*	-0.5083354	0.01480909
MYO1A	-0.7305085	0.00142887	*MAT1A*	-0.4798687	0.00308306
FYCO1	-0.7296306	0.02529655	*KLHL18*	-0.47807	0.01597316
KIF11	-0.7265708	0.04319361	*UHRF1BP1L*	-0.4718866	0.02828334
AFF4	-0.7144026	0.01480909	*NBAS*	-0.4654956	0.01802714
MT-ND2	-0.7137744	0.01457484	*NDUFV2*	-0.4597529	0.02889147
ZZEF1	-0.686694	0.00142887	*UMAD1*	-0.4486598	0.02631827
AZIN1	-0.6861531	0.01480909	*OBSCN*	-0.4407266	0.00283611
ND1	-0.6666074	0.04319361	*NUDCD1*	-0.4390513	0.04859541
PANK3	-0.6619169	0.02529655	*AQR*	-0.4354254	0.04319361
SGCD	-0.6555859	0.00545146	*MTFR1L*	-0.4305051	0.04187513
DENND4A	-0.648696	0.04984122	*PRELID3B*	-0.4298489	0.01597316
YOD1	-0.6405488	0.02868138	*UCHL3*	-0.4212197	0.02631827
MRPL15	-0.6388645	0.02529655	*CLN6*	-0.420795	0.01457484

Table 3. 9. Upregulated genes in the *Pectoralis major* of 10-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement MHA (140MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
FGA	6.85879951	0.01559384	*ENKD1*	0.67692593	0.01843604
FGG	4.91593483	0.02900461	*FBLN5*	0.65930779	0.03398771
ALB	4.79703073	0.00341219	*LONRF3*	0.65301803	0.01029247
APOA4	4.5966113	0.00120166	*CREBL2*	0.56215047	0.00797117
MAL2	2.22315177	0.00639688	*PLPP6*	0.55469789	0.01032603
SATB2	1.77479589	0.03398771	*SLC9A1*	0.54071296	0.00488
KCNE3	1.45326478	0.02150733	*FAM43A*	0.50307326	0.0138408
RGS9BP	1.21445127	0.02113966	*MKNK2*	0.49002114	0.01962077
CREBRF	1.07704221	0.02749451	*MRC2*	0.47651441	0.01029247
SH2B2	1.02684324	0.00797117	*SEC24A*	0.47004027	0.00488
UBE2QL1	1.00362845	0.01882712	*DDX10*	0.43744674	0.01842138
ANKRD9	0.99506819	0.01843604	*CRY2*	0.43203671	0.02165763
TMEM164	0.78888112	0.0416355	*WAC*	0.42602113	0.02294099
ADAMTS2	0.74224997	0.02900461	*ESCO1*	0.39679422	0.03239598
GIT1	0.72220865	0.00488	*GATAD2B*	0.38770536	0.00797117
RFX7	0.71230231	0.04600602	*ARHGAP35*	0.37678315	0.04427409
GJA1	0.70141916	0.00488	*GCN1*	0.37178127	0.03910191
IFT27	0.68280459	0.03368208	*POLR3E*	0.29597056	0.02389873

Table 3. 10. Downregulated genes in the *Pectoralis major* of 10-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement MHA (140MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
SDR42E1	-1.4972831	0.01882712	*EGF*	-0.6815541	0.00488
PPP1R3C	-1.2418328	0.00488	*DHCR7*	-0.6713711	0.02925663
SLC38A4	-1.2154583	0.03094389	*PNRC2*	-0.6313767	0.02389873
LBH	-1.1114228	0.00895023	*MCM8*	-0.5533563	0.00797117
CHRNG	-1.0512445	0.02799597	*MIPEP*	-0.5456906	0.01166719
NQO1	-0.9369416	0.03318887	*GATB*	-0.5410711	0.02625757
NSDHL	-0.8991189	0.00797117	*RCAN1*	-0.5396048	0.01032603
BLNK	-0.891947	0.01184901	*TADA2A*	-0.4824966	0.00488
APLNR	-0.8633069	0.04697298	*FASN*	-0.4588936	0.02150733
STARD4	-0.8027814	0.01515218	*PMPCB*	-0.4404351	0.00067606
TMOD1	-0.7984903	0.00488	*SUCLG2*	-0.4266937	0.01501689
SQLE	-0.7828132	0.01184901	*TARSL2*	-0.4174679	0.04231261
PREPL	-0.755101	0.02199578	*TALDO1*	-0.4081294	0.01184901
HMGCR	-0.7429768	0.00713686	*ALDH7A1*	-0.3995338	0.04664122
SCD	-0.7346	0.01843604	*HMGB3*	-0.3537323	0.0416355
CYP51	-0.7211984	0.01882712	*RASA3*	-0.3222515	0.0416355

Table 3. 11. Top 50 upregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement DLM (100DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
FGA	25.474011	3.23E-13	*LCAT*	1.59316337	0.01374728
GC	23.7908568	1.61E-11	*SCARNA15*	1.52467149	5.56E-05
PIT54	11.4078103	0.00100464	*KCNN1*	1.43168307	0.02195408
ALB	9.71173298	5.03E-06	*TDRKH*	1.29155508	0.02467585
FGG	9.53761142	0.02467585	*DKK3*	1.27362816	0.03825548
APOH	7.44644115	0.044215	*TINAG*	1.01507587	0.00534579
SPP2	6.84027456	0.00510501	*ASB11*	1.01127012	0.01068378
FGB	6.52560704	0.00171762	*P4HA2*	0.99556122	5.56E-05
APOA4	6.01922556	0.0076566	*PPL*	0.99333485	0.00510501
HPX	5.90838421	0.01026627	*E2F7*	0.98156292	0.04717426
VTN	4.85660505	0.00288594	*SLC12A9*	0.96069391	0.02064571
HMGCS2	4.34623166	0.02195408	*GRAMD1B*	0.87515192	0.0076561
SERPIND1	4.33266309	0.01026627	*GLA*	0.85124077	0.00688474
AHSG	4.00386393	0.03208955	*ADAMTS13*	0.84992435	0.01003177
KNG1	3.8062422	0.04321248	*SCN2B*	0.82352744	0.00534579
ALDOB	3.58007637	0.0148389	*PYGO1*	0.77889828	0.02354426
F2	3.08821673	0.0284408	*PKD1*	0.73920927	0.0002748
C5	3.02837765	0.02585549	*SEC14L5*	0.72865414	0.02866958
SERPINC1	3.01464205	0.04451182	*IFT27*	0.71664664	0.04297067
CFI	2.07738463	0.04519598	*HMCN1*	0.70636812	0.03975002
APOD	1.97059005	0.01201005	*KAZALD1*	0.70499189	0.0475285
XDH	1.94450309	0.03975002	*PLAT*	0.69877748	0.00397667
GPR20	1.93222761	0.03138829	*PLA2G15*	0.68311175	0.01013226
ITIH2	1.91714107	0.04116584	*FBXW4*	0.68231286	0.00534579
PLXNA4	1.77884309	0.01214009	*ATP8A1*	0.67863299	0.03116733

Table 3. 12. Top 50 downregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement DLM (100DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
FOS	-2.2387268	0.00847234	*LMCD1*	-0.8849873	0.04451182
HBAD	-2.0477889	0.01630255	*CCDC112*	-0.8776925	0.01026627
NR4A2	-2.0335015	0.02264958	*MYH7B*	-0.8291082	0.00688599
GJD2	-1.8205202	0.02693815	*VAMP1*	-0.8290266	0.00165331
HBAA	-1.7679582	0.01902212	*CDC45*	-0.8218261	5.37E-05
SCNN1A	-1.4042999	8.58E-09	*ASPM*	-0.7934041	0.03116733
OTUD1	-1.3937938	0.00171762	*DNA2*	-0.7899485	0.02467585
CTGF	-1.3047395	0.01026627	*CRYL1*	-0.7853146	0.01289577
ND6	-1.2661401	0.00030735	*CENPK*	-0.7818746	0.01039836
CRCP	-1.1977188	0.00391608	*SPC25*	-0.7470464	0.03361594
GCH1	-1.1517185	0.04519598	*SAT2*	-0.7329598	0.02195408
HSP90AB1	-1.1098982	0.00607475	*SH3KBP1*	-0.6963954	0.02195408
STXBP5L	-1.0799078	0.01026627	*SLC25A12*	-0.6907341	0.04321248
MT-CO2	-1.0750832	0.00017232	*SCNM1*	-0.6822932	0.04868792
JUN	-1.0340453	0.02195408	*COX3*	-0.6704705	0.0475285
IFT57	-1.0274495	0.04451182	*LANCL2*	-0.6655948	0.00586036
TFRC	-1.0261274	0.01026627	*HSDL2*	-0.6629903	0.00038167
ATF3	-1.0165879	0.04710741	*CCNB2*	-0.6611003	0.01790527
NUF2	-1.0009888	0.03733569	*MIPEP*	-0.6461828	0.0113288
GINS2	-0.9861959	0.04305706	*NAP1L4*	-0.6425451	0.03388896
MT-CO1	-0.9794381	0.00385028	*TOB1*	-0.6396988	0.00562077
TFPI	-0.9693278	0.03167372	*MLF1*	-0.6375706	0.04451182
HIST1H2B5	-0.9571973	0.01686679	*E2F1*	-0.6345213	0.02195408
ACTC1	-0.9558649	0.00639919	*PLS3*	-0.6212289	0.00479759
RACGAP1	-0.9506223	0.040376	*ACAD9*	-0.6195313	0.00171762

Table 3. 13. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with at-requirement DLM on day 21 (100DLM).

KEGG Term	Associated Genes
Upregulated	
Regulation of actin cytoskeleton	*ARHGAP35, F2, GSN, PAK3, PFN2, PIK3CA, PIKFYVE, SOS1, SOS2*
Focal adhesion	*ARHGAP35, JUN, PAK3, PIK3CA, PTEN, SOS1, SOS2, VTN*
ErbB signaling pathway	*CDKN1B, ERBB4, JUN, PAK3, PIK3CA, SOS1, SOS2*
Insulin signaling pathway	*FBP2, PHKA1, PIK3CA, PPP1R3D, SOS1, SOS2, SREBF1*
FoxO signaling pathway	*CCNB2, CDKN1B, FOXO3, PIK3CA, PTEN, SOS1, SOS2*
Downregulated	
Oxidative phosphorylation	*ATP5I, COX3, COX4I1, COX6A1, ND6, NDUFA10, NDUFB5, NDUFS3, NDUFS7, PPA1*
Progesterone-mediated oocyte maturation	*ADCY9, CCNA2, CCNB2, CDC25A, HSP90AB1, PIK3CA*
Cell cycle	*CCNA2, CCNB2, CDC25A, CDC45, CDKN1B, E2F1*
Herpes simplex infection	*C5, FOS, HNRNPK, JUN, SRSF1, TAB2*
Cardiac muscle contraction	*ACTC1, COX3, COX4I1, COX6A1, MYH7B*

Table 3. 14. Top 50 upregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement DLM (140DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
PVALB	2.13162018	1.39E-06	*GRAMD1B*	0.83201259	0.00035953
HS3ST2	1.82658445	0.03085327	*CLGN*	0.76325313	0.01408594
GPM6A	1.7709334	0.00886301	*SLC41A3*	0.76013061	0.03163805
BBOF1	1.43723882	4.59E-05	*SLC9A7*	0.750342	0.00200822
SRD5A2	1.41745671	0.03155679	*MAPK13*	0.7466334	0.01663471
MCM9	1.28747268	0.0076729	*CYP2R1*	0.74304712	0.03172684
METTL7A	1.23384933	5.89E-05	*C7*	0.73780705	0.00695257
AANAT	1.19214665	0.0103084	*TINAG*	0.73384864	0.0041322
PRTG	1.17324175	0.00092014	*KAZALD1*	0.7269776	0.02460103
LACTBL1	1.16409622	0.03218084	*ABRA*	0.72535562	0.04652024
RGCC	1.15204969	0.0254128	*ADAMTS13*	0.71524743	0.00765182
PARP16	1.10790661	5.08E-08	*LZTS2*	0.71508716	0.00035519
IGSF11	1.08519898	0.03660541	*CCNG1*	0.69368534	1.14E-05
CDKN1A	1.07011562	0.02828038	*PLEKHG1*	0.67870664	2.33E-06
ATP8B3	1.03064259	0.03345623	*RFX7*	0.67117544	0.00024659
PARVB	1.00631935	0.00449584	*FBLN1*	0.66988498	0.00083139
DCX	0.96927631	0.02044808	*CRY2*	0.6600307	6.43E-10
CHRND	0.93608369	0.00273696	*AASS*	0.65816593	0.0010623
HSF2BP	0.91271052	0.00085008	*CDH23*	0.64827944	0.0162957
CETP	0.90519082	0.01193997	*SEMA6D*	0.64444145	6.92E-05
PSAT1	0.8934259	5.69E-06	*ATOH8*	0.64106388	0.01829545
TMEM233	0.88543859	0.00406955	*SVIP*	0.63639175	0.00663216
SCN2B	0.85988218	1.34E-05	*PARP6*	0.62955668	0.00322058
FREM2	0.85611771	0.02966308	*GLIS2*	0.62561368	0.00062443
PODXL2	0.83617006	0.00087226	*OGN*	0.62228512	0.00171248

Table 3. 15. Top 50 downregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement DLM (140DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	-6.6515869	0.00025839	*JAK3*	-0.962825	0.02496104
GJD2	-2.2924692	1.10E-07	*THSD7B*	-0.9530832	5.12E-06
SLC7A11	-1.709427	0.00625212	*KIFC1*	-0.9397627	0.00422069
ELFN1	-1.7037805	0.00056374	*SCNN1A*	-0.9270106	0.01670349
ND6	-1.6429236	4.11E-33	*PTK2B*	-0.9209661	0.03369558
HBAD	-1.6402941	0.01770955	*TUSC3*	-0.8987307	1.55E-05
FBLN7	-1.5756761	4.17E-05	*SLC16A3*	-0.8834001	0.00093161
HSPB7	-1.5378252	0.01663471	*PLEKHH1*	-0.8755396	0.04448397
ANKRD2	-1.4419132	0.03450158	*ECH1*	-0.875413	0.00249751
MT-CO2	-1.3194722	2.55E-09	*APEH*	-0.8696928	0.0004043
PAQR9	-1.2619696	0.00036346	*PFKFB1*	-0.8496639	0.00171046
MT-CO1	-1.2593206	1.15E-09	*SLC4A1*	-0.8476546	0.00795753
TRIM27.1	-1.2315409	0.03243854	*CA2*	-0.8465553	0.0053459
HSP90AB1	-1.210307	6.94E-07	*ACOT8*	-0.8368079	5.22E-06
SLC25A30	-1.1088163	0.04023676	*TAGLN*	-0.8284519	0.00683618
GRIA3	-1.0967196	0.01612459	*PGPEP1L*	-0.8241045	0.01357251
HPGDS	-1.094416	0.01738099	*P2RY14*	-0.8236632	0.00072437
COX3	-1.0861537	5.75E-06	*OASL*	-0.8150287	0.04023676
HIST4H4	-1.0471825	0.01260077	*NTN1*	-0.8133654	0.04652024
DNASE1L3	-1.0339761	0.00453033	*GLS*	-0.8112973	5.75E-08
CEP55	-1.0064854	0.04210419	*IDH3A*	-0.7809607	1.46E-13
PDCL2	-0.9864196	0.03126828	*SAMSN1*	-0.7809556	0.04714091
GATM	-0.9774487	0.00133705	*HIST2H2AC*	-0.7710817	0.00029757
RPL22L1	-0.9744162	0.00062239	*MME*	-0.769826	0.00266738
SLC16A6	-0.9733851	0.04089474	*DUPD1*	-0.7656374	1.55E-08

Table 3. 16. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with above-requirement DLM on day 21 (140DLM).

KEGG Term	Associated Genes
Upregulated	
Wnt signaling pathway	*CAMK2A, CAMK2D, CCND2, CSNK2A2, FZD2, FZD7, LRP6, TCF7L2*
Tight junction	*CASK, CSNK2A2, EXOC3, MPDZ, MYH11, PPP2R2A, PTEN*
RNA transport	*CYFIP1, EIF4EBP1, GEMIN5, NUP155, NUP85, PABPC1, POP5, THOC7*
FoxO signaling pathway	*CAT, CCND2, CDKN1A, MAPK13, PRKAB2, PTEN, TGFB3*
p53 signaling pathway	*CCND2, CCNG1, CDKN1A, PTEN, RP11-292K15.2, TSC2*
Downregulated	
Oxidative phosphorylation	*ATP5B, ATP5C1, ATP5F1, ATP5G1, ATP5G3, ATP5H, ATP5I, ATP5J2, ATP6, ATP6V0A1, COX3, COX4I1, COX5A, COX6A1, COX7A2, ND1, ND5, ND6, NDUFA10, NDUFA12, NDUFA6, NDUFA8, NDUFS1, NDUFS2, NDUFS4, NDUFS5, NDUFV1, NDUFV2, PPA1, UQCRB, UQCRC1, UQCRC2, UQCRFS1*
Citrate cycle (TCA cycle)	*ACO2, CS, DLAT, DLD, DLST, IDH3A, IDH3B, MDH1, MDH2, PDHB, SUCLA2, SUCLG1*
Cardiac muscle contraction	*COX3, COX4I1, COX5A, COX6A1, COX7A2, TPM1, UQCRB, UQCRC1, UQCRC2, UQCRFS1*
Pyruvate metabolism	*ALDH7A1, DLAT, DLD, LDHD, MDH1, MDH2, ME1, PDHB*
Valine, leucine and isoleucine degradation	*ALDH7A1, AUH, DLD, HADHA, HADHB, HIBADH, MCCC1*

Table 3. 17. Top 50 upregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement MHA (100MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
FOSB	2.72300718	0.00270592	*KAZALD1*	1.09352447	0.0434367
PVALB	2.61386543	9.35E-05	*RORA*	1.05470392	0.00904668
FOS	2.14412517	0.00245074	*KCNN1*	1.05154339	0.00063594
CEBPD	1.97964231	7.71E-05	*SNRPA1*	1.01519876	0.0001694
SYPL1	1.92419555	0.00207952	*TMEM233*	1.00838971	0.01822992
PDK4	1.85257848	2.99E-07	*TCIM*	0.99578044	0.0003298
CTGF	1.71023087	0.02302968	*HSF2BP*	0.96654863	0.00051225
MYO7B	1.65721496	6.33E-05	*JUN*	0.956864	0.0049512
CYR61	1.55370768	0.0003298	*SCN2B*	0.95444164	0.00017598
ZBTB16	1.49017053	0.02031568	*OTUD1*	0.91962799	0.01724479
GPR20	1.4372931	0.01645123	*BTG2*	0.91775281	0.00057015
METTL7A	1.41616918	8.46E-05	*CRHR2*	0.91747886	0.0001694
MYOC	1.35793656	0.0480718	*IL11RA*	0.91161328	2.99E-07
RASD1	1.34906918	0.02100721	*GPR157*	0.89339588	0.00182284
KLF4	1.33808576	0.00293215	*DUSP15*	0.88569457	0.0480718
FGF1	1.32452522	0.01364108	*NFIL3*	0.86356272	0.0001694
ABRA	1.3234647	1.63E-06	*OCLN*	0.85153205	0.02524795
SEMA3B	1.29781387	0.04403468	*KLHDC8B*	0.85032144	0.02993127
APOD	1.26107754	0.00739655	*KLF2*	0.83798577	0.01803209
SCARNA15	1.20511663	0.01672172	*ZNF365*	0.82970972	0.00017598
DDIT4	1.19703288	0.00058319	*WBP1L*	0.82917353	0.02382283
AQP4	1.18725418	0.04928746	*ASB11*	0.81232968	0.00326682
ADAMTS1	1.16491323	0.00207952	*SGK1*	0.79803165	0.00270592
CEBPB	1.16305536	0.0003298	*MYOT*	0.77977668	0.03118687
DKK3	1.13445874	0.04714625	*NUDT18*	0.77723346	0.00125591

Table 3. 18. Top 50 downregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement MHA (100MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	-4.6281727	0.03246844	*GBE1*	-0.7302436	0.01672172
SCUBE2	-1.616101	0.00095738	*THSD7B*	-0.7290858	0.04259045
NR5A2	-1.5825156	0.0395928	*NAP1L4*	-0.728749	0.02100721
CPEB2	-1.5248775	0.01089745	*UHRF1*	-0.7260234	0.02232782
UBE3D	-1.3303848	0.01637615	*PREPL*	-0.7140114	0.02322051
LMCD1	-1.286593	0.00662966	*PDK1*	-0.6969862	0.0001694
NOS1	-1.2171769	0.01957747	*SLC44A5*	-0.695538	0.01641698
ADAM11	-1.150543	0.0480718	*BCAT1*	-0.6880881	0.03829394
MME	-1.0881769	0.03416603	*ABCC1*	-0.6876969	0.0086661
RNF217	-1.0333105	0.04368656	*GUCY1B3*	-0.6579105	0.00026981
FAM160A1	-1.0135872	0.0324478	*CDH13*	-0.6518294	0.00631681
C2orf88	-0.9140535	0.02031568	*PRNP*	-0.6451532	0.00849307
NPM3	-0.9139037	0.01672172	*LAPTM5*	-0.6408986	0.04486516
DUPD1	-0.8914212	0.01478408	*TIGAR*	-0.6323149	0.03984915
ND6	-0.8769751	0.01915107	*GSTK1*	-0.613981	0.03821723
SLC25A24	-0.8736523	0.00157672	*ITPR3*	-0.606744	0.02166697
TFRC	-0.8417867	0.00909941	*BTF3L4*	-0.5984844	0.01672172
GLS	-0.8409355	0.0010767	*LANCL2*	-0.5944155	0.00057015
ISPD	-0.8388298	0.02661224	*ALS2*	-0.593204	0.01645123
FRRS1L	-0.8232078	0.0187921	*PCCB*	-0.5910038	0.03534153
TMEM38B	-0.8168868	0.01491702	*DMXL2*	-0.5670857	0.02610634
ADSSL1	-0.7807527	0.0434367	*ATP2B1*	-0.56618	0.02032782
VWA8	-0.7796538	0.00815541	*WDSUB1*	-0.5627214	0.01645123
CLIP4	-0.7541075	0.04368656	*COTL1*	-0.5612892	0.04732043
COL28A1	-0.7452973	0.01637615	*ETFDH*	-0.5609365	0.02053223

Table 3. 19. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with at-requirement MHA on day 21 (100MHA).

KEGG Term	**Associated Genes**
Upregulated	
MAPK signaling pathway	*DUSP1, ECSIT, FGF1, FOS, JUN, MKNK2, PPP3CB, RELA*
Wnt signaling pathway	*CCND3, CREBBP, CSNK2A2, FZD2, JUN, PPP3CB*
Herpes simplex infection	*CREBBP, CSNK2A2, FOS, JUN, NFKBIA, RELA*
Toll-like receptor signaling pathway	*FOS, JUN, NFKBIA, RELA*
Jak-STAT signaling pathway	*CCND3, CREBBP, OSMR, RP11-195F19.29*
Downregulated	
Calcium signaling pathway	*ATP2B1, CD38, ITPR3, NOS1, PPP3CB*
Phagosome	*ATP6V0A1, NOS1, NOX1, TFRC*
Peroxisome	*ACSL4, GSTK1, PEX6*
Oocyte meiosis	*CPEB2, ITPR3, PPP3CB*
Lysosome	*ASAH1, ATP6V0A1, LAPTM5*

Table 3. 20. Top 50 upregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement MHA (140MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	5.86567442	0.00030252	*G0S2*	0.81460233	0.0184011
MINDY4B	3.0683801	0.03022489	*CETP*	0.80873163	0.00060448
PVALB	2.20432768	2.00E-10	*GOLGA7B*	0.75769819	0.00603423
PI15	2.13037996	0.04371745	*EYA1*	0.75635485	0.01527815
SYPL1	1.79865469	3.39E-06	*SH3PXD2A*	0.75612107	5.17E-07
ISM1	1.63436803	0.00646927	*AASS*	0.73064952	0.02010245
IGSF11	1.56139803	0.00074549	*P4HA2*	0.72488932	0.004576
HGNC:24955	1.28980335	0.02334112	*AGTR1*	0.7247877	0.01336391
FGF1	1.18886622	0.00205139	*CAPN6*	0.70578673	0.00044442
MYOC	1.09163481	0.02005592	*RGCC*	0.7050225	0.04327783
HPCAL4	1.08772219	0.02460679	*KLHL30*	0.70449999	0.03434344
CHRND	1.0351758	1.81E-07	*TPM2*	0.7034811	0.00034631
TMEM233	1.02585035	0.03711443	*WBP1L*	0.69242901	6.14E-05
ASB2	0.99815573	0.02359627	*TMEM201*	0.67599394	0.0154788
DGAT2	0.99348896	3.81E-09	*GRAMD2A*	0.67214164	0.03293775
SIRT4	0.9626098	0.01969422	*FBLN5*	0.65014478	0.00729209
METTL7A	0.94689187	0.01424451	*ABCD4*	0.64386429	0.03022489
ADAMTS13	0.8654211	0.01075321	*GRAMD1B*	0.63351488	0.00260214
ASB11	0.86153594	0.00302253	*EEPD1*	0.62965173	0.00146943
LINGO1	0.83573806	4.16E-05	*PARP6*	0.62402172	0.04460737
CHRNG	0.83150696	0.00146943	*SNRPA1*	0.62320408	0.02460679
KCNN1	0.82932236	0.03904526	*IQCB1*	0.60088611	0.00569328
HSF2BP	0.81711309	0.00299733	*PURG*	0.59762351	0.04116324
SLC41A3	0.81707896	0.00462599	*MAML2*	0.5959318	0.02952064
MB21D2	0.81499396	0.01035903	*CHIC2*	0.57342026	0.03590237

Table 3. 21. Top 50 downregulated genes in the *Pectoralis major* of 21-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement MHA (140MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CSMD1	-2.2394944	1.74E-05	*CENPI*	-0.8698018	0.03064864
STAC	-1.7140078	0.00753691	*METTL21C*	-0.8613139	0.01925179
GSTZ1	-1.5841319	0.00205139	*BUB1*	-0.8556603	0.02015392
RGR	-1.5189298	0.00709943	*PAQR7*	-0.8485256	0.02730558
GATM	-1.4348335	0.00992088	*OMA1*	-0.8385135	0.00248636
UBE3D	-1.2823052	0.02111531	*KIAA1524*	-0.8319302	0.02730558
EPB42	-1.2080472	0.04676977	*AURKA*	-0.8238754	0.02692071
CEP55	-1.1978156	0.00639144	*TK1*	-0.8229822	0.00578759
HBAD	-1.1420694	0.02298986	*DCTD*	-0.8187324	0.04116324
UBE2C	-1.1115584	4.16E-05	*KIF15*	-0.8153611	0.00146943
KCNU1	-1.1104235	0.0002203	*MCM3*	-0.8082126	0.00999631
TRAIP	-1.0839221	0.01779203	*ALAS1*	-0.8005779	1.03E-11
UPP1	-1.0592309	0.01230279	*dAK*	-0.7974365	0.01288144
COX3	-1.0485993	0.0001341	*PLK1*	-0.7956456	0.01008059
GINS2	-1.0320687	0.00421307	*TOP2A*	-0.782018	0.01924901
MT-CO2	-1.0307207	0.00072812	*KIF20A*	-0.7741024	0.01470647
HBAA	-1.0302821	0.01560005	*ZNF367*	-0.7678842	0.00326389
MT-CO1	-0.9966074	0.0041805	*ND6*	-0.7638129	0.00212591
MELK	-0.9521851	0.00986874	*CDC20*	-0.7617079	0.00394895
BIRC5	-0.941653	0.0116729	*NUSAP1*	-0.751434	0.04676977
SMC2	-0.9162483	0.00146943	*SPC25*	-0.7365689	0.01614543
NUF2	-0.915924	0.00194648	*NDC80*	-0.7252373	0.00692761
HSP90AB1	-0.911075	6.96E-06	*MCM5*	-0.7249059	0.00046739
CENPO	-0.9095323	0.03325613	*KPNA2*	-0.7203465	0.01035903
CA2	-0.9063239	0.04099215	*MCM2*	-0.7027737	0.00208837

Table 3. 22. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with above-requirement MHA on day 21 (140MHA).

KEGG Term	**Associated Genes**
Upregulated	
Peroxisome	*ABCD4, CRAT, MPV17L2, PEX6*
Wnt signaling pathway	*CSNK1E, CSNK2A2, DVL1, PPP3CB*
Herpes simplex infection	*ARNTL, CSNK2A2, JAK1, RP11-292K15.2*
Neuroactive ligand-receptor interaction	*AGTR1, CHRND, CHRNG, THRA*
Arginine and proline metabolism	*GATM, P4HA1, P4HA2*
Downregulated	
Oxidative phosphorylation	*ATP5C1, ATP5G3, ATP6, COX3, ND5, ND6, NDUFV2, UQCRC1, UQCRFS1*
Pyrimidine metabolism	*CMPK1, DCTD, POLD3, POLR2B, TK1, UCKL1, UPP1*
Cell cycle	*BUB1, CDC20, E2F1, MCM2, MCM3, MCM5, PLK1*
Oocyte meiosis	*AR, AURKA, BUB1, CDC20, PLK1, PPP3CB*
Citrate cycle (TCA cycle)	*DLAT, DLST, IDH3A, PDHB, SUCLA2*

Table 3. 23. Top 50 upregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement DLM (100DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
SYPL1	5.14856565	2.37E-18	*ADPRHL1*	2.22492293	0.0001836
PVALB	5.00992649	1.44E-05	*CDHR2*	2.2037828	0.00439841
NXNL1	4.68577827	3.03E-12	*C1QTNF12*	2.20094688	0.00398767
FKBP5	4.59644307	7.68E-08	*GATA5*	2.16175497	4.29E-05
ZBTB16	4.34676587	6.85E-06	*DRP2*	2.16175164	0.00027381
ALB	3.48665005	0.0483433	*KCNN1*	2.13039888	3.16E-12
PRTG	3.42276655	1.81E-13	*SLC7A4*	2.10300473	0.01422803
ANKRD55	3.12750787	1.86E-06	*IRS2*	2.09524014	0.00615336
FGB	3.05232867	4.00E-05	*GMEB2*	2.08500093	3.47E-08
U1	2.92940372	0.00042972	*TMEM74*	2.06627557	0.00054196
SLC27A1	2.76294003	5.60E-08	*CYP2R1*	2.06039686	6.62E-21
ABCA4	2.74051938	0.00142476	*ASB11*	2.05021507	2.06E-12
RND1	2.7226755	6.20E-09	*CHADL*	2.03155717	0.00140123
PM20D2	2.70204266	0.00017146	*HSF2BP*	2.02149054	1.32E-10
APOA4	2.67437738	0.0290974	*KLHL38*	2.00251747	0.00417262
FGF1	2.54925667	9.21E-10	*Lrrn1*	1.99615109	0.00315328
NFIL3	2.53619736	0.00102217	*KLF9*	1.99519931	4.55E-10
PDE10A	2.51674494	3.50E-08	*CHRND*	1.99363612	1.92E-06
GPR182	2.4908522	0.00046491	*BMP7*	1.97840641	0.00142634
SLC47A2	2.40746173	0.0001232	*SCNN1G*	1.95820753	0.00010254
WBP1L	2.39314154	1.66E-17	*SOX10*	1.95531892	3.59E-05
KNG1	2.37617951	0.00064777	*ACE*	1.91752605	2.59E-06
BMP3	2.26872397	0.04317278	*VEGFD*	1.90588076	5.27E-06
UTS2R	2.23474561	0.00320613	*HAO1*	1.89426701	0.0291717
CFAP99	2.22860847	0.00649174	*ACBD7*	1.84980518	0.0019726

Table 3. 24. Top 50 downregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement DLM (100DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	-5.5810443	0.00072533	*IL20RA*	-2.9256175	7.56E-06
TGM4	-5.2126947	3.99E-08	*BARX2B*	-2.9093268	7.76E-05
PHGDH	-4.4427832	3.79E-08	*C14orf37*	-2.8861048	4.89E-08
CSMD1	-3.9245814	1.60E-10	*CENPF*	-2.8855922	7.96E-14
GAP43	-3.7728459	5.60E-06	*JCHAIN*	-2.8775686	4.72E-09
MYL10	-3.6499731	4.72E-09	*SBK2*	-2.8638579	2.32E-10
BCAN	-3.5427732	2.57E-05	*EGFL6*	-2.827739	1.18E-14
SLC7A11	-3.5060651	0.0009542	*DNA2*	-2.8270311	1.59E-12
HPGDS	-3.3727083	2.47E-15	*MDK*	-2.8270011	9.19E-08
CENPM	-3.3073503	7.03E-10	*KPNA2*	-2.7966495	1.07E-57
DCSTAMP	-3.2941401	3.54E-05	*CCLi7*	-2.7891899	0.00048442
PSTPIP2	-3.2763402	2.34E-06	*BRSK2*	-2.7856925	0.00903647
PLK1	-3.2132921	1.52E-26	*C1QL2*	-2.7851313	0.03708312
GGCL1	-3.193745	1.83E-13	*SHCBP1*	-2.7768106	8.15E-10
DIAPH3	-3.1830153	1.71E-07	*PBK*	-2.7496532	2.64E-15
CYBB	-3.0824098	9.98E-08	*NUSAP1*	-2.7352817	9.54E-29
CDK1	-3.0769739	3.68E-20	*SLC6A9*	-2.7248235	9.46E-14
DCDC2	-3.0641534	0.01586435	*ZP1*	-2.7236099	0.00192044
IRG1	-3.0499968	3.09E-05	*NDC80*	-2.7063256	3.12E-18
TRAIP	-3.0265261	5.34E-07	*CXCL14*	-2.678885	7.10E-09
MELK	-3.0119352	8.18E-11	*CACNG7*	-2.662831	4.42E-05
CDCA3	-3.002792	2.86E-13	*NUF2*	-2.6375159	2.37E-18
ASNS	-2.9883615	8.38E-05	*CDC20*	-2.624763	3.12E-18
SMC2	-2.9598176	1.66E-10	*TOP2A*	-2.5779461	1.52E-26
EXO1	-2.9371611	1.01E-05	*GIPC3*	-2.5746687	0.00061548

Table 3. 25. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with at-requirement DLM on day 35 (100DLM).

KEGG Term	Associated Genes
Upregulated	
Protein processing in endoplasmic reticulum	*AMFR, BCL2, CRYAB, DNAJB12, DNAJC1, HSP90AA1, HSPA4L, HSPH1, MAPK8, NSFL1C, RAD23B, RPN1, SEC24A, SEC24B, SEC24C, SEC62, SEC63, SSR2, STUB1, TRAF2, UBE2D1, UBE2D2, UBE2E3, UBE2G1, UBE2G2, UBE2J2, UBE4B, UBQLN4, UBXN6, VIMP*
RNA transport	*CASC3, CLNS1A, DDX20, EIF3A, EIF3B, EIF3E, EIF3H, EIF4A2, EIF4E, EIF4E2, EIF4G2, EIF5, EIF5B, GEMIN2, GEMIN5, NMD3, NUP155, NUP37, NUP54, NUP58, NUP88, NUP98, NXT2, PABPC1, PHAX, RANGAP1, SUMO3, TGS1, THOC2, TPR, TRNT1, UBE2I, UPF1, XPOT*
Ubiquitin mediated proteolysis	*ANAPC10, ANAPC2, ANAPC5, BTRC, CUL3, CUL4B, DET1, FBXW11, ITCH, NEDD4, PIAS1, PIAS2, PIAS4, PPIL2, STUB1, TRIP12, UBA3, UBE2B, UBE2D1, UBE2D2, UBE2E3, UBE2G1, UBE2G2, UBE2I, UBE2J2, UBE2O, UBE2Q1, UBE3B, UBE4B, UBOX5, UBR5*
Spliceosome	*AQR, CCDC12, CDC40, CDC5L, CHERP, CRNKL1, CWC15, DDX23, DDX46, DHX8, EFTUD2, HNRNPU, LSM5, PHF5A, PPIH, PPIL1, PRPF3, PRPF4, PRPF40A, PRPF6, PRPF8, RBM25, SF3A1, SMNDC1, SNRNP200, SNRNP27, SNRPA1, SNRPC, SNW1, SRSF10, TCERG1, THOC2, U2AF1, U2SURP*
Purine metabolism	*ADCY9, AK6, ENPP4, ENTPD4, ENTPD5, GART, GMPS, ITPA, NME6, PAPSS2, PDE10A, PDE4D, PDE8B, PNPT1, POLD3, POLR2D, POLR2F, POLR2I, POLR3B, POLR3E, TWISTNB*
Downregulated	
Focal adhesion	*AC113404.1, ACTG1, ACTN1, ACTN2, ACTN4, CAPN2, CAV1, COL1A2, COL4A1, COL4A2, COL6A1, COL6A2, COL6A3, CTNNB1, EGF, EGFR, FLNB, FN1, FYN, ITGA2, ITGA3, ITGA4, ITGA6, ITGA8, ITGB1, LAMB4, MYL10, MYL2, MYLK, PDGFB, PDGFRB, PIK3CD, PIK3R5, PPP1CB, PPP1R12A, PRKCB, PTK2, PXN, RAC2, SHC2, THBS4, TLN1, TNC, VAV2, ZYX*
Regulation of actin cytoskeleton	*ACTG1, ACTN1, ACTN2, ACTN4, ARHGEF6, ARPC1B, ARPC5, CFL2, CHRM4, CYFIP1, DIAPH3, EGF, EGFR, FGF12, FGFR2, FN1, IQGAP2, ITGA2, ITGA3, ITGA4, ITGA6, ITGA8, ITGB1, ITGB2, MYL10, MYL2, MYLK, NCKAP1L, PDGFB, PDGFRB, PIK3CD, PIK3R5, PPP1CB, PPP1R12A, PTK2, PXN, RAC2, RDX, SCIN, TIAM1, TMSB4X, VAV2*
Cell cycle	*ABL1, ANAPC13, BUB1, BUB1B, BUB3, CCNB2, CDC20, CDC25A, CDC45, CDC7, CDK1, CDK2, CDK6, CDKN2C, CHEK1, CHEK2, E2F1, E2F3, HDAC2, MCM2, MCM3, MCM5, MCM6, MDM2, MYC, ORC1, PLK1, RAD21, WEE1, YWHAQ, YWHAZ*
Phagosome	*ACTG1, ATP6V1C2, ATP6V1D, ATP6V1G1, BLB1, C3, COLEC12, CTSS, CYBB, DMB2, ITGA2, ITGB1, ITGB2, LOC771876, MARCO, MRC2, NCF1, NCF2, NCF4, PLA2R1, RAB7B, SEC61A1, STX7, TAP1, THBS4, TLR2A, TLR4, TUBAL3, TUBB, TUBB6, VAMP3*
Oxidative phosphorylation	*ATP5B, ATP5C1, ATP5F1, ATP5G1, ATP5G3, ATP5I, ATP5J, ATP5J2, ATP6, ATP6V1C2, ATP6V1D, ATP6V1G1, COX15, COX3, COX5A, COX6A1, COX7A2, ND1, ND5, ND6, NDUFA1, NDUFA12, NDUFA2, NDUFA5, NDUFA6, NDUFB2, NDUFB3, NDUFS3, NDUFS5, NDUFV2, PPA1, UQCRB, UQCRC1, UQCRC2, UQCRFS1, UQCRQ*

Table 3. 26. Top 50 upregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement DLM (140DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
PVALB	5.03552081	1.01E-27	*SLC35F3*	1.70543528	0.00085297
PDK4	3.09433293	0.00588355	*ATOH8*	1.6899065	9.27E-10
ZBTB16	3.06073878	1.03E-06	*WBP1L*	1.68361557	1.41E-06
FKBP5	3.01852131	0.00013413	*C7*	1.61283759	1.27E-05
CXCR5	2.80066217	1.39E-05	*FAM196A*	1.58787812	0.00109481
SYPL1	2.75152098	1.57E-07	*ISM1*	1.57212172	0.00081782
GPM6A	2.58652688	0.00038816	*IGSF11*	1.56072241	2.41E-05
MAP3K15	2.50954044	2.15E-06	*SLC22A23*	1.55001873	1.26E-06
GRM4	2.45588158	0.00578131	*GATA5*	1.53066428	0.01949848
APOD	2.32523392	6.87E-09	*ASB2*	1.50649132	3.88E-11
FGF1	2.23020526	1.33E-07	*TOM1L1*	1.49736775	0.00295095
GDAP1	2.16244549	0.02359777	*NGEF*	1.49639707	0.00030817
ATP8B3	2.13014562	4.14E-06	*ASPA*	1.49521718	0.01297317
NXNL1	2.08957603	0.00071765	*ALKAL2*	1.48388847	0.00190449
KLHL38	2.08538807	0.00039451	*HPGD*	1.47217832	0.00666695
CEBPD	2.03028683	7.16E-08	*TINAG*	1.47205235	0.00014223
GABRA1	1.94535604	0.02201383	*MYO7B*	1.46617214	0.01160351
PRTG	1.91677146	1.61E-05	*NFIL3*	1.44359811	2.18E-06
KLF9	1.88811541	1.35E-06	*PLA2G15*	1.43735373	0.00088103
KCNN1	1.87524683	2.05E-05	*TDRKH*	1.39853123	2.38E-05
ASB11	1.83943329	1.37E-07	*FBXO32*	1.39189736	0.00285748
PPL	1.83642115	1.04E-07	*GMEB2*	1.37067142	1.29E-07
METTL7A	1.79552106	7.53E-05	*HPSE2*	1.36608339	0.02202132
GPR157	1.71522942	3.88E-11	*SCARNA15*	1.36093339	0.04443989
PLCH1	1.71455608	0.02225892	*CIT*	1.34818376	2.51E-07

Table 3. 27. Top 50 downregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement DLM (140DLM).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
ZP1	-3.6570307	5.00E-06	*GRIA3*	-1.8468185	0.00037497
CSMD1	-3.6308169	0.00023549	*IL21R*	-1.8389127	0.01996597
SPIRE2	-3.3114237	0.02800983	*CIITA*	-1.8128334	6.56E-08
WNT16	-3.1915896	1.60E-05	*CEP55*	-1.7936902	0.00549295
OASL	-3.1361804	5.98E-05	*DCLK3*	-1.7857352	0.00208751
RSAD2	-3.0136351	0.00012443	*C1QB*	-1.7381007	0.0009926
GSTZ1	-2.6965776	0.00161605	*CYBB*	-1.7057548	0.02514178
ASNS	-2.6366831	1.89E-09	*C3*	-1.6871774	0.02029759
PHGDH	-2.6265586	0.0001923	*BCL11A*	-1.6798002	1.69E-05
FSHR	-2.5628344	1.72E-07	*ZNFX1*	-1.6797341	4.95E-05
CYP11A1	-2.5084812	0.00021912	*APLNR*	-1.6789577	0.00033911
SBK2	-2.5073346	0.00016136	*FANCB*	-1.6736412	1.47E-07
CACNG7	-2.4542029	0.03212565	*MT-CO1*	-1.6623072	1.24E-08
CCL19	-2.4336759	0.00027645	*MPEG1*	-1.6475817	0.0013082
SLC7A1	-2.2793488	1.04E-05	*SCNN1A*	-1.6364681	8.76E-07
CA2	-2.1664879	5.15E-10	*SDK1*	-1.633835	6.12E-06
JCHAIN	-2.1505941	0.02553002	*CST7*	-1.6263308	0.0242539
IL18R1	-2.1368982	0.00013307	*BCO1*	-1.6231395	1.23E-07
SLC16A3	-2.0364392	2.40E-07	*k123*	-1.6148269	0.03830168
TUSC3	-2.017203	4.98E-09	*GJD2*	-1.6134171	0.00537772
SCUBE2	-1.9358643	5.13E-09	*MELK*	-1.6039117	0.00409807
CDH1	-1.8988133	0.00473738	*SLC10A4*	-1.6031054	0.00719056
EPSTI1	-1.8976518	9.22E-05	*GATM*	-1.5993081	0.02504957
IRF6	-1.8928429	0.03071168	*KY*	-1.5771334	0.00207941
PGR	-1.8626795	0.00670569	*FGD4*	-1.5750416	0.00315093

Table 3. 28. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with above-requirement DLM on day 35 (140DLM).

KEGG Term	Associated Genes
	Upregulated
RNA transport	*CASC3, CLNS1A, DDX20, EIF2B2, EIF2B5, EIF3A, EIF3B, EIF3D, EIF3H, EIF4E2, EIF4G2, EIF5, GEMIN4, GEMIN5, NUP155, NUP54, NUP88, NUP98, RANGAP1, RBM8A, SUMO3, TGS1, TPR, TRNT1, UBE2I, UPF1, XPOT*
Ubiquitin mediated proteolysis	*ANAPC10, ANAPC2, KEAP1, NEDD4, PIAS1, PIAS4, RFWD2, STUB1, UBA3, UBE2B, UBE2D2, UBE2E3, UBE2G1, UBE2H, UBE2I, UBE2O, UBE2S, UBE3B, UBE4B, UBOX5*
Autophagy	*AKT1, ATG14, ATG16L2, ATG9A, HRAS, IRS2, LAMP1, MTMR3, NRBF2, PIK3CA, PIK3R4, PRKCQ, RAB1B, RB1CC1, RPTOR, RRAS, SMCR8, SUPT20H, TSC2, WIPI2*
Ribosome	*MRPL12, MRPL17, MRPL24, MRPS11, MRPS2, MRPS21, RPL12, RPL21, RPL23A, RPL30, RPL34, RPL37, RPL37A, RPL5, RPL6, RPL7, RPL7A, RPL8, RPS12, RPS19, RPS20, RPS26*
Spliceosome	*CCDC12, CDC40, CDC5L, CHERP, DDX23, DHX38, DHX8, EFTUD2, HNRNPU, PPIL1, PRPF3, PRPF4, PRPF40A, PRPF6, PRPF8, RBM8A, SNRNP200, SNRNP27, SNRPA1, SRSF10, TCERG1*
	Downregulated
Regulation of actin cytoskeleton	*ARPC5, CDC42, CFL2, CXCR4, CYFIP1, EGF, EGFR, INSRR, ITGA1, ITGA4, ITGA6, ITGB1, ITGB2, ITGB5, LPAR4, PDGFB, PDGFC, PIK3CD, PPP1CB, PPP1R12A, PTK2, RAC2, RDX, ROCK1, ROCK2, TMSB4X, VAV3*
Focal adhesion	*ARHGAP5, CAPN2, CDC42, CTNNB1, EGF, EGFR, ITGA1, ITGA4, ITGA6, ITGB1, ITGB5, PDGFB, PDGFC, PIK3CD, PPP1CB, PPP1R12A, PRKCB, PTK2, RAC2, RAP1B, ROCK1, ROCK2, THBS4, VAV3*
Cell cycle	*ANAPC13, BUB1, CCNA2, CCNB1, CDC20, CDC45, CDC7, CDK1, CDK6, CDKN2C, HDAC1, HDAC2, MCM3, MCM5, ORC1, PLK1, RAD21, WEE1*
Lysosome	*AP1S2, AP3B1, AP3S2, ARSB, ATP6AP1, CLTB, CTSH, CTSK, CTSO, CTSS, DNASE2B, GUSB, HEXB, LAPTM5, MANBA*
Vascular smooth muscle contraction	*ADCY7, CALD1, GNAQ, GUCY1A3, GUCY1B3, ITPR3, KCNMB1, MYL6, PPP1CB, PPP1R12A, PRKCB, PRKCD, PRKG1, RAMP2, RAMP3, ROCK1, ROCK2*

Table 3. 29. Top 50 upregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement MHA (100MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
PVALB	5.35672403	3.75E-35	*SESN1*	1.62098486	2.30E-08
BRSK2	3.43035149	0.00034092	*PLA2G15*	1.60953317	4.63E-09
ZBTB16	3.42282939	2.06E-11	*LINGO1*	1.60668992	6.37E-06
MINDY4B	3.35152774	0.00746503	*PCOLCE2*	1.5342092	1.45E-16
SYPL1	2.4709168	2.42E-07	*Lrrn1*	1.52695685	0.0009335
TINAG	2.11020365	2.66E-06	*RND1*	1.52555502	2.93E-05
FKBP5	2.08832084	3.14E-07	*UCP3*	1.47160649	0.04966767
PLP1	2.03750719	0.00020607	*SCN2B*	1.46249428	1.11E-06
FGF1	2.03146054	6.01E-10	*WNT5B*	1.45221756	3.30E-07
GMEB2	1.93856446	7.07E-15	*SMAD6*	1.4406561	3.14E-06
ASB2	1.93556798	1.32E-11	*LZTS2*	1.43895553	3.09E-14
ASB11	1.88485685	2.31E-06	*ACE*	1.43245325	0.00483766
KCNN1	1.88480322	8.14E-07	*TBC1D22B*	1.41630837	1.51E-16
MYOC	1.87520418	7.82E-11	*ACVR1C*	1.40402211	0.02247636
SLC35F4	1.87210037	0.0131254	*ADAMTS13*	1.39515524	6.97E-13
MPZ	1.86507544	0.00409122	*UNC5C*	1.3926809	0.02987165
TDRKH	1.85269681	4.44E-10	*WBP1L*	1.39063873	3.13E-09
KLF9	1.84588842	3.40E-05	*GDNF*	1.3656914	4.95E-05
ATOH8	1.7717693	2.62E-33	*PURG*	1.34161564	8.89E-05
PRTG	1.76247327	4.84E-06	*CACHD1*	1.33783694	2.13E-07
PROKR2	1.75056499	0.00590347	*OCLN*	1.33265861	0.00010409
GREM2	1.70217614	0.0218407	*ZNF142*	1.30650563	2.04E-11
PLCH1	1.65048752	0.00493052	*SIRT4*	1.30620312	0.00092533
AGTR1	1.64765623	2.22E-07	*PRDM4*	1.29166099	1.81E-05
CHRNB2	1.63853158	0.00607826	*SMAD9*	1.28386136	0.00120888

Table 3. 30. Top 50 downregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with at-requirement MHA (100MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
MLANA	-6.745478	0.03324216	*PTX3*	-3.3779853	0.0114016
CALB2	-5.5343859	0.00091965	*MYL3*	-3.3523114	0.01025223
MMP7	-4.9383655	9.37E-05	*JCHAIN*	-3.3458067	1.58E-07
CCL19	-4.8731519	2.44E-07	*DSG2*	-3.3455855	0.0116561
CTHRC1	-4.8635679	2.76E-05	*MMP27*	-3.3349712	0.00364692
WNT16	-4.5921191	0.00507639	*XDH*	-3.3092385	0.00044428
ZP1	-4.5453623	3.43E-08	*IRG1*	-3.2942084	6.59E-05
k123	-4.3395407	4.19E-06	*RSAD2*	-3.2912337	0.00687292
RASL10B	-4.3183186	0.00023308	*CCLi7*	-3.2891443	1.79E-05
MDK	-4.2835083	0.00123582	*TGM4*	-3.2714792	0.00032249
ARC	-4.1821121	9.86E-06	*TLR15*	-3.2701357	8.71E-09
SCN5A	-4.0760346	0.00029041	*MSX1*	-3.2011554	0.02523214
DCSTAMP	-4.0003809	3.49E-05	*MMP9*	-3.1883025	0.02598219
DCDC2	-3.9881544	0.02848495	*IL1R2*	-3.1420181	0.00107555
OASL	-3.8171952	3.28E-05	*PCDH15*	-3.1311232	0.00072358
CTLA4	-3.7717024	3.28E-05	*TNC*	-3.1194255	0.00079233
BCAN	-3.7430194	0.01531986	*TNNI1*	-3.0816244	0.00650875
MYL10	-3.6743164	0.00695918	*CSF3R*	-3.0746565	4.04E-08
HPX	-3.5641683	0.00100184	*IL20RA*	-3.0711672	0.00017419
TNFAIP6	-3.5492696	0.00314969	*GGCL1*	-3.0381719	4.32E-10
MYBPC1	-3.5215696	0.02137032	*MASP1*	-3.0368118	0.00395403
EPSTI1	-3.4209354	7.48E-08	*CYBB*	-3.0253427	1.74E-06
PSTPIP2	-3.4098652	6.24E-05	*SYNDIG1L*	-3.0166505	0.00406099
KIF26B	-3.3880509	0.00303767	*KCNA3*	-2.9787014	0.00034925
DUSP5	-3.3786232	0.00262019	*NRCAM*	-2.9681098	0.00597676

Table 3. 31. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with at-requirement MHA on day 35 (100MHA).

KEGG Term	**Associated Genes**
Upregulated	
Ubiquitin mediated proteolysis	*ANAPC10, ANAPC2, BTRC, CDC27, CDC34, CUL3, CUL4B, DDB1, DET1, FBXW11, FBXW8, NEDD4, NHLRC1, PIAS1, STUB1, UBA3, UBE2D2, UBE2E3, UBE2F, UBE2G1, UBE2H, UBE2J1, UBE2J2, UBE2O, UBE2Q1, UBE3B, UBE4B, UBOX5, WWP1*
RNA transport	*AAAS, CASC3, CLNS1A, DDX20, EIF1, EIF2B2, EIF3A, EIF3E, EIF3H, EIF4EBP1, EIF4EBP2, EIF4G1, EIF4G2, EIF5, GEMIN2, GEMIN4, GEMIN5, GEMIN8, KPNB1, NUP153, NUP155, NUP88, NUP98, SNUPN, SUMO3, TRNT1, UPF1, XPOT*
Wnt signaling pathway	*AXIN1, BTRC, CACYBP, CAMK2D, CAMK2G, CCND3, CREBBP, CSNK2A2, DVL1, FBXW11, FZD2, FZD8, FZD9, MAPK8, NFATC1, NLK, NOTUM, PPARD, PRKACB, PRKCA, WNT5B*
Spliceosome	*CCDC12, CWC15, DDX23, DDX46, DHX38, DHX8, EFTUD2, HNRNPU, PHF5A, PPIL1, PRPF3, PRPF40A, PRPF6, PRPF8, SNRNP200, SNRNP27, SNRPA1, SNW1, SRSF10, TCERG1*
Ribosome	*MRPL12, MRPL18, MRPL2, MRPL24, MRPL9, MRPS11, MRPS15, MRPS2, MRPS21, RPL23A, RPL30, RPL37A, RPL3L, RPS12, RPS26, RPS29, RPSA, UBA52*
Downregulated	
Endocytosis	*ARAP2, ARF5, ARFGAP3, ARPC1B, ARPC2, ARPC3, ARPC4, ARPC5, ASAP1, ASAP2, CAV3, CCR5, CDC42, CHMP2B, CHMP4B, CLTA, CLTC, CXCR4, CYTH1, CYTH4, DNAJC6, F2R, GRK4, IL2RG, MET, PDCD6IP, PIP5K1B, RAB11FIP4, RAB31, RET, RHOA, SH3GL3, SNX1, SNX3, SNX6, TFRC, VPS35, WIPF1*
Regulation of actin cytoskeleton	*ACTN1, ARHGEF12, ARHGEF6, ARPC1B, ARPC2, ARPC3, ARPC4, ARPC5, BDKRB1, CDC42, CHRM4, CYFIP1, DIAPH3, EZR, F2R, FGF12, FN1, IQGAP2, ITGA3, ITGA4, ITGA6, ITGA8, ITGB1, ITGB2, ITGB5, MYL10, MYLK, NCKAP1, NCKAP1L, PDGFRB, PFN2, PIK3CB, PIK3CD, PIK3R5, PIP5K1B, PPP1CB, RAC2, RHOA, ROCK1, SCIN, TIAM1, TMSB4X, VAV3*
Focal adhesion	*AC113404.1, ACTN1, CAPN2, CAV3, CCND2, CDC42, COL6A1, CTNNB1, FLNB, FN1, FYN, HGF, ITGA3, ITGA4, ITGA6, ITGA8, ITGB1, ITGB5, MET, MYL10, MYLK, PARVA, PARVG, PDGFRB, PIK3CB, PIK3CD, PIK3R5, PPP1CB, PRKCB, RAC2, RHOA, ROCK1, THBS2, THBS4, TLN1, TNC, VAV3, VTN, ZYX*
Cell cycle	*ANAPC13, BUB1, BUB1B, BUB3, CCNB2, CCND2, CCNE2, CDC20, CDC45, CDC7, CDK1, CDK2, CDK6, CHEK1, E2F1, HDAC1, HDAC2, MAD2L1, MCM2, MCM3, MCM5, MCM6, MYC, ORC1, ORC5, ORC6, PLK1, RAD21, RB1, RBL1, TTK, WEE1, YWHAB, YWHAQ, YWHAZ*
Herpes simplex infection	*BLB1, C3, CASP3, CD74, CDK1, CDK2, DMB2, EIF2AK2, FADD, FAS, HNRNPK, IFIH1, IFNAR1, IFNAR2, IKBKE, IL15, IRF7, MYD88, PPP1CB, SOCS3, STAT1, TAP1, TLR2A, TLR3, TNFRSF1A, TRAF1, TRAF5*

Table 3. 32. Top 50 upregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement MHA (140MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
GPNMB	6.76057862	0.01750995	*CFAP99*	2.00568822	0.03659782
PVALB	4.18823645	3.72E-34	*KCNH4*	1.98156668	0.01304353
SYPL1	3.96662908	6.55E-07	*TAAR1*	1.97316001	0.03153401
PRTG	3.40042384	8.81E-07	*C1QTNF12*	1.95800742	0.04631484
ZBTB16	3.20373368	0.00020603	*IRS2*	1.93795411	0.00093981
SLC22A3	2.99805734	0.02405846	*HSPH1*	1.92344932	6.63E-05
UTS2R	2.98038916	0.00735859	*CEBPD*	1.88620439	0.0112901
FKBP5	2.97795556	0.00544942	*KLHL38*	1.88526007	0.00052572
PDK4	2.84314188	0.03411695	*BAIAP2L2*	1.87807918	0.03171028
CA12	2.64702685	0.01009973	*GATA5*	1.84901895	0.00095837
ABCA4	2.58493236	0.0048638	*CHRND*	1.84720063	1.41E-11
CPXM2	2.54872908	0.01004121	*KAZALD1*	1.8390468	0.02268433
BMP5	2.51026483	0.00088812	*U6*	1.83138431	4.15E-07
ANKRD55	2.45887797	0.00034438	*TACSTD2*	1.8248128	0.02675026
BEST4	2.40978247	0.01462819	*PDE10A*	1.79547855	6.14E-06
GPM6A	2.37826135	0.00361656	*UPP2*	1.79141124	2.07E-09
KRT80	2.37249244	0.00779825	*NSUN7*	1.77537622	0.02115302
GABRA1	2.21020903	0.00882604	*GRM4*	1.76736391	0.02354479
ROS1	2.18481238	0.00867784	*GMEB2*	1.76022949	8.82E-14
FGF1	2.14659666	0.00017869	*ATP8B3*	1.7086319	2.34E-05
GDAP1	2.10271206	0.01837499	*HSF2BP*	1.69190182	0.00043617
ST6GALNAC1	2.0966808	0.00034438	*ADPRHL1*	1.69174162	0.01198549
PPRC1	2.07545712	7.67E-05	*PLCH1*	1.68101563	0.02794686
METTL7A	2.01660724	1.46E-08	*FAM83H*	1.63993102	0.02638813
KCNN1	2.00660438	1.45E-08	*ASB11*	1.63868485	0.00193497

Table 3. 33. Top 50 downregulated genes in the *Pectoralis major* of 35-day-old broiler chickens reared at 35°C whose diets were supplemented with above-requirement MHA (140MHA).

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	-6.444569	1.34E-05	*SLC7A1*	-2.045519	0.00018708
TRPC3	-3.483881	9.16E-06	*SLBP*	-2.0368171	6.48E-05
CSMD1	-3.2712386	0.0003377	*C1QB*	-1.9634566	1.34E-14
SBK2	-3.2411274	5.82E-19	*ACKR4*	-1.9474954	0.000362
GIPC3	-3.0520211	3.82E-07	*SHCBP1*	-1.9393284	7.50E-06
IRF6	-3.0105676	2.08E-07	*EPSTI1*	-1.9294041	1.35E-05
KCNU1	-2.7441361	3.08E-06	*IL18R1*	-1.9187946	1.47E-06
RSAD2	-2.6684593	0.00117026	*MPEG1*	-1.9073092	1.08E-07
OASL	-2.6173608	2.66E-06	*CENPM*	-1.9070938	0.00445007
CA2	-2.5694303	7.06E-08	*SLC16A3*	-1.8990899	1.52E-13
ZP1	-2.4679155	0.00037025	*CLEC19A*	-1.8949049	0.03411695
SCNN1A	-2.4242561	8.32E-18	*SLC16A6*	-1.8917083	2.59E-11
GABRB2	-2.4202468	0.0255603	*MT-CO1*	-1.8694085	7.26E-06
GZMA	-2.2969384	0.00039504	*FLT3*	-1.8676671	0.0253323
LDLRAD1	-2.2579907	6.37E-06	*MYO16*	-1.8620098	0.00047354
SBK3	-2.2423421	0.01802007	*JCHAIN*	-1.8505425	0.00081212
KY	-2.2408567	0.00616689	*CACNG7*	-1.8457526	0.00276008
ASNS	-2.1994124	0.00612391	*WFIKKN2*	-1.8221651	0.00433525
SMPX	-2.1874138	2.43E-11	*RASL11A*	-1.8163344	0.00339869
RGS16	-2.165518	0.0253373	*IL21R*	-1.7593671	0.01226841
STMN2	-2.1571186	0.00052328	*HTR1A*	-1.7408819	0.02676103
HPGDS	-2.1529933	1.16E-05	*SSTR2*	-1.6896353	0.00963174
GRIA3	-2.1414128	6.85E-06	*DDO*	-1.6881313	0.0011914
PHGDH	-2.0802182	0.00023361	*GATM*	-1.6855995	0.00116679
MTERF2	-2.0483953	3.30E-08	*MCEE*	-1.6557343	0.00173823

Table 3. 34. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C whose diets were supplemented with above-requirement MHA on day 35 (140MHA).

KEGG Term	Associated Genes
	Upregulated
Ribosome	*MRPL18, MRPL2, MRPL9, MRPS11, MRPS21, RPL12, RPL14, RPL21, RPL22, RPL23, RPL23A, RPL27A, RPL30, RPL31, RPL32, RPL34, RPL36, RPL37, RPL37A, RPL38, RPL3L, RPL4, RPL5, RPL6, RPL7, RPL7A, RPL8, RPLP2, RPS12, RPS17, RPS20, RPS21, RPS24, RPS25, RPS26, RPS29, UBA52*
RNA transport	*CASC3, CLNS1A, DDX20, EEF1A1, EIF2B5, EIF3A, EIF3B, EIF3D, EIF3E, EIF3G, EIF3H, EIF4A3, EIF4E2, EIF5, GEMIN5, NUP155, NUP54, NUP88, NUP98, PABPC1, SUMO3, THOC2, TPR, TRNT1, UBE2I, UPF1, XPO5, XPOT*
Spliceosome	*AQR, CCDC12, CDC5L, CHERP, CRNKL1, DDX23, DDX46, DHX8, EFTUD2, EIF4A3, HNRNPU, PPIH, PPIL1, PRPF3, PRPF4, PRPF40A, PRPF6, PRPF8, PUF60, SF3B4, SNRNP200, SNRNP27, SNRPA1, SNRPB, SNW1, SRSF10, TCERG1, THOC2, U2AF1*
FoxO signaling pathway	*AKT1, CAT, CREBBP, CSNK1E, EP300, FBXO32, FOXO3, FOXO4, G6PC3, GRB2, HRAS, INSR, IRS2, MAPK13, PDPK1, PIK3CA, PRKAG2, SOS1, STAT3, USP7*
Wnt signaling pathway	*AXIN1, CACYBP, CREBBP, CSNK1A1, CSNK1E, CSNK2A2, CTBP1, CTC-554D6.1, DVL1, DVL3, EP300, FZD1, FZD2, NFATC1, NOTUM, PPARD, PRKACB, PRKCA, WNT5B*
	Downregulated
Regulation of actin cytoskeleton	*ACTG1, ACTN2, ACTN4, ARHGEF12, CDC42, CFL2, DIAPH3, EGF, F2, FGFR2, ITGA1, ITGA4, NCKAP1, PDGFB, PIK3R5, PPP1CB, PPP1R12A, PTK2, RAC2, RDX, TMSB4X*
Influenza A	*ACTG1, BLB1, DMB2, EIF2AK3, FDPS, IFIH1, KPNA2, MAP2K6, PIK3R5, RP11-292K15.2, RSAD2, STAT1, TLR3, TLR4, TLR7, TRIM25*
Oxidative phosphorylation	*ATP5B, ATP5C1, ATP5G1, ATP5G3, ATP5I, ATP6, COX15, COX3, COX5A, COX6A1, ND1, ND5, ND6, NDUFA12, NDUFB2, NDUFS1, NDUFS3, NDUFV2, UQCRB, UQCRC1, UQCRC2, UQCRFS1*
Cell cycle	*ANAPC13, BUB1B, BUB3, CCNB2, CDC14B, CDC20, CDC45, CDK1, CDKN2C, CUL1, E2F3, MCM6, PLK1, RAD21, YWHAQ*
Oocyte meiosis	*ADCY7, ANAPC13, AURKA, CAMK2A, CCNB2, CDC20, CDK1, CUL1, PLK1, PPP1CB, PPP2R5C, PPP3CA, PPP3CB, RPS6KA3, YWHAQ*

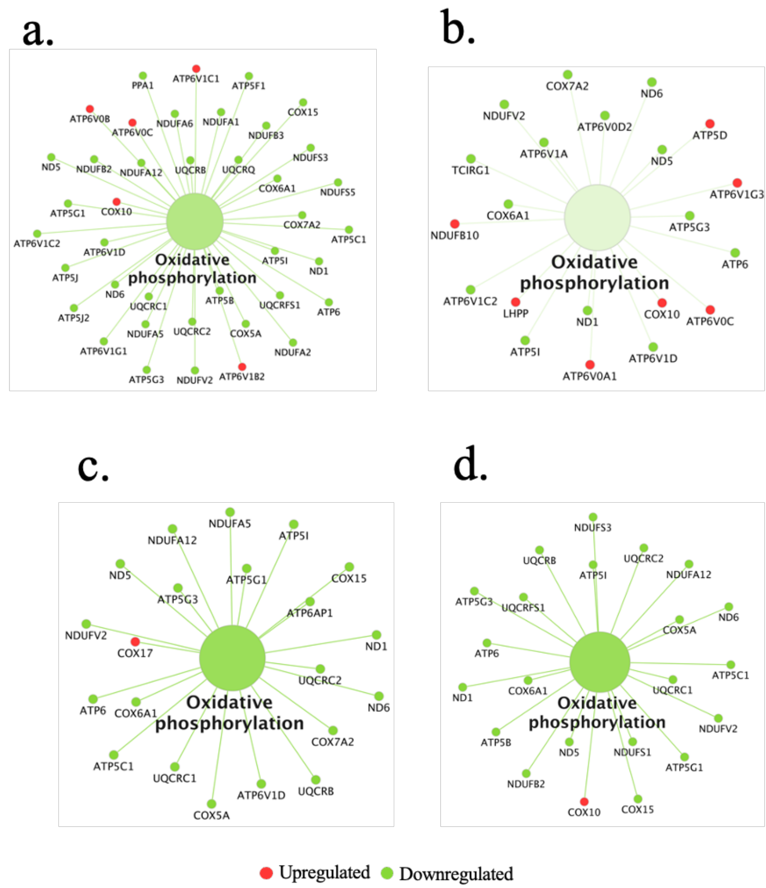

Figure 3. 7. Network of differentially expressed genes related to oxidative phosphorylation in heat-stressed broilers fed (**a**) at-requirement D,L-methionine (100DLM), (**b**) at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), (**c**) above-requirement D,L-methionine (140DLM), or (**d**) above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA) on day 35.

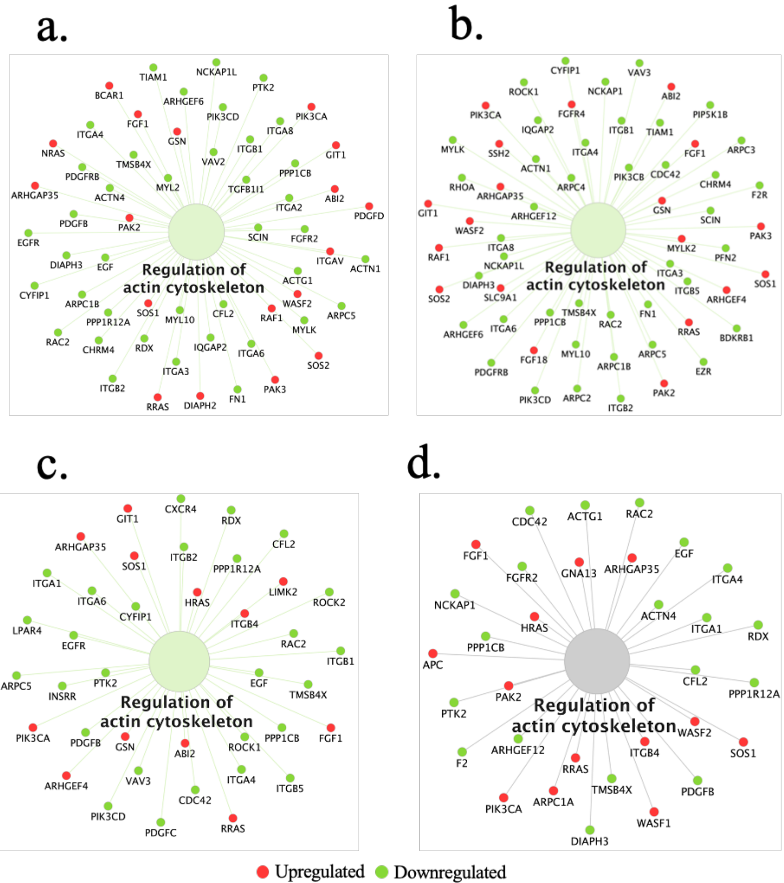

Figure 3. 8. Network of differentially expressed genes related to regulation of the actin cytoskeleton in heat-stressed broilers fed (**a**) at-requirement D,L-methionine (100DLM), (**b**) at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), (**c**) above-requirement D,L-methionine (140DLM), or (**d**) above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA) on day 35.

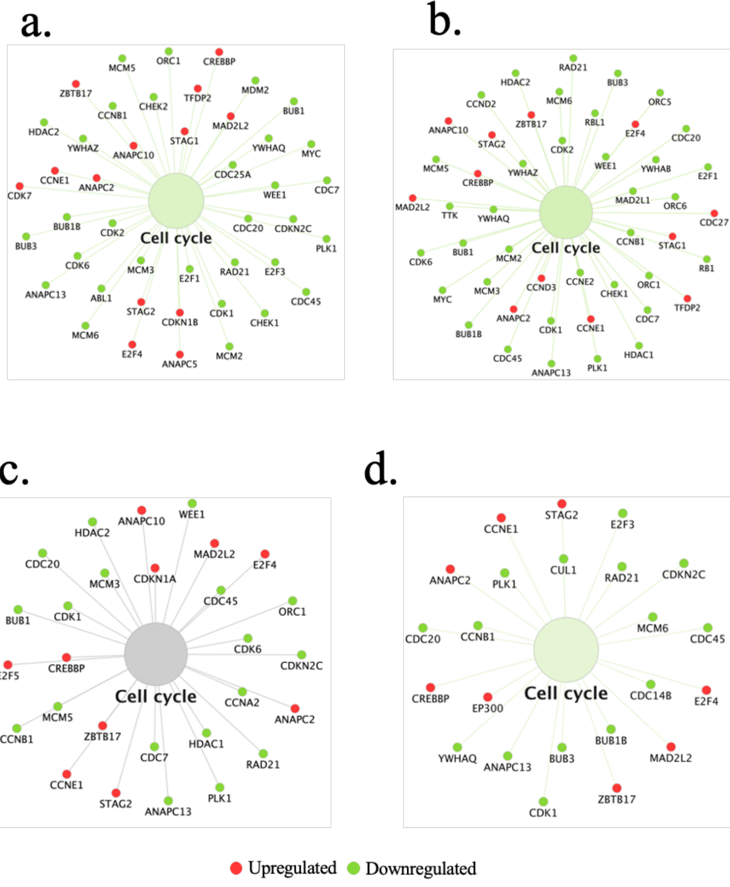

Figure 3. 9. Network of differentially expressed genes related to the cell cycle in heat-stressed broilers fed (**a**) at-requirement D,L-methionine (100DLM), (**b**) at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), (**c**) above-requirement D,L-methionine (140DLM), or (**d**) above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA) on day 35.

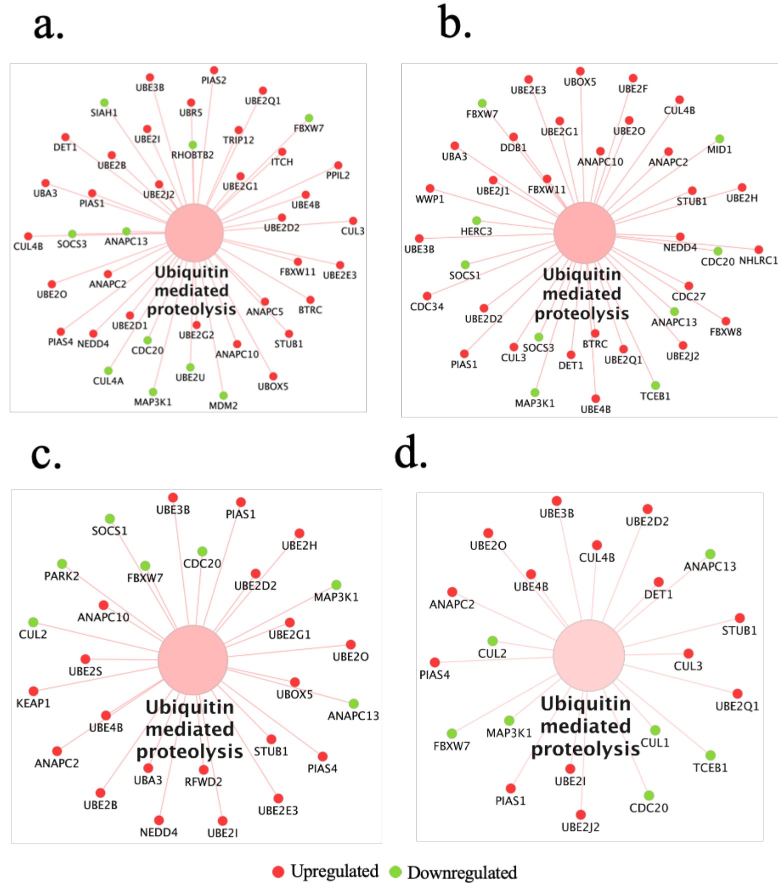

Figure 3. 10. Network of differentially expressed genes related to ubiquitin mediated proteolysis in heat-stressed broilers fed (**a**) at-requirement D,L-methionine (100DLM), (**b**) at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), (**c**) above-requirement D,L-methionine (140DLM), or (**d**) above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA) on day 35.

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CHAPTER 4

AGE AND DIETARY METHIONINE ALTER THE *PECTORALIS MAJOR* TRANSCRIPTOME OF BROILER CHICKENS REARED IN A HOT ENVIRONMENT

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ABSTRACT

The objective of this study was to compare how heat stress affects the transcriptome of a broiler when applied during just one of the three phases of broiler production. Additionally, this work aims to identify the effects of dietary content and source of methionine on the broiler transcriptome during heat stress. Day-old broiler chicks were randomly divided into 16 groups arranged in a 4x2x2 factorial design: environmental condition (thermoneutral or heat stress during the starter, grower, or finisher phase), dietary methionine source (DL-methionine or DL-2-hydroxy-(4-methylthio)butanoic acid), and dietary inclusion of methionine (100% or 140% of requirement). The *Pectoralis major* of broilers was sampled on days 10, 21, and 35 from the 4 groups reared in the thermoneutral environment and from 4 groups just ending their heat stress treatment at the conclusion of the starter (day 10), grower (day 21), and finisher (day 35) phases. RNA-sequencing was performed and mapped to the *Gallus gallus* (4.0, version 81, Ensembl) reference genome. Performance results showed rearing in a hot environment during the starter phase did not influence body weight gain or feed intake. When heat-stress during the grower phase, broiler performance is impaired, though increasing dietary methionine does increase weight gain. When heat-stressed during the finisher phase, dietary methionine had no effect on performance. The transcriptome of the breast muscle revealed heat stress had a great influence on oxidative phosphorylation, possibly contributing to reactive oxygen species generation. This increased oxidative stress could impact protein deposition, though increasing dietary methionine could reduce proteolysis during the grower phase. Dietary methionine had little effect on proteolysis when heat stress was applied during the finisher phase, and the transcriptome was more similar regardless of diet. Genes regulating the actin cytoskeleton were more downregulated when heat was applied from days 22 to 35, affecting focal adhesions, cell signaling, and other processes vital for cell survival. In all,

this study showed the age in which a broiler is subjected to heat stress determines its molecular response, and methionine source and inclusion in the diet additionally affect this response.

INTRODUCTION

A significant stressor in the poultry industry is heat. Heat stress is induced when the amount of energy produced by the bird is greater than the energy exiting the bird. This imbalance creates a pooling of energy within the bird in the form of heat (Lucas and Marcos 2013). Upon experiencing a larger heat load, blood flow is diverted from the viscera to the periphery to facilitate heat exchange, cooling the bird. This diversion of blood flow during heat stress leads to hypoxia and nutrient deficiency in various tissues including the liver, muscle, and intestines (Hall et al. 1999). Numerous studies have determined heat stress is detrimental to broiler performance (Hurwitz et al. 1980; Attia et al. 2011; Sohail et al. 2012; Awad et al. 2018; Goo et al. 2019; Lin Law et al. 2019). Additionally, heat stress inhibits proper immune function (Trout and Mashaly 1994; Bartlett and Smith 2003; Borges et al. 2004; Mashaly et al. 2004). The immune system of a broiler is still developing during the first weeks post-hatch (Mast and Goddeeris 1999), and heat stress weakens immune response at a young age (Juul-Madsen et al. 2004). However, work has shown heat stress at a young age may benefit the bird by molding the bird's development (Vinoth et al. 2015). This thermal conditioning could additionally benefit performance of broilers later in life (Yahav and Hurwitz 1996; Yahav and Plavnik 1999; Meteyake et al. 2020).

Heat stress' effect on broiler performance changes with age (Andretta et al. 2021). Performance is less affected by heat stress when broilers are in the starter or grower phases of production. When a broiler is older than 21 days, heat stress has a more profound effect on weight gain and feed conversion. This could be attributed to young birds having a higher heat tolerance (Chowdhury et al. 2012). The modern broiler reaches market weight at a much younger age (Havenstein et al. 2003), impacting the development and thermotolerance of the bird. Fast growing broilers have diminished immune development and increased proteolysis when heat stressed

(Zhang et al. 2017). This has led poultry producers to alter the diet of broilers under heat stress to help ease the deleterious effects.

Under thermoneutral temperatures, NRC (1994) recommends broiler diets contain 0.50%, 0.38%, and 0.30% methionine for starter, grower, and finisher phases, respectively. However, under suboptimal conditions, these recommendations may not be adequate to fulfill the bird's requirement for methionine to mitigate performance loss and impaired immunity. Previous research has shown increasing only essential amino acids, including methionine, benefit broilers subjected to heat stress (Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005). Ojano-Dirain and Waldroup (2002) have shown under moderately hot temperatures, methionine and lysine requirements for broilers are more than requirements suggested by NRC (1994). Suganya et al. (2015) recommends a 1-2% reduction in crude protein with a 5-10% increase of critical amino acids under heat stress temperatures. In chronically heat stressed broilers, the doubling of methionine inclusion in the diet increased broiler weight gain (Balnave et al. 1999). Soares et al. (2020) reared broilers under cyclical heat stress while feeding diets differing in protein content during 22-42 days of age. They found weight gain and FCR were improved in heat stressed broilers supplemented with 220 g/kg protein compared to those supplemented with 160 g/kg protein.

Today, synthetic methionine is the primary source of methionine in broiler diets. Synthetic methionine can be supplemented in several forms, including D-, L-, or DL-methionine (DLM), or as a methionine analogue. Only L amino acids are biologically available, therefore the conversion of the D enantiomorph or methionine analogue must occur for proper utilization (Dibner and Knight 1984). Results disagree regarding broiler performance in relation to methionine source. Some research shows under heat stress conditions, broilers supplemented with DL-2-hydroxy-(4-methylthio)butanoic acid (MHA) have better performance and lower mortality than broilers

supplemented with DLM (Swick and Pierson 1988). Studies have additionally shown DLM improves broiler performance while MHA does not (Balnave and Oliva 1990). However, it has also been shown methionine supplement source has no effect on broiler performance under both thermoneutral conditions or heat stress conditions (Ribeiro et al. 2001).

Age affects poultry nutrient deposition (Sakomura et al. 2005). It is well known a broiler's protein requirement decreases with age while its energy requirement increases. Methionine's requirement additionally changes with age; however, the dietary source and environmental stressors may change the broiler's requirement and utilization of methionine (Ojano-Dirain and Waldroup 2002). Moreover, heat stress will affect poultry differently depending on the age of the bird (Andretta et al. 2021). Because age is a major factor for both nutrient requirement and heat stress, this study subjected broiler chickens to heat stress during different phases of production. Through RNA-sequencing, we investigated the transcriptome of broilers heat stressed at different ages while fed different diets to further resolve the molecular response of broilers to heat stress.

MATERIALS AND METHODS

Experimental design

This study consisted of 16 groups in a 4x2x2 factorial design comprised of environmental condition (thermoneutral or heat stress during the starter, grower, or finisher phase), dietary methionine source (DLM or MHA), and dietary inclusion of methionine (100% or 140% of requirement). At hatch, chicks were placed on pine shaving-covered floor pens (0.072 m^2/bird) with ambient temperature set to 32^oC. Each group consisted of 6 replicate pens with 20 birds per replicate. Ambient temperature was gradually reduced over the next 7 days in accordance with industry standards. Environmental condition was initiated for the starter (days 7-10), grower (days 11-21), and finisher (days 22-35) phases, and broilers were subjected to a hot environment (35^oC)

for only one phase of production. Broilers kept at a thermoneutral environment remained at 25°C for all three phases (Figure 4.1).

Feed and water were provided *ad libitum*. Broiler body weight and feed weight were taken on days 0, 10, 21, and 35. The 4 unique diets were formulated for broiler starter (day 0-10), grower (day 11-21), and finisher (22-35) phases from a common basal diet (Table 3.1). For each phase, diets containing 100% and 140% of Cobb500 methionine requirement were formulated using DLM. Then, diets containing MHA were formulated to remain equimolar to 100% DLM and 140% DLM, respectively. Starter diets contained 22% crude protein (CP) and 12.4 MJ apparent metabolizable energy (AME). The 100% DLM and 140% DLM starter diets contained 0.653% and 0.792% methionine and 0.997% and 1.131% total sulfur amino acids (TSAA), respectively. Grower diets for 100% DLM and 140% DLM contained 20% CP and 12.8 MJ AME with 100% DLM and 140% DLM diets containing 0.573 and 0.669% methionine, and 0.880 and 0.975% TSAA, respectively. Finisher diets 100% DLM and 140% DLM contained 18% CP and 12.8 MJ AME. The 100% DLM and 140% DLM finisher diets contained 0.511 and 0.630% methionine, and 0.880 and 0.918% TSAA, respectively (Table 3.2).

Replicate total pen body weight and feed weight were measured at days 0, 10, 21, and 35. The sampling timeline is outlined in figure 4.1. On day 10, 5 birds from the 4 groups reared in a thermoneutral environment were euthanized and *Pectoralis major* tissue samples were collected. Additionally, 5 birds from the 4 groups reared at 35°C for the starter phase were euthanized and samples collected. On day 21, 5 birds from the 4 groups reared in a thermoneutral environment and from the 4 groups reared at 35°C for the grower phase were euthanized and samples collected. Similarly, on day 35, 5 birds from the 4 groups reared in a thermoneutral environment and from

the 4 groups reared at 35°C for the finisher phase were euthanized and samples collected. Tissue from the *Pectoralis major* were flash frozen in liquid nitrogen and stored at -80°C.

RNA preparation and RNA-sequencing

Sampled *Pectoralis major* tissue was coarsely ground in liquid nitrogen and further broken down via lysing matrix tubes (MP Biomedicals, Santa Ana, CA). Total RNA was extracted using TRIzol reagents (Invitrogen, Carlsbad, CA), then cleaned with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA was treated with RNase-Free DNase (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. Purified RNA was resuspended in RNase-free water. RNA concentration and purity were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm an $OD_{260/280}$ ratio greater than 1.9. cDNA libraries were prepared with 4 μg total RNA using TruSeq RNA sample Preparation Kit to obtain cDNA fragments of 229 bp average size, or 355 bp including adapter sequences. All cDNA libraries were sequenced with 150 bp paired-end read chemistry using the Illumina HiSeq 2000 system.

Analysis of RNA-sequencing data and gene ontology

FastQC was used to identify the quality of raw reads. Low-quality bases and adapter sequences were trimmed using Trimmomatic v.0.36 (Bolger et al. 2014) and Flexbar v.2.4 (Dodt et al. 2012). Reads were aligned to the chicken reference genome (*Gallus gallus* 4.0, version 81, Ensembl) using STAR aligner v.2.5.2b (Dobin et al. 2013). Hit counts were counted using featureCounts v.1.5.2 (Liao et al. 2014), using only unique reads moving forward. DESeq2 (Anders and Huber 2010; Love et al. 2014) was used for differential gene expression analysis. For pairwise analysis, four comparisons were devised for each sampling day: non-stressed broilers fed 100% DLM versus heat stressed broilers fed 100% DLM (100DLM), non-stressed broilers fed 100% MHA versus heat stressed broilers fed 100% MHA (100MHA), non-stressed broilers fed

140% DLM versus heat stressed broilers fed 140% DLM (140DLM), non-stressed broilers fed 140% MHA versus heat stressed broilers fed 140% MHA (140MHA). Counts were normalized using the transcripts per million method. Normalized counts were subjected to $\log_2$ fold change based on the comparisons outlined using the Wald test to generate p-values. An adjusted p-value for false discovery rate (FDR) was generated using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). Genes are considered differentially expressed when the FDR $\leq$ 0.05 and fold change $\geq$ 1.2 or $\leq$ -1.2.

The ClueGO Plugin v2.5.4 (Bindea et al. 2009) was used for gene ontology (GO) within Cytoscape v3.7.2. Only genes considered to be differentially expressed were used for analysis. The majority of analysis was done using Kyoto Encyclopedia of Genes and Genomes (KEGG) terms, however molecular and biological GO pathways/networks were used, when stated.

Quantitative reverse transcription-PCR verification of RNA-sequencing data

Aliquots from all RNA samples used for RNA-sequencing were retained and used for RT-qPCR validation of genes. Nine differentially expressed genes identified by RNA-sequencing were used for validation. These included *CHRNG*, *FBLN5*, *GSN*, *PVALB*, *GLO1*, *CDK1*, *PLK3*, *ATP5G1*, and *PEAK1* (Table 4.1). cDNA was synthesized from two micrograms of total RNA using high-capacity cDNA Reverse Transcription Kit in accordance with the manufacture's protocol (Applied Biosystems, Foster City, CA) and using a thermocycler (Eppendorf, Hauppage, NY) with the following settings: 10 min at 25°C, 120 min at 37°C, five min at 85°C and final cycle at 4°C. Newly synthesized cDNA was stored at -20°C until further use. cDNA was diluted so two ng would be used for each reaction during RT-qPCR, additionally using 0.3 µL of forward primer (10 µM), 0.3 µL of reverse primer (10 µM), 8.4 µL of RNase-DNase-free water, and 10 µL of SYBR Green Master Mix (Applied Biosystems, Carlsbald, CA). Each biological sample was run

in triplicate for each gene. Conditions for RT-qPCR were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds using a StepOnePlus (Applied Biosystems, Carlsbald, CA). Chicken *β-actin* was used as an endogenous control and differential expression was measured using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001), with relative expression comparisons identical to the comparisons used for RNA-sequencing.

Statistical analysis

Statistical analysis for body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) results for each sampling day were determined using the GLMMIX procedure in SAS (SAS Institute, Cary, North Carolina). All tests were performed with a 5% level of significance. Environment, methionine source, and methionine level were considered as main effects. Least square means was used to compare effects using PDIFF in SAS when found to be significant. Probability values were generated using analysis of variance for main and interaction effects. For validation of differentially expressed genes (DEGs), linear regression using PROC REG (SAS Institute, Cary, North Carolina) tested the strength of RNA-sequencing $\log_2$ fold change predicting RT-qPCR $\log_2$ fold change.

RESULTS

Performance results are displayed in tables 4.2, 4.3, and 4.4. Heat stress during the starter phase did not have a significant effect on body weight gain (BWG), feed intake (FI), or feed conversion ratio (FCR). All 4 dietary treatments performed similarly when subjected to high temperatures from days 8 to 10. When broilers were stressed from days 11 to 21 however, all 4 dietary treatments showed reduced BWG than their thermoneutral counterparts. Heat-stressed broilers in 100DLM and 100MHA showed significant reduction in BWG whereas those in 140DLM and 140MHA did not gain significantly less wait than thermoneutral broilers. Because

feed intake was numerically reduced for all heat-stressed broilers, FCR during the grower phase was similar between all 8 groups.

Increased dietary methionine did not increase BWG of heat-stressed broilers during the finisher phase. In fact, BWG was slightly higher in stressed broilers fed at-requirement methionine. Thermoneutral broilers fed at-requirement methionine and above-requirement DLM gained significantly more weight than any heat-stressed group. This aided in improving FCR for those groups. Feeding above-requirement MHA resulted in the lowest BWG of all non-stressed groups, resulting in statistically similar BWG to stressed broilers in 100DLM, 100MHA, and 140DLM.

Transcriptome

A total of 3.98 billion reads were obtained from the *Pectoralis major* from all three sampling days. This resulted in an average of 34,232,789 reads per sample on day 10, 33,085,448 on day 21, and 32,089,123 on day 35. On day 10, 88.8% of all reads were mapped to the reference genome, 84.1% being uniquely mapped. On day 21, 88.6% of all reads were mapped and 83.6% uniquely mapped. During the final sampling on day 35, 89.3% of reads were mapped to the reference genome with 84.4% uniquely mapped. A total of 43 data-points were selected for validation via RT-qPCR based on significance, comparison, and sampling time. Regression analysis revealed strong correlation ($p < 0.0001$, $R^2 = 0.9137$) between the $\log_2$ fold change of significantly DEGs determined by RNA-sequencing and RT-qPCR (Figure 4.2).

Heat stress during the starter phase

Differential gene expression of heat stressed broilers supplemented with at-requirement DLM (100DLM)

After exposure to a hot environment from days 7 to 10 post-hatch, broilers in 100DLM upregulated 60 genes and downregulated 66 genes (Figure 4.3a). The genes *ADPRHL1*, *PDK4*,

CHAC1, *CEBPD*, and *DDIT4* were most upregulated (Table 3.7) while *KCNU1*, *CHADL*, *PEAK1*, *FAM126B*, and *UBE2C* were most downregulated (Table 3.8). GO reveals oxidative phosphorylation was associated with the most downregulated genes, including *ATP5G1*, *ATP6V1C1*, *COX6A1*, *COX7A2*, *ND1*, and *NDUFV2*. mRNA surveillance pathway, spliceosome, and cysteine and methionine metabolism were all KEGG terms associated with multiple downregulated genes in 100DLM. Influenza A, herpes simplex infection, alanine, aspartate and glutamate metabolism, FoxO signaling pathway, and Wnt signaling pathway were all associated with upregulated genes.

Differential gene expression of heat stressed broilers supplemented with at-requirement MHA (100MHA)

When broilers were fed at-requirement MHA, heat stress differentially regulated only 9 genes (Figure 4.3a). *RPL3L*, *GRIN3B*, *MINDY4B*, and *BRSK2* were upregulated and *FABP1*, *CELSR1*, *CPOX*, *SDF2*, and *PEAK1* were all downregulated. Both *BRSK2* and *PEAK1* code for serine/threonine kinases, *FABP1* codes for fatty acid binding protein 1, and *CPOX* for the mitochondrial protein coproporphyrinogen oxidase.

Differential gene expression of heat stressed broilers supplemented with above-requirement DLM (140DLM)

Only one gene, *TCIM*, was differentially regulated in 140DLM at day 10 (Figure 4.3a). This upregulated gene codes for a positive regulator of the NIK/NF-kappaB signaling.

Differential gene expression of heat stressed broilers supplemented with above-requirement MHA (140MHA)

A total of 71 genes were differentially regulated in 140MHA at day 10 (Figure 4.3a); 36 positively and 35 negatively expressed (Tables 3.9 and 3.10). The genes *ALB*, *FGA*, and *FGG* code

for albumin, fibrinogen alpha chain, and fibrinogen gamma chain, respectively, and were all upregulated in the comparison. *ARHGAP35*, *GIT1*, *SLC9A1* were additionally upregulated and are associated with the regulation of the actin cytoskeleton. KEGG term steroid biosynthesis was associated with the 4 downregulated genes *CYP51A1*, *DHCR7*, *NSDHL*, and *SQLE*.

Heat stress during the grower phase

Differential gene expression of heat stressed broilers supplemented with at-requirement DLM (100DLM)

When reared in a hot environment from day 11 to 21, broilers in 100DLM upregulated 824 genes while downregulating 700 genes (Figure 4.3b). The top positively regulated genes were *TUBA8B*, *HIST1H4J*, *APOD*, *PAX3*, and *PVALB* (Table 4.4) and the most negatively regulated genes were *MINDY4B*, *GJD2*, *CSMD1*, *RGR*, and *KCNK3* (Table 4.5). KEGG pathway enrichment showed 24 upregulated genes associated with autophagy, 20 with MAPK signaling, 19 with endocytosis, and 13 with ubiquitin mediated proteolysis. Additionally, the lysosome and mTOR signaling had a large number positively regulated genes associated with those terms. The most downregulated pathways included the cell cycle, regulation of the actin cytoskeleton, focal adhesion, and cellular senescence (Table 4.6). Oxidative phosphorylation was additionally associated with negatively regulated genes, similar to 100DLM on day 10 (Figure 4.4a).

Differential gene expression of heat stressed broilers supplemented with at-requirement MHA (100MHA)

Broilers fed at-requirement MHA upregulated 253 genes and downregulated 274 genes after being subjected to heat stress during the grower phase (Figure 4.3b). The genes *ALDH1A1*, *SLC11A1*, *PVALB*, *AQP4*, and *ATP2C2* were most upregulated (Table 4.7) while *CCK*, *CHAC1*, *CPEB2*, *JCHAIN*, and *KPNA7* were most downregulated (Table 4.8). Similar to 100DLM, broilers

in 100MHA upregulated a majority of genes related to autophagy. Neuroactive ligand-receptor interaction, purine metabolism, and TGF-beta signaling pathway were additional KEGG terms associated with positively expressed genes. Oxidative phosphorylation, oocyte meiosis, protein processing in endoplasmic reticulum, the cell cycle, and cellular senescence were all associated with primarily downregulated genes in 100MHA at day 21 (Table 4.9). The cell cycle, regulation of the actin cytoskeleton, and focal adhesions were not as strongly related to negatively expressed genes in 100MHA as 100DLM on day 21. Additionally, mTOR signaling was unaffected by heat stress in 100MHA, whereas heat stress influenced mTOR regulation in 100DLM (Figure 4.4a, Figure 4.4b).

Differential gene expression of heat stressed broilers supplemented with above-requirement DLM (140DLM)

Increasing the inclusion of DLM in the diet of stressed broilers reduced the number of DEGs to 774, with 392 being upregulated and 382 downregulated (Figure 4.3b). *PVALB* was again among the most upregulated genes, along with *PDK4*, *BMP5*, *MIM1*, and *SLC47A2* (Table 4.10). The genes *GJD2*, *FOS*, *RGS1*, *APOLD1*, and *CDH6* were most downregulated in 140DLM (Table 4.11). The KEGG term oxidative phosphorylation was related to 30 downregulated genes, most of any comparison at day 21. Similar to broilers fed at-requirement methionine, MAPK signaling was most associated with upregulated genes, along with protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, endocytosis, and TGF-beta signaling pathway (Table 4.12).

Differential gene expression of heat stressed broilers supplemented with above-requirement MHA (140MHA)

Increasing MHA content in the diet of heat stressed broilers during days 11 to 21 resulted in the upregulation of 406 genes and the downregulation of 336 genes (Figure 4.3b). Again, *PVALB*

was the most upregulated gene in heat stressed broilers. The genes *BMP5*, *PDE10A*, *UTS2R*, and *CETP* were also highly expressed (Table 4.13). *MIM1*, *RGR*, *CSRP3*, *ELFN1*, and *PAQR7* were most negatively regulated in 140MHA during the grower phase (Table 4.14). Unlike the previous comparison at day 21, regulation of the actin cytoskeleton and focal adhesions were associated with upregulated genes in 140MHA. The KEGG terms cell cycle, oxidative phosphorylation, and oocyte meiosis were related to downregulated genes (Table 4.15).

Heat stress during the finisher phase

Differential gene expression of heat stressed broilers supplemented with at-requirement DLM (100DLM)

When heat stress was initiated during the finisher phase, broilers fed at-requirement DLM upregulated twice the number of genes as when stressed during the grower phase. Additionally, 1,158 genes were downregulated in those same broilers (Figure 4.3c). The genes *NOG*, *CLDN3*, *PTX*, *SLC4A9*, and *HTR3A* were most upregulated (Table 4.16) and *CCK*, *MARCH11*, *NXPH1*, *GAP43*, and *FMO4* most downregulated (Table 4.17). Endocytosis, FoxO signaling pathway, RNA transport, protein processing in endoplasmic reticulum, and the spliceosome were KEGG terms most associated with upregulated genes. The terms oxidative phosphorylation, calcium signaling pathway, cell adhesion molecules, vascular smooth muscle contraction, and pyruvate metabolism were most related to downregulated genes in 100DLM (Table 4.18). When stressed during the grower phase, 100DLM downregulated many genes related to the cell cycle. Upon experiencing heat stress in the finisher phase, broilers fed at-requirement DLM upregulated a majority of genes related to the cell cycle (Figure 4.4a). RNA transport and mRNA surveillance pathways were additionally regulated in this way between days 21 and 35.

Differential gene expression of heat stressed broilers supplemented with at-requirement MHA (100MHA)

Heat stressed broilers upregulated 1,398 genes and downregulated 1,564 genes when fed a diet containing at-requirement MHA during the finisher phase (Figure 4.3c). *FAM135B*, *PVALB*, *ZBTB16*, *U11*, and *SLC26A4* were the most upregulated genes in these broilers (Table 4.19). The genes *MMP27*, *MLANA*, *DCDC2*, *MMP13*, and *CCL19* were most negatively regulated in 100MHA (Table 4.20). The spliceosome and RNA transport were associated with many positively expressed genes, similar to 100DLM. The KEGG terms ubiquitin mediated proteolysis, autophagy, and ribosome were additionally associated with upregulated genes in 100MHA. Focal adhesion, regulation of actin cytoskeleton, and endocytosis were the three KEGG terms containing the most negatively expressed genes in 100MHA at day 35 (Table 4.21). Focal adhesion and the regulation of the actin cytoskeleton saw a large increase in the number of DEGs between days 21 and 35 in 100MHA (Figure 4.4b). Ubiquitin mediated proteolysis additionally saw an increase in the number of DEGs when broilers were stressed in the finisher phase.

Differential gene expression of heat stressed broilers supplemented with above-requirement DLM (140DLM)

Unlike the grower phase, increasing DLM inclusion did not reduce the number of differentially expressed genes in the breast muscle when stressed during the finisher phase. A total of 2,783 genes were differentially expressed in 140DLM; 1,405 positively and 1,378 negatively expressed (Figure 4.3c). The genes *U11*, *SNORA12*, *PVALB*, *SYPL1*, and *PPIL6* were most upregulated (Table 4.22) while *MMP9*, *NMU*, *GAP43*, *CCK*, and *CNTNAP5* were most downregulated (Table 4.23). GO analysis revealed ribosome, autophagy, RNA transport, Ubiquitin mediated proteolysis, and Wnt signaling pathway to be most associated with upregulated genes in

heat stressed broilers. Focal adhesion and regulation of actin cytoskeleton were most related to downregulated genes (Table 4.24). These processes were far less regulated by heat stress in the grower phase, in addition to ubiquitin mediated proteolysis (Figure 4.4c).

Differential gene expression of heat stressed broilers supplemented with above-requirement MHA (140MHA)

Above-requirement MHA supplementation during heat stress also resulted in the upregulation of *PVALB* and *U11* in broilers (Table 4.25). A total of 1,807 genes were upregulated in this comparison, along with 1,728 downregulated genes (Figure 4.3c). *CCK* was again one of the most downregulated genes, along with SPIRE2, SMPD3, OSTN, and MMP7 (Table 4.26). Similar to other comparisons, KEGG enrichment revealed ribosome, RNA transport, spliceosome, Wnt signaling pathway, and ubiquitin mediated proteolysis to be most associated with upregulated genes in 140MHA. Focal adhesion and regulation of actin cytoskeleton were again highly associated with downregulated genes when broilers were heat stressed during the finisher phase (Table 4.27).

DISCUSSION

Heat stress greatly limits broiler performance (Hurwitz et al. 1980; Attia et al. 2011; Sohail et al. 2012; Awad et al. 2018; Goo et al. 2019; Lin Law et al. 2019). These consequences are primarily seen in older broilers, as high environmental temperatures do not have a profound effect on the performance of young chicks (Inês Müller Fernandes et al. 2013). In the current study, heat stress during 7 to 10 days of age did not affect BWG, FI, or FCR of broilers. This is unsurprising, as younger broilers have a higher heat tolerance (Chowdhury et al. 2012). Hot environmental temperatures do induce heat stress in older broilers, however, impairing growth and performance (Andretta et al. 2021). Heat stress from days 11 to 21 reduced weight gain of broilers, though

increasing methionine inclusion in the diet aided BWG. The largest proportion of protein in a broiler is in the skeletal muscle, and maturing birds require more protein and amino acids for growth (Fisher 2013). The diversion of methionine from growth to defense against reactive oxygen species (ROS) and immune activation limits growth under heat stress (Cuthbertson 1931; Aquilani et al. 2005; Zeitz et al. 2020b). Increased dietary methionine benefits heat stressed broilers, aiding in performance and health (Balnave et al. 1999; Ojano-Dirain and Waldroup 2002; Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005; Soares et al. 2020). This was only seen during the grower phase in the current study, and increased dietary methionine did not aid in the growth of broilers during the finisher phase. Similar to previous work (Bonnet et al. 1997; Tesseraud and Temim 1999; Pope and Emmert 2002; Habashy et al. 2017), older broilers performed worse under heat stress. In addition, excess protein in broiler diets also increase the heat load on the bird, compounding the effects of a hot environment (MacLeod 1997; Musharaf and Latshaw 1999; Bell et al. 2002). Lower requirement for methionine, slower growth, and elevated protein turnover of older broilers could explain why increased dietary methionine did not aid broiler performance in the finisher phase (Tesseraud et al. 1996; Northcutt et al. 1998; Gasparino et al. 2018).

The mitochondria and oxidative stress

Under heat stress, the diversion of blood flow to the periphery gives rise to a hypoxic environment (Hall et al. 1999). Under normal conditions, electron leak in the mitochondria's electron transport chain (ETC) generates superoxide (Turrens 2003). In a hypoxic environment, this generation of superoxide and other ROS reaches uncontrollable levels (Pamplona and Costantini 2011). After experiencing hot environmental temperatures from days 7 to 10, broilers in 100DLM downregulated the genes *ATP5G1*, *ATP6V1C1*, *COX6A1*, *COX7A2*, *ND1*, and *NDUFV2* related to oxidative phosphorylation. Heat stress is known to induce mitochondrial

dysfunction (Downs and Heckathorn 1998; Monti et al. 2001; Zhao et al. 2006), and the downregulation of genes coding for certain subunits of complexes along the ETC could result in impaired function. The genes *ND1* and *NDUFV2* code subunits of complex I, which is the main source of mitochondria-derived ROS (Murphy 2008). Similarly, *COX6A1* and *COX7A2* code for subunits of complex IV of the ETC. Complex IV catalyzes the transfer of electrons to oxygen, and the downregulation of two genes coding for subunits may prevent proper function. Zhang et al. (2019) saw the downregulation of *COX6A1* in heat stressed broilers, which may have contributed to the production of ROS. If there is an increase of ROS production, the antioxidant system of the broiler should respond to mitigate damage. However, no genes coding for any major antioxidant systems were differentially expressed in any comparison at day 10, suggesting heat stress at a younger age does not require a robust antioxidant response. This is consistent with other studies that have seen younger animals have higher capacity for oxidative stress (Sandercock et al. 2001; Del Vesco et al. 2017).

When heat stressed from days 11 to 21, broilers in the 100DLM and 100MHA comparisons each downregulated 12 genes associated with oxidative phosphorylation. When methionine content of the diet was increased, broilers in 140DLM and 140MHA downregulated 30 and 10 genes, respectively, related to oxidative phosphorylation. Increasing methionine supplementation increases the flux through the ETC and aids in mitochondria function (Lozoya et al. 2018; Tripodi et al. 2018). At first, it is surprising heat stressed birds in 140DLM downregulated a larger number of genes related to the ETC. However, if thermoneutral broilers in 140DLM have increased flux through the ETC, heat stress could introduce a larger drop in flow, displayed as more pronounced downregulation. Additionally, it may be increasing methionine inclusion in the diet is detrimental for mitochondrial function. Work has shown methionine restriction benefits oxidative status by

reducing ROS (Naudí et al. 2007; Perrone et al. 2010). However, Swennen et al. (2011) has shown neither methionine source nor inclusion level affects antioxidant activity or oxidative status of broilers at 4 weeks or age. At 6 weeks, broilers fed above-requirement MHA had lower circulating malondialdehyde levels and higher SOD activity than those fed DLM (Swennen et al. 2011).

Age and body weight factor heavily on a bird's ability to cope with heat stress (Sandercock et al. 2001). At 21 days of age, heat stressed broilers have lower H_2O_2 production and elevated antioxidant activity than broilers at an age of 42 days (Del Vesco et al. 2017). In the current study, heat stress during the finisher phase resulted in 27 negatively expressed genes in 100DLM related to oxidative phosphorylation. Increased downregulation of oxidative phosphorylation increases oxidative damage and cell death, as well as reduces activity of glutathione peroxidase (GPx) (Chin et al. 2008). Glutathione (GSH), a vital antioxidant derived from cysteine, is essential for the control of ROS (Habashy et al. 2019). GPxs use GSH to neutralize H_2O_2, protecting the cell from damage (Chu et al. 2020). Catalase is another antioxidant responsible for the neutralization of H_2O_2 (Cao et al. 2003). In some instances, GPxs and catalase compete for the job of protecting the cell against H_2O_2, and the activity of one antioxidant far outweighs the other (Cao et al. 2003; Habashy et al. 2018). When stressed from days 22 to 35, 100DLM downregulated 5 genes (*ANPEP*, *CHAC2*, *GPX7*, *HPGDS*, *MGST3*) related to GSH metabolism. Those broilers additionally downregulated *DERA*, *FBP1*, *GPI*, and *PRPS2*, related to the pentose phosphate pathway (PPP). The PPP generates NADPH (Stine et al. 2014; Litwack 2018), which is utilized by the GSH cycle for antioxidant defense. Increased H_2O_2 production can lead to reduced GPx activity (Islam et al. 1997), and Habashy et al. (2018) saw higher expression of catalase than GPx when birds were subjected to heat stress. Indeed, 100DLM did upregulate the gene *CAT* when heat stressed during the finisher phase. However, these broilers additionally upregulated *GPX2*, a Se-dependent GPx

(Surai 2006). The additional upregulation of *GCLC*, coding for glutamate-cysteine ligase, indicates some antioxidant activity of GSH in 100DLM. This disagreement could be contributed to selenium availability, as GPx7 is Se-independent (Flohé and Brigelius-Flohé 2016), or because antioxidant activity can fluctuate based on age and tissue (Habashy et al. 2019). *GPX7* was additionally downregulated in 100MHA, 140DLM, and 140MHA, however 140DLM did not differentially regulate any other genes related to GSH metabolism. The activity of GSH is positively correlated with methionine supplementation (Waschulewski and Sunde 1988; Tchantchou et al. 2008; Del Vesco et al. 2014; Zeitz et al. 2020a), so the increased inclusion of methionine in 140DLM could be sufficient to not require a significant response when heat stressed.

Methionine metabolism

GSH is a sulfur-containing amino acid derived from cysteine via methionine metabolism (James et al. 2008; Lu 2009). With so few DEGs related to GSH metabolism in 140DLM at day 35, regulation of methionine metabolism may also be unaffected by heat stress. Still, *BCAT1*, *GOT2*, *MAT1A*, *MPST*, *MTR*, and *PHGDH* were all downregulated in 140DLM when heat stressed during the finisher phase. The genes *GOT2*, *MPST*, *BCAT1*, and *PHGDH* are all involved with the utilization of cysteine for metabolic processes other than GSH production. The enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase, coded by *MTR*, is responsible for the re-methylation of homocysteine to replenish methionine in the methionine cycle (Stover 2009). This action is in direct contrast with transsulfuration which utilizes homocysteine for GSH synthesis. So, the downregulation of *MTR* could indicate reduced re-methylation of homocysteine while cysteine is spared for GSH synthesis.

The genes *GOT2*, *BCAT1*, and *PHGDH* were additionally downregulated in 100DLM at day 35, as well as *AHCY*. Adenosylhomocysteinase, coded by *AHCY*, metabolizes S-

adenosylhomocysteine to homocysteine. *AHCY* expression is positively corelated with methionine supplementation (Yin et al. 2018; Farinha et al. 2021), and heat-resilient poultry show higher expression of *AHCY* (Fleming et al. 2017). The downregulation of *MAT1A* in 100DLM also indicates methionine availability is low when broilers are heat stressed during the finisher phase, requiring remethylation (Pillai et al. 2006). However, the gene *MTR* was downregulated in 100DLM, indicating reduced activity of 5-methyltetrahydrofolate-homocysteine methyltransferase. Interestingly, the genes *ENOPH1*, *MRI1*, and *SRM* were upregulated in 100DLM. These genes code for enolase-phosphatase E1, methylthioribose-1-phosphate isomerase, and spermidine synthase, respectively, which are a part of the methionine salvage pathway (Albers 2009). Methionine salvage allows for the conservation of methionine without the threat of degradation (Belda et al. 2013; Sekowska et al. 2019). It may be metabolites used in the folate cycle for the remethylation of homocysteine are unavailable, reducing *MTR* expression and activating methionine salvage.

When heat stressed in the grower phase, broilers in 100DLM and 100MHA downregulated only 4 genes each related to methionine metabolism, and GSH metabolism appears unaltered. As mentioned, broilers fed above-requirement levels of methionine did not show differential regulation of genes related to GSH metabolism at day 21. The lack of DEGs in 140DLM and 140MHA related to methionine metabolism shows increasing methionine in the diet benefits broiler methionine metabolism during heat stress from 11 to 21 days of age. Additionally, heat stress at this younger age has less of an effect on GSH and methionine metabolism.

mTOR signaling

The mechanistic target of rapamycin (mTOR) pathway links nutrient availability with cellular processes like protein synthesis and growth (Jewell et al. 2013). When heat stressed,

corticosterone can inhibit this pathway, reducing protein synthesis (Pazini et al. 2016; Ma et al. 2021). ROS (Lou et al. 2017) and mitochondrial dysfunction (Tokunaga et al. 2004) additionally reduce mTOR signaling in birds. mTOR signaling is important for cell survival (Ferrara-Romeo et al. 2020). During times of low nutrient availability, cells stimulate autophagy to conserve and salvage nutrients crucial for cell survival (Kuma et al. 2004). In some cases, age can be a larger determining factor for mTOR activity than ROS (Blagosklonny 2008). The link between mTOR and energy production is thought to increase cell lifespan. Processes initiated by mTOR require lots of ATP (Buttgereit and Brand 1995), and reduced TORC1 activity in yeast induced differential regulation of oxidative phosphorylation (Pan and Shadel 2009). Inhibition of Eukaryotic translation initiation factors (eIFs) (Tohyama et al. 2008) and mTOR (Harrison et al. 2009) extend the life of organisms, possibly through more efficient mitochondrial function. In the current study, heat-stressed broilers in 100DLM upregulated 15 genes related to mTOR signaling in 100DLM at day 21. Expression of genes related to mTOR does not necessary corelate to increased protein synthesis and growth, however. For example, *STK11* codes for serine threonine kinase 11, which serves as an activator for AMPK, inhibiting mTORC1 (Meijer et al. 2015). The gene *DEPDC5* codes for a subunit of GAP activities Towards Rags 1 complex (GATOR1), which also inhibits mTORC1 action (Panchaud et al. 2013). So, it may be the upregulation of genes associated with mTOR in 100DLM at day 21 have more to do with the inhibition of cell growth than with protein synthesis.

After experiencing heat stress from days 22 to 35, broilers in 140MHA upregulated genes associated with protein synthesis and mTOR signaling. Genes coding for eIF 3, 4, and 5 were upregulated in these stressed broilers, as well as 29 genes related to mTOR signaling. Through phosphorylation of p70S6K by mTORC1, eIF4B is activated, initiating the translation of proteins

(Shahbazian et al. 2006). The additional inhibitor action of mTORC1 on 4EBP1 and eEF2K allows for translation via eIF4E and eEF2 (Hay and Sonenberg 2004). The survival of more essential cells may require the degradation of less-crucial cells to provide metabolites for more important processes or components (Kuma et al. 2004).The upregulation of *AKT*, *PIK3CA*, *PTEN*, *HRA5*, *RPTOR*, and *RRAGC* in 140MHA are associated with autophagy. Heat stressed broilers in 140MHA may therefore be displaying signs of cell survival, where cell death is initiated to conserve resources, and translation is initiated to ensure survival of vital cells and function. A similar transcriptome response was seen in 100DLM, 100MHA, and 140DLM when heat stressed during the finisher phase, whereas heat stress in the grower phase did not elicit such a response. It seems the age in which a bird is stressed plays into mTOR signaling, oxidative phosphorylation, protein synthesis, and cell survival.

Cell cycle

After heat stress during the grower phase, broilers in 100DLM downregulated 30 genes related to the cell cycle. mTOR signaling takes part in controlling progression through the cell cycle (Fingar et al. 2004). ROS generation inhibits function of the mTOR pathway, as well as induces apoptosis through cellular arrest at the G2/M check point (Jiang et al. 2018). Under heat stress, the checkpoints at G1/S and G2/M transitions are compromised (Elledge 1996). The genes *CCNA2*, *CDC25A*, *CDC7*, *CDK1*, *CHEK1*, *PLK1*, and *RAD21* are related to G2/M phase transition while *CCNA2*, *CCNE2*, *CDC45*, *CDKN2C*, *E2F1*, and *RBL1* are related to the G1/S phase transition. The downregulation of these genes, along with others related to the cell cycle, indicate heat stress during the grower phase induces cellular arrest in 100DLM. Broilers in 100MHA also downregulated genes related to the cell cycle; however, this included only the genes *BUB1B*, *CCNA2*, *CCNB1*, *CDK1*, *MCM3*, *PLK1*, and *YWHAG*. It may be MHA is more beneficial for cell

survival when heat stressed, as no genes related to apoptosis were differentially regulated in 100MHA on day 21. In fish, MHA supplementation reduces apoptosis and improves the oxidative status in the intestine compared to DLM supplementation (Pan et al. 2017). Increasing methionine supplementation additionally aids in reducing apoptosis (Yalçınkaya et al. 2009). In the current study, increasing DLM supplementation lowered the number of DEGs related to the cell cycle, and no genes related to apoptosis were differentially expressed on day 21 in 140DLM.

Interestingly, 100DLM upregulated 16 genes and downregulated 9 genes related to the cell cycle at day 35. On the other hand, 100MHA, 140DLM, and 140MHA downregulated the majority of genes related to the cell cycle when stressed during the finisher phase. The genes *GADD45A* and *GADD45B* were upregulated in only 100DLM, and code for growth arrest and DNA-damage-inducible protein 45, a part of the response to DNA damage (Liu et al. 2009). Diets deficient in sulfur amino acids induce the expression of *GADD45B* (Rao et al. 2001), and its expression is linked with oxidative damage (Barger et al. 2012). The genes *CDKN1B*, *RB1*, and *ZBTB17* are additionally associated with cellular arrest, and their upregulation in 100DLM indicate heat stressed broilers are suffering DNA damage, possibly initiating cell death.

Proteolysis

Protein degradation is strongly correlated to cellular arrest and apoptosis (Bastians et al. 1999; He et al. 2009). ROS also initiate proteolysis through amino acid oxidation, damaging proteins (Zhang et al. 2013). The ubiquitin proteasome pathway (UPP) and the 26s proteasome are a major contributor to proteolysis (Baumeister et al. 1998; Li et al. 2003). Proteasomes can be activated upon mTOR inactivation (Zhao et al. 2015), a phenomenon seen in 100DLM when stressed during the grower period. Those broilers upregulated genes related to the inhibition of mTORC1, while additionally upregulating 13 genes related to the UPP. If ROS derived from the

mitochondria were to induce proteolysis, we could expect broilers in 100MHA to also upregulate the UPP, as 100DLM and 100MHA each downregulated 12 genes associated with the ETC during the grower phase. However, 100MHA did not differentially regulate any genes related to the UPP during this time. Moreover, heat stressed broilers in 100DLM or 100MHA did not show significantly lower BWG during the grower period from each other, suggesting protein loss should be similar. One answer why 100MHA did not differentiate genes related to proteolysis may have to do with nutrient intake. Both stressed and non-stressed broilers in 100MHA ate a similar amount of feed during the grower period, whereas stressed broilers in 100DLM at numerically less feed than their thermoneutral counterparts. Although feed intake was not significantly different during this phase, these differences could indicate the discrepancy of the transcriptome. mTOR signaling plays a major part in nutrient intake (Richards and Proszkowiec-Weglarz 2007), so the lack of DEGs in 100MHA related to mTOR signaling and proteolysis indicate heat stress during the grower phase does not affect proteolysis when broilers are fed at-requirement MHA.

When fed above-requirement methionine, broilers reared in a hot environment from days 11 to 21 upregulated only a small number of genes related to proteolysis via the UPP. Those broilers also gained more weight during the grower phase than heat stressed broilers fed at-requirement methionine. When supplemented with excess methionine, protein degradation is reduced in heat stressed birds (Del Vesco et al. 2015). Increasing essential amino acid supplementation leads to improved performance and weight gain under heat stress (Balnave et al. 1999; Ojano-Dirain and Waldroup 2002; Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005; Soares et al. 2020), similar to the grower phase of this study. However, increasing methionine supplementation did not improve the weight gain of broilers when heat stressed from days 22 to 35. Methionine content in the diet has a larger effect on broiler performance at a younger age

(Gasparino et al. 2018). This, combined with the increase of proteolysis in older birds (Tesseraud et al. 1996; Northcutt et al. 1998), may explain why the UPP was more upregulated when broilers were stressed during the finisher phase and why amount of methionine had no influence on weight gain. Both 100DLM and 100MHA upregulated 22 genes each while 140DLM and 140MHA upregulated 23 and 30 genes, respectively, related to the UPP. Ten genes (*ANAPC10*, *BTRC*, *DET1*, *PIAS1*, *STUB1*, *UBA3*, *UBE2D2*, *UBE2Q1*, *UBE2S*, *UBE4B*) were commonly regulated between all 4 comparisons during the finisher phase. These genes are all associated with ubiquitin ligase activity, which is highly active during times of oxidative stress (Li et al. 2003; Medicherla and Goldberg 2008; Lee et al. 2010; Manohar et al. 2019). Oxidative phosphorylation was downregulated in all 4 comparisons during the finisher phase, indicating possible mitochondrial dysfunction leading to ROS production. This increased oxidative stress could damage proteins and induce proteolysis, leading to the expression of genes related to the UPP. Increasing dietary essential amino acids does not always benefit chronically stressed broilers (Attia et al. 2011). The duration of heat stress, as well as age, also effects birds differently (Azad et al. 2010; Xie et al. 2014; Del Vesco et al. 2017). This could explain why increasing methionine content of broilers stressed during the grower phase lowers DEGs, while heat stress during the finisher phase results in similar DEGs and performance between all comparisons.

Actin cytoskeleton

The cytoskeleton of a cell is comprised of microtubules, intermediate filaments, and microfilaments. Actin is the primary protein of microfilaments and makes up a large portion of protein in skeletal muscles (Anderson 1976; Murakami and Uchida 1985). Seven methionine residues are prone to oxidation in actin (Dalle-Donne et al. 2002; Dalle-Donne et al. 2003), and heat stress has been shown to induce proteolysis of actin (Nakashima et al. 2004). ROS and

oxidative damage to the cytoskeleton results in the disassembly of actin structures, while additionally promoting organization and re-assembly (Zhu et al. 2005; Farah et al. 2011). When heat stressed during both the starter and grower phase, 140MHA upregulated genes associated with the regulation of the actin cytoskeleton. Coincidently, regulation of the UPP had few related DEGs in 140MHA during the starter and grower phase, indicating heat stress did not induce proteolysis when fed above-requirement MHA. This was also seen in 100MHA and 140DLM at days 10 and 21, where neither regulation of the actin cytoskeleton or the UPP were significantly differentially regulated. However, heat stressed broilers in 100DLM did downregulate 17 genes related to the regulation of the actin cytoskeleton after heat stress in the grower phase, in addition to increasing regulation of the UPP. The rearrangement of the cytoskeleton aids in the survival of cells during heat stress (Gavrilova et al. 2013); however, the additional upregulation of 18 genes related to autophagy indicates at-requirement DLM does not protect against cytoskeletal damage when heat stressed from days 11 to 21.

Heat stressed broilers in all comparisons downregulated a majority of genes related to the actin cytoskeleton on day 35. Heat exposure from age 21 to 35 days induces differential expression of proteins associated with the cytoskeleton in broilers (Kang and Shim 2020). Proteolysis is more prevalent in older birds (Tesseraud et al. 1996; Northcutt et al. 1998), as seen in this study. As mentioned, protein degradation in the muscle is reduced when dietary methionine is increased (Del Vesco et al. 2015), and S-adenosyl-methionine enacts protection of the cytoskeleton from xenobiotics (Ramírez et al. 2003). In the current study, however, increased methionine supplementation resulted in increased downregulation of the actin cytoskeleton during the finisher phase. In 140DLM and 140MHA, 35 and 42 genes were downregulated, respectively, related to regulation of the actin cytoskeleton. Interestingly, DEGs related to actin regulation in 100DLM

were more equally positively and negatively regulated, with 20 genes being upregulated and 25 downregulated. The genes *CDC42*, *CFL2*, *CXCR4*, and *ITGB1* were downregulated in all 4 comparisons. Cofilin 2, coded by *CFL2*, is expressed during acute heat stress, aiding in actin stability (Cruzen et al. 2015). It may be surprising *CFL2* is downregulated in the current study, however the chronic stress broilers were experiencing could bring about sweeping changes in the regulation of actin. To this point, *LIMK1* was additionally downregulated in 140DLM and 140MHA. The gene *LIMK1* codes for LIM kinase 1, an enzyme that regulates actin dynamics by means of cofilin inhibition (Gorovoy et al. 2005). As with mTOR signaling, the large number of DEGs associated with regulation of the actin cytoskeleton points to the importance of actin during hyperthermia, and the somewhat contradicting regulation of genes indicates massive disconnect of processes overwhelming the bird.

CONCLUSION

Heat stress from days 8 to 10 post-hatch does not influence broiler weight gain, FI, or FCR. Amount of dietary methionine additionally does not influence the performance of broilers during the starter phase, regardless of environmental temperature. Increasing dietary methionine does aid weight gain during the grower phase, especially when broilers are experiencing heat stress from days 11 to 21 post-hatch. This benefit is not seen when heat stressed during the finisher phase, however. The expression of genes related to proteolysis are similar between all heat stressed broilers from days 22 to 35. This could be in response to increased ROS generation derived from the mitochondria, as genes associated with the ETC were downregulated during this time. Actin is a major protein in the breast muscle of broilers, and the oxidation of methionine and sulfur residues could lead to increased proteolysis. The UPP appears to be less active when broilers are heat stressed in the starter and grower phases, likely because of increased thermotolerance of younger

birds as well as more robust antioxidant defense. Interestingly, it seems a broiler's requirement for either DLM or MHA depends on the age when experiencing heat stress, as molecular processes are regulated differently depending on the source and level of methionine. This should be considered when addressing nutrition of stressed broilers, as dietary methionine could greatly influence on these processes.

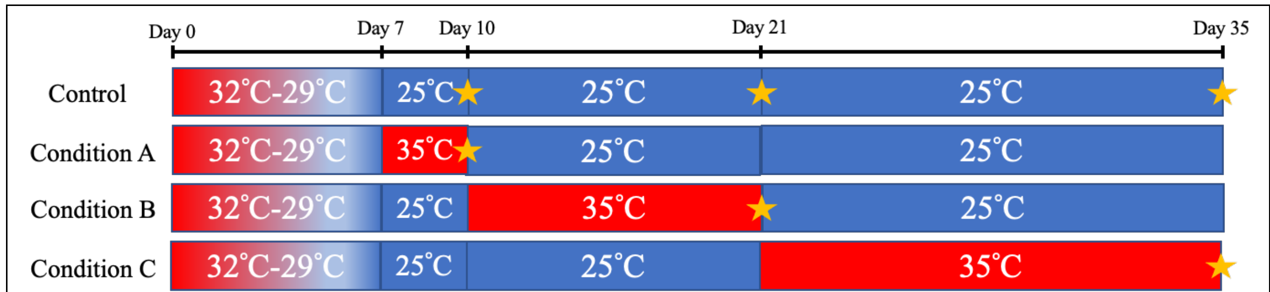

Figure 4. 1. Heat exposure and sampling timeline in days (d) for broilers. A star signifies a sampling point. Eight groups were sampled during each sampling day: 4 groups from the control and 4 groups from a specific condition. Each group sampled consisted of 5 replicate birds.

Table 4. 1. Forward and reverse primers used for RT-qPCR validation of RNA-sequencing.

Gene	Forward Primer	Reverse Primer
CHRNG	5′ GCTGGAGAAGATTGGACGTG 3’	5′ AAGAGCGAGGCCATGATGAA 3’
FBLN5	5′ AAGATCGTGCCAAGACGTGA 3’	5′ AATAGGCTCCAGGGTAGCGA 3’
ATP5G1	5′ GGACACGGCAAGTAATAGG 3’	5′ CATCAAACAGAAGAGACCCA 3’
GSN	5′ GAGGAGGGAGAAGCCCATTTG 3’	5′ CACCACTGATAGATGTTACTGCC 3’
PVALB	5′ TGGGAGCCTTTTCAGCAGC 3’	5′ CGTCCTTATCTCCAGCAGCC 3’
PLK3	5′ AAGGCCAAGAAGGGCTCTCT 3’	5′ CGGCAGATGGACGTCTTCA 3’
PEAK1	5′ TTCAGCGAGGCGAAATCAGA 3’	5′ ATCGGGCACTTGACTGGGG 3’
GLO1	5′ CCGAGGATTTGGGCACATT 3’	5′ ATGCTCCTTGCTGAAGCTCAT 3’
CDK1	5′ TACCCACAGCCATGGAGGAT 3’	5′ GGGTGTCCCTAAAGCTCTGA 3’
β-actin	5′ AGACATCAGGGTGTGATGGTTGGT 3’	5′ TCCCAGTTGGTGACAATACCGTGT 3’

Table 4. 2. Body weight gain of broiler chickens housed at either 25ºC (TN) or 35ºC (HS) during the starter (days 0-10), grower (days 11-21), or finisher (days 22-35) phase.

Environment	Source[1]	Level[2]	Starter	Grower	Finisher	Total
TN-TN-TN	DLM	100	172.0^{abc}	600.6^{abcd}	1,412.1^{ab}	2,184.6^{a}
TN-TN-TN	DLM	140	168.4^{abc}	614.7^{abc}	1,417.1^{a}	2,200.2^{a}
TN-TN-TN	MHA	100	157.3^{cd}	609.5^{abc}	1,407.7^{ab}	2,174.3^{a}
TN-TN-TN	MHA	140	163.7^{abcd}	632.9^{ab}	1,257.8^{abcde}	2,054.3^{a}
HS-TN-TN	DLM	100	160.5^{abcd}	542.3^{abcde}	1,336.8^{abc}	2,039.4^{ab}
HS-TN-TN	DLM	140	164.9^{abcd}	540.0^{abcde}	1,295.7^{abcd}	2,000.4^{ab}
HS-TN-TN	MHA	100	166.8^{abcd}	551.1^{abcde}	1,290.8^{abcd}	2,008.6^{ab}
HS-TN-TN	MHA	140	158.3^{bcd}	524.7^{bcde}	1,328.1^{abc}	2,011.0^{ab}
TN-HS-TN	DLM	100	174.6^{a}	492.2^{de}	1,182.5^{bcdef}	1,849.4$_{bc}$
TN-HS-TN	DLM	140	169.0^{abc}	523.0^{bcde}	1,093.5^{defg}	1,785.4^{cd}
TN-HS-TN	MHA	100	167.7^{abcd}	480.5^{e}	1,120.7^{cdefg}	1,768.4^{cd}
TN-HS-TN	MHA	140	162.8^{abcd}	514.1^{cde}	1,055.9^{efgh}	1,732.7^{cd}
TN-TN-HS	DLM	100	174.3^{ab}	608.0^{5abc}	828.6^{h}	1,621.3^{d}
TN-TN-HS	DLM	140	170.8^{abc}	639.9^{a}	926.1^{gh}	1,736.8^{cd}
TN-TN-HS	MHA	100	164.9^{abcd}	597.5^{abcd}	966.9^{fgh}	1,729.2^{cd}
TN-TN-HS	MHA	140	151.9^{d}	600.4^{abcd}	922.3^{gh}	1,674.6^{cd}
SEM			3.2	22.2	46.9	40.5
Main effect of environment						
TN-TN-TN			165.4	614.4^{a}	1,373.6^{a}	2,153.4^{a}
HS-TN-TN			162.6	539.5^{b}	1,312.8^{a}	2,014.9^{b}
TN-HS-TN			168.5	502.5^{b}	1,113.1^{b}	1,784.1^{c}
TN-TN-HS			165.5	611.6^{a}	913.5^{c}	1,690.5^{d}
SEM			1.6	11.1	23.40	20.3
Main effect of methionine source						
DLM			169.3^{a}	570.1	1,187.8	1,927.2
MHA			161.7^{b}	563.8	1,168.8	1,894.2
SEM			1.1	7.9	16.6	14.3
Main effect of methionine level						
100			167.3^{a}	560.3	1,194.5	1,922.0
140			163.7^{b}	573.7	1,162.1	1,899.4
SEM			1.1	7.9	16.6	14.3
			Probability			
Model			<0.0001	<0.0001	<0.0001	<0.0001
Environment			0.0900	<0.0001	<0.0001	<0.0001
Source			<0.0001	0.5726	0.4191	0.1076
Level			0.0320	0.2302	0.1703	0.2697
Environment x Source			0.0246	0.6670	0.1606	0.2550
Environment x Level			0.1847	0.5045	0.3443	0.4424
Source x Level			0.3761	0.6491	0.3286	0.1489
Environment x Source x Level			0.0598	0.9077	0.2200	0.1511

[1]DLM = DL-methionine; MHA = D,L-2-hydroxy-(4-methylthio) butanoic acid

[2]100 = at-requirement methionine; 140 = above-requirement methionine

Mean values within a row with no common superscript differ significantly ($p<0.05$)

Table 4. 3. Feed intake of broiler chickens housed at either 25ºC (TN) or 35ºC (HS) during the starter (days 0-10), grower (days 11-21), or finisher (days 22-35) phase.

Environment	Source[1]	Level[2]	Starter	Grower	Finisher	Total
TN-TN-TN	DLM	100	137.9[a]	907.9[abc]	2,423.1[a]	3,468.9[a]
TN-TN-TN	DLM	140	133.8[ab]	911.4[abc]	2,439.2[a]	3,484.4[a]
TN-TN-TN	MHA	100	120.6[ab]	873.3[abcd]	2,415.3[a]	3,409.3[a]
TN-TN-TN	MHA	140	127.1[ab]	919.4[ab]	2,295.8[ab]	3,342.3[a]
HS-TN-TN	DLM	100	120.1[ab]	797.2[cde]	2,363.1[ab]	3,282.8[ab]
HS-TN-TN	DLM	140	126.2[ab]	857.1[abcde]	2,340.0[ab]	3,320.0[a]
HS-TN-TN	MHA	100	120.7[ab]	836.7[abcde]	2,347.1[ab]	3,312.3[a]
HS-TN-TN	MHA	140	118.4[ab]	808.7[bcde]	2,347.3[ab]	3,274.7[ab]
TN-HS-TN	DLM	100	127.9[ab]	791.3[de]	2,151.1[bc]	3,070.3[bc]
TN-HS-TN	DLM	140	128.1[ab]	826.0[abcde]	2,074.8[c]	3,028.9[c]
TN-HS-TN	MHA	100	125.4[ab]	889.7[abcd]	2,058.0[cd]	3,073.1[bc]
TN-HS-TN	MHA	140	121.9[ab]	742.7[e]	2,038.2[cde]	2,902.9[cd]
TN-TN-HS	DLM	100	135.8[ab]	927.5[a]	1,747.5[f]	2,809.8[d]
TN-TN-HS	DLM	140	125.6[ab]	896.9[abcd]	1,873.7de[f]	2,896.2[cd]
TN-TN-HS	MHA	100	130.8[ab]	901.7[abcd]	1,848.6[ef]	2,881.2[cd]
TN-TN-HS	MHA	140	114.9[b]	883.1[abcd]	1,780.3[f]	2,778.2[d]
SEM			4.3	23.0	38.70	42.7
Main effect of environment						
TN-TN-TN			129.8	903.0[a]	2,393.3[a]	3,426.2[a]
HS-TN-TN			123.2	824.9[b]	2,349.4[a]	3,297.5[b]
TN-HS-TN			125.8	812.4[b]	2,080.5[b]	3,018.8[c]
TN-TN-HS			126.5	902.3[a]	1,812.5[c]	2,841.3[d]
SEM			2.2	11.5	19.30	21.3
Main effect of methionine source						
DLM			129.2[a]	864.4	2,176.6	3,170.2[a]
MHA			123.5[b]	856.9	2,141.3	3,121.8[b]
SEM			1.5	8.1	13.7	15.1
Main effect of methionine level						
100			128.6[a]	865.7	2,169.2	3,163.5
140			124.1[b]	855.7	2,148.7	3,128.4
SEM			1.5	8.1	13.7	15.1
			Probability			
Model			0.0238	<0.0001	<0.0001	<0.0001
Environment			0.1910	<0.0001	<0.0001	<0.0001
Source			0.0099	0.5153	0.0725	0.0260
Level			0.0421	0.3869	0.2906	0.1048
Environment x Source			0.2020	0.8490	0.3498	0.4198
Environment x Level			0.1354	0.0528	0.4214	0.2849
Source x Level			0.5597	0.0217	0.1098	0.0067
Environment x Source x Level			0.3446	0.0035	0.0679	0.7665

[1]DLM = DL-methionine; MHA = D,L-2-hydroxy-(4-methylthio) butanoic acid
[2]100 = at-requirement methionine; 140 = above-requirement methionine
Mean values within a row with no common superscript differ significantly ($p<0.05$)

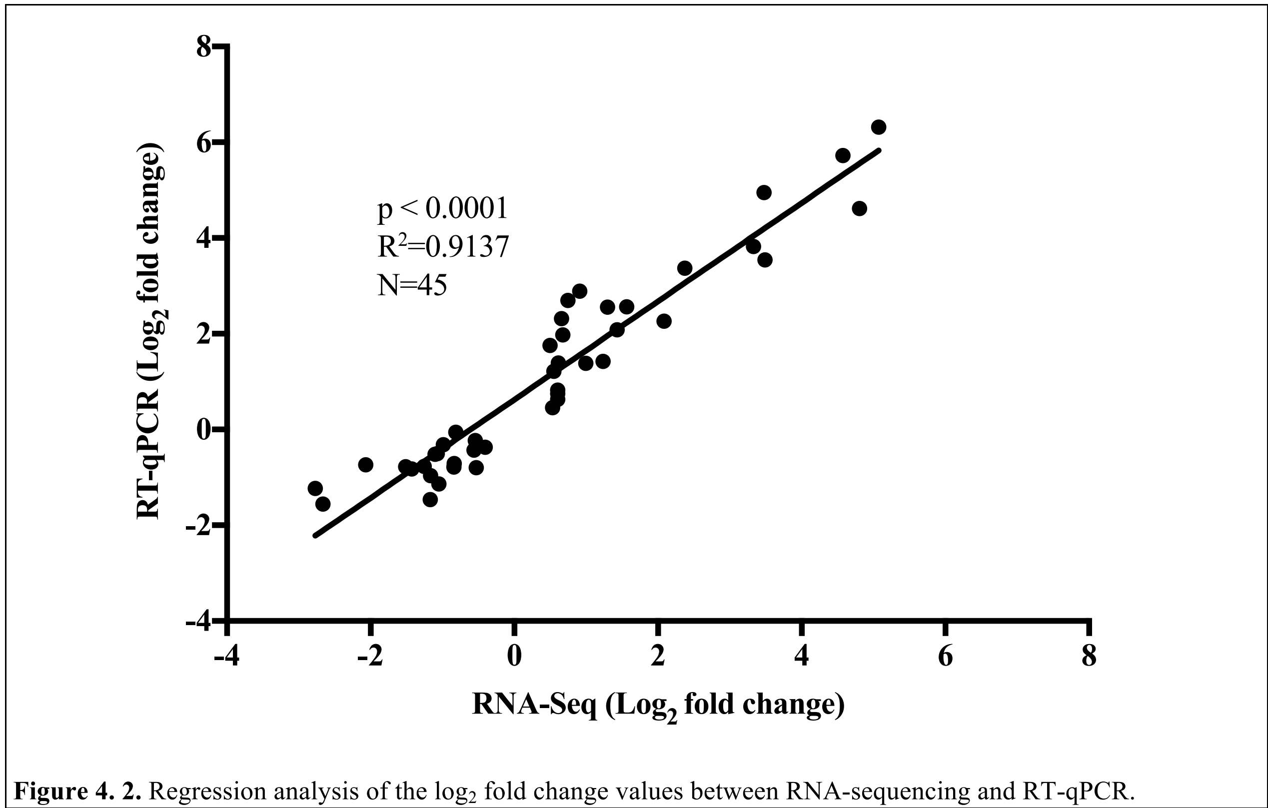

Figure 4. 2. Regression analysis of the log_2 fold change values between RNA-sequencing and RT-qPCR.

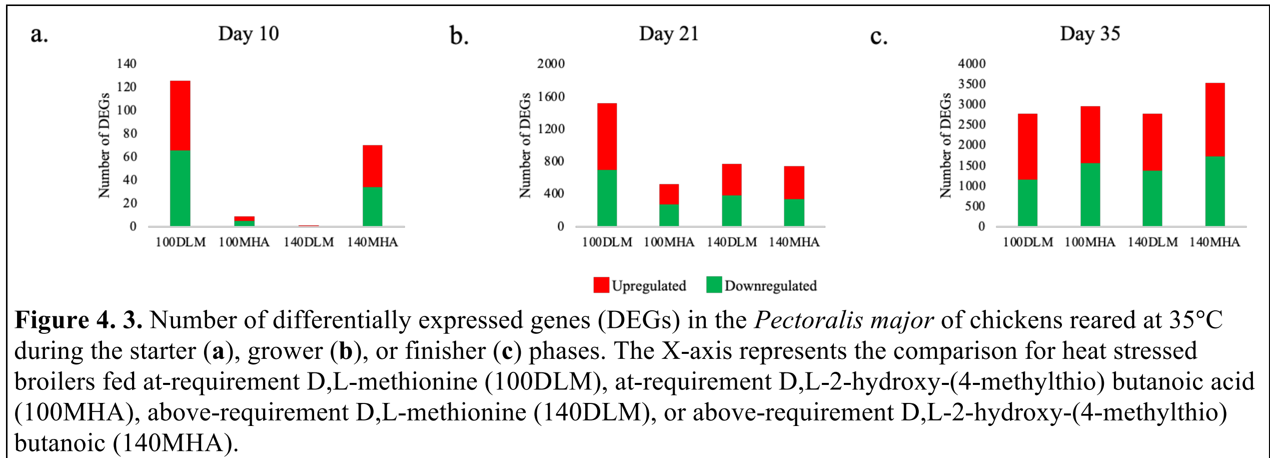

Figure 4. 3. Number of differentially expressed genes (DEGs) in the *Pectoralis major* of chickens reared at 35°C during the starter (**a**), grower (**b**), or finisher (**c**) phases. The X-axis represents the comparison for heat stressed broilers fed at-requirement D,L-methionine (100DLM), at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), above-requirement D,L-methionine (140DLM), or above-requirement D,L-2-hydroxy-(4-methylthio) butanoic (140MHA).

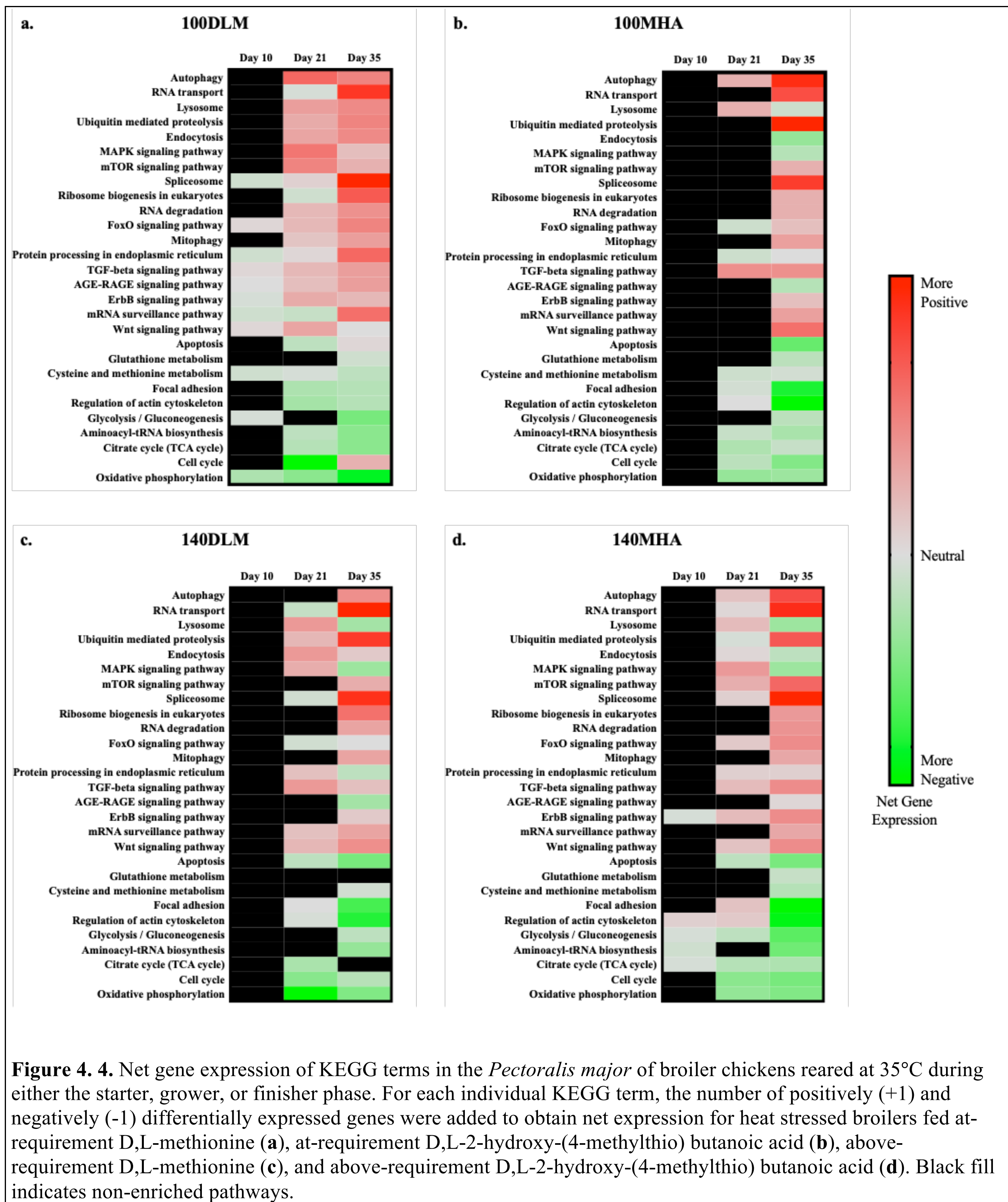

Figure 4. 4. Net gene expression of KEGG terms in the *Pectoralis major* of broiler chickens reared at 35°C during either the starter, grower, or finisher phase. For each individual KEGG term, the number of positively (+1) and negatively (-1) differentially expressed genes were added to obtain net expression for heat stressed broilers fed at-requirement D,L-methionine (**a**), at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (**b**), above-requirement D,L-methionine (**c**), and above-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (**d**). Black fill indicates non-enriched pathways.

Table 4. 4. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with at-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
TUBA8B	4.86320603	0.00294215	*GPM6A*	1.37079705	0.04210858
HIST1H4J	2.7358984	0.04519031	*PACSIN1*	1.36257099	0.01460564
APOD	2.63420723	1.95E-14	*WBP1L*	1.34959513	0.04506749
PAX3	2.63005013	0.00071716	*METTL7A*	1.31729424	0.00025415
PVALB	2.37533376	0.00469509	*CHRNG*	1.29898489	0.03091663
PRTG	2.17459803	1.96E-05	*GATA5*	1.29639204	0.03204559
SYPL1	2.08406795	0.00055036	*SV2B*	1.27866154	0.01693951
GREM1	1.9651611	0.00277436	*TINAG*	1.24678418	3.20E-11
SCARNA15	1.9374451	1.12E-08	*NSG2*	1.21764565	0.02522977
MYO7B	1.93601284	5.13E-05	*IQCA1*	1.20398835	0.04472486
FGF1	1.90452868	1.36E-07	*AGXT2*	1.19765092	0.0378201
ALDH1A1	1.84054868	0.0003128	*ATP8B3*	1.18537903	0.01693951
C7	1.66621741	0.01110407	*MYOC*	1.18073299	8.53E-05
ASB2	1.63388969	0.00687853	*Lrrn1*	1.1601647	0.0081091
CHRND	1.63249724	0.00141408	*GTSF1*	1.15769135	1.89E-05
TDRKH	1.60271304	6.16E-06	*PRKCH*	1.14359085	0.00139585
KLHL38	1.58437987	0.00500168	*RFWD2*	1.12347751	0.02557832
PPL	1.55799748	2.86E-07	*PARP6*	1.12267603	2.95E-08
GPR20	1.54079568	0.00037147	*PLA2G15*	1.1195388	8.03E-08
ASB11	1.53973821	2.56E-11	*TTC7A*	1.11655427	0.00166349
NETO2	1.52186589	0.00105749	*B4GALNT3*	1.11099787	0.0178506
SAG	1.46535788	0.0232927	*RGCC*	1.11030687	0.04519031
ATP2C2	1.43608879	0.00014552	*ANKRD1*	1.08527286	0.03111339
KCNN1	1.41118824	9.77E-06	*KLHDC8B*	1.07852008	0.00011871
SCN2B	1.40792172	1.39E-06	*ATOH8*	1.07280188	0.00050623

Table 4. 5. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with at-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
MINDY4B	-2.7204775	0.01032807	*KIF15*	-1.3639631	5.97E-05
GJD2	-2.6219209	1.96E-07	*CENPW*	-1.3609512	0.00261841
CSMD1	-2.3591647	0.04937735	*DNA2*	-1.359939	0.00062324
RGR	-2.253705	0.02302585	*UBE2C*	-1.355746	0.00039956
KCNK3	-2.0835393	0.01407218	*CDCA3*	-1.3435825	0.00284869
CDH6	-2.0402248	0.02117238	*GCH1*	-1.341003	0.00412038
DEPDC1B	-1.6854656	0.00020812	*TRAIP*	-1.3324243	0.00114267
SCNN1A	-1.6520497	4.20E-11	*ANGPTL4*	-1.3322919	0.01254958
SLC6A9	-1.6306287	0.03034335	*CDH19*	-1.3303999	0.03347925
EXO1	-1.6168934	0.00884167	*CCNE2*	-1.3168734	0.01541533
HBAD	-1.6105393	0.00721649	*CENPF*	-1.3153428	0.00126776
HSP90AB1	-1.599859	1.28E-10	*SMC2*	-1.3135646	0.01338713
ND6	-1.5206176	1.98E-09	*GRIA3*	-1.2949471	0.00011985
CDK1	-1.5121444	0.0002327	*dAK*	-1.2909942	0.00022114
SAT2	-1.505509	1.85E-15	*TOP2A*	-1.2874323	0.01623388
CENPA	-1.4979524	0.02585282	*SHCBP1*	-1.2859868	0.00068703
NUF2	-1.494751	0.00012486	*CENPM*	-1.2789379	0.02682528
NDC80	-1.4901364	0.00067567	*KIF18B*	-1.2723197	0.00191277
CEP55	-1.4618087	0.01722853	*HBAA*	-1.2709631	0.01736084
FSTL4	-1.4608971	0.0002803	*NUSAP1*	-1.2665386	0.00076074
ERCC6L	-1.4429027	0.00065275	*PLK1*	-1.2651537	0.00780433
DIAPH3	-1.4253937	0.01974767	*BIRC5*	-1.260141	0.00073355
RPL22L1	-1.3904806	6.99E-12	*KPNA2*	-1.2458284	0.00055047
CENPI	-1.376578	0.00029412	*NEK2*	-1.227652	0.0001941
MELK	-1.3760127	0.0031756	*NCAPG2*	-1.2162929	0.00067822

Table 4. 6. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the grower phase whose diets were supplemented with at-requirement DLM.

KEGG Term	Associated Genes
Upregulated	
Autophagy	*ATG101, ATG14, ATG16L2, ATG2A, ATG9A, BNIP3, HRAS, IRS2, LAMP1, MTMR3, NRBF2, PIK3R4, PRKCQ, RAF1, RB1CC1, RPTOR, RUBCN, SMCR8, STK11, STX17, SUPT20H, TRAF6, TSC2, UVRAG*
Endocytosis	*ARFGEF1, BF1, CHMP6, CHMP7, EEA1, EPN3, GIT1, HRAS, IGF2R, KIAA0196, LDLRAP1, NEDD4, RABEP1, RNF41, SMURF2, TRAF6, VPS25, VPS37C, WASHC4*
MAPK signaling pathway	*CACNG1, DUSP3, ECSIT, ERBB4, FGF1, FGF22, HRAS, INSR, LOC101748851, MAPKAPK2, MAPT, MKNK2, NFKB1, PPM1A, RAF1, SOS1, SOS2, TAB2, TAOK3, TRAF6*
mTOR signaling pathway	*DEPDC5, DVL1, FNIP1, FZD2, HRAS, INSR, LAMTOR1, RAF1, RPTOR, SOS1, SOS2, STK11, TSC2, WDR24, WNT5B*
Lysosome	*ACP2, AP1B1, ENTPD4, GGA1, GLA, GNS, IGF2R, LAMP1, MCOLN1, NPC1, PLA2G15, SMPD1, SORT1, SUMF1*
Downregulated	
Cell cycle	*ANAPC13, BUB1, BUB1B, BUB3, CCNA2, CCNB1, CCNE2, CDC20, CDC25A, CDC45, CDC7, CDK1, CDK6, CDKN2C, CHEK1, E2F1, ESPL1, HDAC2, MCM2, MCM3, MCM5, MCM6, ORC1, PLK1, RAD21, RBL1, WEE1, YWHAG, YWHAQ, YWHAZ*
Cellular senescence	*CCNA2, CCNB1, CCNE2, CDC25A, CDK1, CDK6, CHEK1, E2F1, MYBL2, PPID, PPP1CB, RBL1, SLC25A4, SLC25A5, TRAF3IP2, TRPM7*
Regulation of actin cytoskeleton	*ACTN1, ARHGEF12, ARPC4, ARPC5, CXCL12, CYFIP1, DIAPH3, ITGA2, ITGA6, LPAR4, MYLK, PDGFA, PFN2, PPP1CB, ROCK1, TMSB4X*
Oocyte meiosis	*ANAPC13, AURKB, BUB1, CCNB1, CCNE2, CDC20, CDK1, CPEB2, ESPL1, PLK1, PPP1CB, YWHAG, YWHAQ, YWHAZ*
Focal adhesion	*ACTN1, ARHGAP5, CHAD, COL4A1, CTNNB1, ITGA2, ITGA6, MYLK, PDGFA, PPP1CB, RAP1B, ROCK1*

Table 4. 7. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with at-requirement MHA.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
ALDH1A1	2.25212343	0.02230888	*EYA1*	1.0934952	0.00011312
SLC11A1	2.09164822	0.00663473	*TDRKH*	1.08908225	0.01243017
PVALB	2.08501873	0.04825888	*ASPA*	1.05804759	0.03938207
AQP4	1.87971668	1.25E-05	*PTN*	1.04492554	0.01606704
ATP2C2	1.56641827	1.74E-05	*TTC7A*	1.02027369	0.00364138
SYPL1	1.5208665	1.02E-05	*DFNA5*	0.98378238	0.0001891
ASB2	1.50882623	0.0007971	*MYOC*	0.96478624	0.03744015
FSHR	1.50094545	0.04995812	*ZDHHC4*	0.96249605	0.02638618
SPDYA	1.4854581	0.01920388	*GRIN3B*	0.94792665	0.00020153
GREM2	1.47139603	0.00023355	*METTL7A*	0.93563446	0.00673207
FHL2	1.46859315	0.00878484	*FGF1*	0.9224216	0.03919228
ROS1	1.43757643	0.01489503	*IL11RA*	0.9198441	8.13E-10
MYO7B	1.39748737	4.34E-05	*SYNJ2*	0.89919046	0.0402988
ISM1	1.3703343	0.0052835	*PARP6*	0.89699968	3.54E-05
HPSE2	1.35630076	0.00895877	*GPR157*	0.8822329	1.10E-07
LRRC30	1.26149386	0.01552514	*ITGB4*	0.87808036	0.03968431
IQCA1	1.22859966	0.03368083	*IRAK1BP1*	0.87167634	0.02159769
GRAMD2A	1.22800983	6.58E-10	*CHST9*	0.86404144	0.03120284
HSF2BP	1.21605256	2.30E-08	*TAPBPL*	0.84400265	0.00125239
ZBTB16	1.20211606	0.01144583	*ASB11*	0.83531757	0.00878484
CETP	1.17051518	0.00047788	*SMAD6*	0.83341363	0.00101766
DKK3	1.14998769	8.20E-06	*OCLN*	0.83159959	0.01910915
APOD	1.14262635	0.00364089	*CADM1*	0.83120419	0.00010431
COL8A2	1.11555337	0.03775562	*B9D1*	0.82806256	0.00293488
TMEM233	1.10011863	0.01920388	*ABRA*	0.81859654	0.00665301

Table 4. 8. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with at-requirement MHA.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	-4.5763449	0.0402988	*TUSC3*	-0.9717321	0.01558128
CHAC1	-2.5844617	0.02251687	*PRKX*	-0.9411785	0.04261962
CPEB2	-2.1902842	1.85E-05	*NDC80*	-0.928445	0.01437052
JCHAIN	-2.0220928	0.04892453	*CENPW*	-0.9260779	0.03250487
KPNA7	-1.5675996	0.03629017	*KPNA2*	-0.887603	0.00821503
LMCD1	-1.5301794	3.33E-05	*MOXD1*	-0.8845311	0.02512308
FGD4	-1.5036238	0.01510563	*SMC2*	-0.8823946	0.02463502
GCH1	-1.485357	2.47E-07	*NPM3*	-0.873485	0.01209868
SMPX	-1.3704692	0.01156626	*NR2E1*	-0.8487502	0.02898899
HBAD	-1.331637	0.02512308	*ACER3*	-0.8485049	0.04024444
SLC25A30	-1.2672405	8.69E-06	*TRAIP*	-0.8483107	0.02960298
ND6	-1.2499276	3.84E-15	*PPP1R12A*	-0.8469442	5.47E-05
FAM196A	-1.234636	0.00113923	*ABCC1*	-0.8456066	4.93E-08
KIF11	-1.1217047	0.00085902	*CDK1*	-0.8429746	0.0423789
HBAA	-1.0866002	0.00020067	*IDH3A*	-0.8379986	2.21E-05
FRS2	-1.0822819	0.00391831	*DNAJA1*	-0.8214568	9.37E-05
RPL22L1	-1.0771771	0.00032372	*PLK1*	-0.8192531	0.03175846
DIAPH3	-1.0279178	0.03516494	*UBE2C*	-0.8027623	0.00885602
MELK	-1.0233178	0.03934458	*NAP1L4*	-0.7897027	5.76E-07
PAQR7	-1.0199198	0.04877678	*MT-ND2*	-0.7879535	0.00206059
MTR	-1.0196294	2.17E-06	*DUPD1*	-0.7875979	0.00062427
GLS	-0.9930781	0.00014818	*LMNB2*	-0.7765983	0.00263607
ABCC4	-0.9900062	0.00015001	*FREM2*	-0.7715866	0.04874664
DOCK2	-0.9852992	0.00995744	*FAM126B*	-0.7714574	0.04877678
GTSE1	-0.982355	0.0083982	*SLC4A4*	-0.7640864	0.00700481

Table 4. 9. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the grower phase whose diets were supplemented with at-requirement MHA.

KEGG Term	Associated Genes
Upregulated	
Neuroactive ligand-receptor interaction	*CHRNA4, CHRND, FSHR, GRIN3B, THRA*
Purine metabolism	*ADCY9, ENTPD4, GART, PDE4B*
TGF-beta signaling pathway	*ACVR1B, E2F4, GREM2, NEO1, SMAD6*
Various types of N-glycan biosynthesis	*B4GALNT3, B4GALT2, CHST9, ST3GAL3*
Autophagy	*ATG9A, CTSB, NRBF2, PIK3R4*
Downregulated	
Oxidative phosphorylation	*ATP5C1, ATP5F1, ATP5G1, COX6A1, ND1, ND6, NDUFA10, NDUFA12, NDUFS3, NDUFV2, UQCRB, UQCRC1*
Oocyte meiosis	*CCNB1, CDK1, CPEB2, ITPR3, PLK1, PPP1CB, PPP3CB, SLK, YWHAG*
Protein processing in endoplasmic reticulum	*CANX, DNAJA1, HSP90AB1, HYOU1, TUSC3, XBP1*
Cell cycle	*BUB1B, CCNA2, CCNB1, CDK1, MCM3, PLK1, YWHAG*
Cellular senescence	*CCNA2, CCNB1, CDK1, ITPR3, PPP1CB, PPP3CB, SLC25A4*

Table 4. 10. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with above-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
PVALB	3.33078031	1.92E-14	*ISM1*	1.1674138	0.01251989
PDK4	2.65969257	0.02563961	*UPP2*	1.13747379	0.006003
BMP5	2.32641742	1.33E-05	*BBOF1*	1.12315762	0.04968292
MIM1	2.08839746	0.02754408	*CHODL*	1.11662732	0.03862578
SLC47A2	1.93621852	0.00104035	*CHST9*	1.10902966	0.01329856
CBLN1	1.89891161	0.01304983	*MAPK13*	1.09595268	0.01510338
RGCC	1.86421875	0.00034375	*COL19A1*	1.08233868	0.001721
HS3ST2	1.69297929	0.00507429	*HSF2BP*	1.06992183	0.00654421
METTL7A	1.6341109	0.0008752	*SLC9A7*	1.06534935	0.00040501
CHRND	1.61770105	0.00014108	*PARP16*	1.05101683	0.0093617
AQP4	1.54685978	0.04437735	*TMEM233*	1.03269849	0.00259958
ZBTB16	1.49361759	0.00015211	*KCNN1*	1.01782385	0.02122846
GREM1	1.48113005	0.00150929	*LHFPL4*	1.00733492	0.00426288
CHRNG	1.42926026	0.04267159	*COMP*	1.00329941	0.00013224
ATOH8	1.39656962	8.45E-09	*WBP1L*	0.97159522	7.53E-06
SLC41A3	1.38486467	0.01385525	*MATK*	0.96924954	0.00825104
CETP	1.36469239	0.02910856	*SLC18A1*	0.96754564	6.64E-05
DKK3	1.3156867	0.00011793	*IL13RA2*	0.96416282	0.02693894
IGSF11	1.26848089	0.02170753	*COL8A2*	0.95893063	0.02112776
SRD5A2	1.23316545	0.00771557	*ITGB4*	0.94610015	0.03156496
C7	1.21880105	5.29E-05	*TIGD5*	0.92176711	0.03086571
ADAMTS13	1.21099122	7.53E-06	*TSPAN15*	0.91713306	0.0004266
SCN2B	1.20788799	2.36E-13	*SIRT4*	0.90071626	0.00654421
PRTG	1.19223818	0.01901905	*TDRKH*	0.89837226	0.00330415
FGF1	1.17549759	9.07E-06	*PDLIM3*	0.8951511	0.00318748

Table 4. 11. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with above-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
GJD2	-2.8925815	3.42E-15	*CDK1*	-1.10431	3.44E-05
FOS	-2.332284	0.00050678	*HSP90AB1*	-1.10346	0.00012046
RGS1	-2.0240426	0.00591098	*NEK2*	-1.095029	0.00099018
APOLD1	-1.9440713	0.00147942	*SLC16A6*	-1.0949646	0.00104035
CDH6	-1.9418755	0.00839066	*TK1*	-1.0900166	7.61E-07
SBK3	-1.8132772	0.00092453	*HSPB7*	-1.082836	0.01941471
FOSB	-1.7567386	0.03131594	*CENPM*	-1.0799869	0.01014792
WNT6	-1.7420423	0.04363402	*DNA2*	-1.0744186	0.00056411
SLC7A11	-1.616443	0.00831024	*KIF2C*	-1.0743478	1.55E-05
ND6	-1.5653266	1.89E-30	*NDC80*	-1.0713878	0.00042088
ELFN1	-1.5281853	0.01650571	*ADAMTS1*	-1.0431653	0.00157908
EGR1	-1.4325453	0.04137334	*PLK1*	-1.0298363	0.00039994
TRIM27.1	-1.4272065	0.00790364	*MXD3*	-1.0297702	0.00700888
GRIA3	-1.3985394	4.90E-05	*KIF11*	-1.0267877	0.00382452
MELK	-1.3207816	0.00049452	*CSTA*	-1.0163304	0.0398544
CEP55	-1.3081866	0.02115206	*SMC2*	-1.010407	0.00012472
GATM	-1.3013097	0.0052535	*CDYL2*	-1.008721	0.00019022
KCNU1	-1.2069785	0.01184106	*CTGF*	-1.0082225	0.01123179
UBE2C	-1.1933117	4.01E-07	*NUF2*	-0.987104	0.00016239
KIF18B	-1.1925094	3.94E-05	*GEM*	-0.9766948	4.03E-06
PAQR9	-1.1909478	0.00316856	*TOP2A*	-0.9642605	4.06E-05
CPEB2	-1.1732228	0.00010429	*KPNA2*	-0.9616895	0.00106148
SHCBP1	-1.1221503	0.01094773	*DMTN*	-0.9570595	0.00414313
CENPF	-1.1094044	0.000201	*GCH1*	-0.9562527	0.02041277
GINS2	-1.1069385	0.00273545	*CNTN4*	-0.9492948	0.03703616

Table 4. 12. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the grower phase whose diets were supplemented with above-requirement DLM.

KEGG Term	Associated Genes
Upregulated	
Protein processing in endoplasmic reticulum	*AMFR, CRYAB, MBTPS1, RPN1, SEC24A, TRAF2, UBE2J1*
MAPK signaling pathway	*FGF1, MAPK13, MKNK2, NFKB2, PDGFD, PDGFRA, TGFB3, TRAF2*
Ubiquitin mediated proteolysis	*DET1, FBXW11, KEAP1, NEDD4, NHLRC1, RFWD2, UBE2J1*
Endocytosis	*CHMP6, EEA1, EHD3, IGF2R, NEDD4, PDGFRA, RNF41, WASHC4*
TGF-beta signaling pathway	*ACVR1B, AMH, BMP5, GREM1, SMAD1, SMAD6, SMAD9, TGFB3*
Downregulated	
Oxidative phosphorylation	*ATP5B, ATP5C1, ATP5F1, ATP5G1, ATP5G3, ATP5H, ATP5I, ATP5J2, ATP6, COX4I1, COX5A, COX6A1, COX7A2, ND1, ND5, ND6, NDUFA10, NDUFA12, NDUFA6, NDUFB3, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFV2, PPA1, UQCRB, UQCRC1, UQCRC2, UQCRFS1*
Cell cycle	*ANAPC13, BUB1, BUB1B, CCNB1, CDC20, CDK1, CDK2, E2F1, E2F3, ESPL1, MCM2, MCM3, MCM5, PLK1, RAD21*
Oocyte meiosis	*ANAPC13, BUB1, CCNB1, CDC20, CDK1, CDK2, CPEB2, ESPL1, PLK1*
Cardiac muscle contraction	*ACTC1, COX4I1, COX5A, COX6A1, COX7A2, UQCRB, UQCRC1, UQCRC2, UQCRFS1*
Progesterone-mediated oocyte maturation	*ANAPC13, BUB1, CCNB1, CDK1, CDK2, CPEB2, HSP90AB1, PLK1*

Table 4. 13. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with above-requirement MHA.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
PVALB	3.48821205	0.00619659	*KL*	0.9294426	0.03947302
BMP5	2.80307352	2.89E-10	*SLC41A3*	0.92255499	5.51E-05
PDE10A	2.41582673	0.03099181	*PLA2G15*	0.9222576	0.02101509
UTS2R	2.15277049	0.00826265	*LINGO1*	0.90523925	0.00130317
CETP	1.80860202	1.22E-06	*GRAMD2A*	0.88509145	5.58E-05
TG	1.80652748	0.01078812	*MID1IP1*	0.83988842	9.61E-05
METTL7A	1.7397971	0.01758704	*NCMAP*	0.82814514	0.00698612
SLC47A2	1.7189794	0.00126449	*CD38*	0.82603206	0.00133598
FGF1	1.68258897	5.01E-11	*SAMD11*	0.82449184	0.01042582
ISM1	1.63210488	0.00027009	*TMEM201*	0.82332003	0.00756451
ASB2	1.5775171	0.00452221	*AQP4*	0.82195679	0.01055183
RGCC	1.53324749	8.23E-09	*SYNE3*	0.81755244	0.01381973
ZBTB16	1.52928987	0.02728633	*SVEP1*	0.81656508	6.36E-08
IGSF11	1.41306702	0.00374501	*WNT5B*	0.81517337	0.00755222
RGS11	1.26191865	0.01469811	*HABP2*	0.81305397	0.01187098
ASB11	1.17059196	9.85E-09	*EYA1*	0.78113889	0.01670106
ADAMTS13	1.14792242	3.43E-07	*WDCP*	0.77698134	0.01663687
KCNN1	1.13962954	0.02024757	*TINAG*	0.77458457	0.02215618
MYOC	1.13732018	0.02502897	*CYB561A3*	0.77259174	0.00715624
ATOH8	1.07932502	0.00331811	*ITGB4*	0.77138854	0.0099373
PRTG	1.06985719	0.04046396	*GALNT15*	0.75939143	0.03214695
TMEM233	1.05802023	0.02943976	*LZTS2*	0.75850613	3.23E-05
SCN2B	1.01900876	0.00056355	*RAMP1*	0.753601	0.00431049
C7	0.93480018	0.01351607	*EEPD1*	0.75327079	1.32E-08
TDRKH	0.93312561	0.02003153	*USF3*	0.74530943	0.00052859

Table 4. 14. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the grower phase whose diets were supplemented with above-requirement MHA.

Gene	Log2 fold change	Adjusted P-value	Gene	Log2 fold change	Adjusted P-value
MIM1	-3.50493	0.00752105	*PAQR9*	-1.0771794	0.01988538
RGR	-3.0042393	7.67E-05	*CENPL*	-1.0771645	0.01141873
CSRP3	-1.944492	0.01591429	*VDR*	-1.0753684	0.03795658
ELFN1	-1.5168792	0.01279958	*HSP90AB1*	-1.0741519	6.08E-05
PAQR7	-1.375369	2.58E-05	*UBE2C*	-1.0580027	0.00995702
MELK	-1.3321405	0.00068716	*ERCC6L*	-1.0431514	0.0016082
GJD2	-1.2574073	0.04416255	*CEP55*	-1.0396039	0.0145392
EPB42	-1.2338153	0.03972266	*BUB1*	-1.0188356	0.00281021
BIRC5	-1.215653	5.42E-07	*NEK2*	-1.0142681	0.00142313
Enpp2	-1.2056545	8.27E-07	*NDC80*	-1.0134833	0.03074104
SCNN1A	-1.1934518	0.00011592	*F13A1*	-1.011225	0.01163898
GINS2	-1.1744628	0.00057535	*PLK1*	-1.0102634	0.00152568
TRAIP	-1.1634525	0.00203656	*KPNA2*	-1.0087212	8.03E-05
CPEB2	-1.1611533	0.00798646	*TOP2A*	-0.9996416	0.00074607
SFXN5	-1.1405463	0.04566719	*KIF15*	-0.9941818	0.02321589
APCDD1L	-1.1227385	0.00376331	*ARHGEF39*	-0.9910677	0.03637504
GPR158	-1.1122689	0.04732675	*dAK*	-0.9883452	0.00192557
SHCBP1	-1.1107834	0.01140035	*CDK1*	-0.9881082	0.0049921
SLC6A9	-1.1102191	0.0142309	*PDCL2*	-0.977881	0.03459841
ADRA2A	-1.0911392	0.012015	*HIST1H2B5*	-0.9762222	0.01170327
SMC2	-1.0875674	0.01128021	*GRIA3*	-0.9715132	0.03150732
DTL	-1.0874052	0.00603703	*CENPI*	-0.9623265	0.00308683
CSTA	-1.0809688	0.00335152	*KIF2C*	-0.9377349	0.00563339
DNA2	-1.0803814	0.00083397	*MCM3*	-0.9352298	0.00011563
TK1	-1.0773168	0.0011529	*CENPF*	-0.9222822	0.01140035

Table 4. 15. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the grower phase whose diets were supplemented with above-requirement MHA.

KEGG Term	Associated Genes
Upregulated	
MAPK signaling pathway	*CACNA1S, CACNG1, DUSP3, ERBB4, FGF1, INSR, LOC101748851, MAPT, MKNK2, SOS1, TAB2*
Ribosome	*MRPL34, MRPS21, RPL12, RPL30, RPL3L, RPL8, RPLP0, RPS17, RPS20, RPS26, RPS29*
Focal adhesion	*ARHGAP35, COL4A5, COMP, ITGA7, ITGB4, LAMA2, SOS1*
Lysosome	*ARSG, IGF2R, LAMP1, NPC1, PLA2G15, SCARB2, SORT1*
Autophagy	*ATG9A, BNIP3, LAMP1, PRKCQ, SH3GLB1, SUPT20H*
Downregulated	
Cell cycle	*BUB1, BUB1B, CCNB1, CDC20, CDK1, CDKN2C, E2F1, MCM2, MCM3, PLK1, RAD21, TTK, YWHAZ*
Oxidative phosphorylation	*ATP5C1, ATP5G3, ATP5J, COX6A1, COX7A2, ND6, NDUFA8, NDUFV2, PPA1, UQCRC1*
Oocyte meiosis	*AURKB, BUB1, CCNB1, CDC20, CDK1, CPEB2, PLK1, PPP2CA, YWHAZ*
Vascular smooth muscle contraction	*ARHGEF12, CALD1, GUCY1A3, GUCY1B3, MYL6, MYLK, PPP1R12A*
Progesterone-mediated oocyte maturation	*AURKB, BUB1, CCNB1, CDK1, CPEB2, HSP90AB1, PLK1*

Table 4. 16. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with at-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
NOG	5.92163134	0.03043647	*XKR7*	4.24021636	0.00481293
CLDN3	5.51983577	3.43E-07	*RIPPLY2*	4.18820808	0.00854174
PTX3	5.37755464	0.00037394	*UTS2R*	4.17223761	0.0007125
SLC4A9	5.3035271	0.01331199	*TERC*	4.04093148	0.04189993
HTR3A	5.20708309	0.01268589	*VRTN*	4.0248822	0.0007535
SLC15A1	5.0583843	0.03935437	*SYPL1*	3.96868098	1.48E-11
FKBP5	4.91192301	2.49E-14	*FoxN4*	3.87871124	0.01734684
PMP2	4.85463314	5.58E-05	*ATP10B*	3.81542722	0.0130309
GPNMB	4.80951374	0.04212351	*U11*	3.81115063	0.00117418
PVALB	4.80446519	5.00E-11	*CASP17*	3.80863103	0.00771195
NKX6-1	4.7956045	0.00142547	*Pax-2*	3.74506047	0.0240873
FAT2	4.78106443	0.00074054	*NXNL1*	3.74050011	4.95E-06
CSTA	4.75946189	0.00037041	*TBR1*	3.72120999	0.04884161
ZBTB16	4.75852416	3.75E-10	*PADI1*	3.65065819	0.00933979
FAM166A	4.71526517	0.01185472	*MAP7*	3.64432987	0.00366633
SLC2A2	4.67591684	0.02339515	*U1*	3.6257903	0.00067013
ATP6V0D2	4.62978378	0.00038801	*NEU4*	3.60680021	0.04810346
SLC38A8	4.61554805	0.01761963	*IRS2*	3.60208732	4.85E-05
PDK4	4.61240521	0.00089217	*ADAMTS4*	3.57870916	0.00111553
ARTN	4.57645801	0.01655609	*SCG2*	3.5457445	0.00253404
APOA5	4.46355091	0.00011408	*C9orf172*	3.53453478	0.00043926
VGLL1	4.44203657	0.00203771	*ELF3*	3.46947085	0.00037853
APOA4	4.42594511	0.00075228	*TMIGD1*	3.46743846	0.03241057
TBPL2	4.35658725	0.01733673	*CHRM5*	3.41260428	0.04539521
RRH	4.30600438	0.04023669	*FGF19*	3.39702642	0.02406272

Table 4. 17. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with at-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	-5.2180813	0.00096124	*DUPD1*	-2.8389712	0.00026831
MARCH11	-4.8405526	0.00028194	*ALB*	-2.8084867	0.0005184
NXPH1	-4.226813	0.01747538	*NTF3*	-2.7896948	0.0089119
GAP43	-4.1324681	1.39E-05	*EGFL6*	-2.7715791	0.00039204
FMO4	-4.1087928	0.00030049	*KRT19*	-2.7647255	0.02774823
CSMD1	-3.9595035	7.66E-07	*JCHAIN*	-2.7550782	2.08E-07
IRX6	-3.8331235	0.01087244	*LDLRAD1*	-2.7249068	2.98E-09
RGR	-3.7097149	0.00690864	*GFRA2*	-2.6828122	1.34E-05
SBK2	-3.7089839	2.25E-06	*MCEE*	-2.6747929	1.43E-15
SLITRK1	-3.528092	0.00746268	*PNOC*	-2.6719875	0.00128879
SLC7A11	-3.4432752	7.14E-05	*EPYC*	-2.6446943	0.04898115
TSPAN8	-3.2871451	0.02287695	*SCNN1A*	-2.6416308	6.28E-09
MBL2	-3.2366362	0.02497539	*WNT2*	-2.6013947	0.01587111
THBS4	-3.1906261	2.82E-15	*ESM1*	-2.5901229	0.00012303
KCNU1	-3.1747793	1.08E-12	*PHGDH*	-2.5845957	0.0030308
LRIT1	-3.1543536	0.00488926	*TNIP3*	-2.5820843	0.03901865
LRRC19	-3.1251786	0.01263339	*TFCP2L1*	-2.557163	4.01E-05
PCK1	-3.0537384	0.02206163	*C14orf37*	-2.5409337	0.01781431
PAH	-3.0012566	0.018274	*DCUN1D5*	-2.5270677	8.69E-05
GSTZ1	-2.9940962	0.00020296	*GATM*	-2.4847546	0.00088222
SEMA3E	-2.9727569	0.00377307	*FSTL4*	-2.4630352	0.00331469
WNT16	-2.9166472	0.00036847	*CYP11A1*	-2.4106071	0.00013206
APLNR	-2.9147122	4.15E-06	*ASNS*	-2.3929574	1.83E-05
CA2	-2.9029938	8.06E-10	*FGFR2*	-2.367087	8.02E-05
INSC	-2.8671849	0.00054701	*ADAM19*	-2.362	0.00075228

Table 4. 18. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the finisher phase whose diets were supplemented with at-requirement DLM.

KEGG Term	Associated Genes
Upregulated	
Endocytosis	*ARFGAP1, ARFGEF1, ARPC1A, ARPC5L, CHMP5, CHMP6, EEA1, HGS, HRAS, IGF2R, KIAA0196, PARD6B, RAB22A, RAB35, RAB4A, RABEP1, RNF41, SNF8, SPG20, STAM2, VPS25, VPS26A, VPS37C, VPS4B, WASHC4, WIPF2*
FoxO signaling pathway	*AKT1, CAT, CDKN1B, CREBBP, CSNK1E, FBXO32, FOXO3, FOXO4, GADD45A, GADD45B, GRB2, HRAS, INSR, IRS2, MAPK13, NRAS, PIK3CA, PRKAG2, S1PR4, SIRT1, SKP2, SOS1, STAT3, STK4, USP7*
RNA transport	*CASC3, CLNS1A, DDX20, EIF2S1, EIF3B, EIF3H, EIF4A3, EIF4E2, EIF5, GEMIN4, GEMIN5, NUP107, NUP155, NUP54, NUP58, NUP88, NUP98, NXT2, PABPC1, PYM1, RNPS1, RPP30, SUMO3, TGS1, TPR, TRNT1, UBE2I, UPF1, XPO5, XPOT*
Protein processing in endoplasmic reticulum	*ATF4, CALR, CRYAA, DERL2, DNAJB12, EIF2S1, HSP90AA1, HSPA4L, HSPH1, MOGS, RAD23B, RPN1, SEC24A, SEC24B, SEC24C, SEC62, SSR2, STUB1, TRAF2, UBE2D2, UBE2G2, UBE2J1, UBE2J2, UBE4B, UBXN6, UGGT1*
Spliceosome	*AQR, CCDC12, CDC40, CHERP, CRNKL1, DDX23, DHX38, DHX8, EFTUD2, EIF4A3, HNRNPU, NWD1, PPIH, PPIL1, PRPF3, PRPF4, PRPF40A, PRPF6, PRPF8, SF3A2, SF3A3, SF3B4, SF3B6, SMNDC1, SNRNP200, SNRNP27, SNRPA1, SNU13, SNW1, SRSF10, U2AF1*
Downregulated	
Oxidative phosphorylation	*ATP5B, ATP5C1, ATP5F1, ATP5G1, ATP5G3, ATP5I, ATP5J, ATP5J2, ATP6, ATP6V1D, COX5A, COX6A1, ND1, ND5, ND6, NDUFA10, NDUFA12, NDUFA5, NDUFS1, NDUFS3, NDUFS6, NDUFV2, UQCRB, UQCRC1, UQCRC2, UQCRFS1, UQCRQ*
Calcium signaling pathway	*ATP2B2, CACNA1H, CAMK2A, CXCR4, GNAQ, MYLK, NOS1, NOS2, P2RX7, PDGFRB, PHKA1, PHKG1, PLCD3, PLCG1, PPP3CB, STIM1*
Cell adhesion molecules (CAMs)	*ALCAM, BLB1, CD99, CDH5, CNTNAP1, DMB2, ESAM, ITGB1, JAM3, NCAM1, NECTIN3, NRXN1, PTPRF, PTPRM*
Vascular smooth muscle contraction	*CALD1, GNAQ, GUCY1A3, GUCY1B3, KCNU1, MRVI1, MYLK, PPP1R12A, PRKCH, RAMP1, RAMP2, RAMP3, ROCK2*
Pyruvate metabolism	*ACACA, ACAT2, ACSS2, ALDH7A1, DLAT, GLO1, GRHPR, LDHD, MDH1, MDH2, ME1, ME3, PCK1, PDHB, PKLR*

Table 4. 19. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with at-requirement MHA.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
FAM135B	4.79058166	0.00220459	*PLP1*	2.21272754	0.00014546
PVALB	4.57292507	2.08E-14	*CXR*	2.18581235	0.00694834
ZBTB16	3.88639621	1.31E-15	*GMEB2*	2.16637971	4.82E-21
U11	3.87470758	0.00095778	*ADMP*	2.16364449	0.03568702
SLC26A4	3.52225305	0.01002511	*WBP1L*	2.15685864	1.50E-06
SYPL1	3.48089241	2.75E-12	*MPZ*	2.12543868	0.00439302
PDK4	3.47497476	0.00099429	*NOXRED1*	2.10324234	0.003207
FETUB	3.40410135	0.03037054	*CFAP61*	2.08991584	0.01721703
GPR182	3.20205791	0.00061371	*PRTG*	2.04612211	1.57E-08
CLDN3	3.13882161	0.00428403	*KLF9*	1.99881708	9.21E-05
SP8	3.12497784	0.02540301	*MYOC*	1.99650146	5.07E-08
BRSK2	3.09536341	0.00563886	*RHOBTB3*	1.97589044	0.02143969
TRPC3	2.99691472	0.0003238	*TINAG*	1.97030366	3.44E-05
FKBP5	2.93879303	1.43E-07	*MSMP*	1.96712994	0.02540301
RXFP3	2.91713379	0.02013008	*NXNL1*	1.96203963	0.00794854
U1	2.8645058	0.00012974	*RND1*	1.95810234	1.84E-06
FGF1	2.75829337	9.89E-10	*SLC35F4*	1.87466926	0.04329593
PCSK4	2.52889054	0.0025642	*SPACA9*	1.84464798	0.02683816
PRSS56	2.47567021	0.03613371	*DIRAS2*	1.84404143	0.01747667
ROS1	2.39653547	0.00253945	*CEBPD*	1.8393782	1.23E-11
HPCAL4	2.38222414	0.00011748	*PLA2G15*	1.79485606	1.74E-07
GPM6A	2.36248121	0.01672656	*UTS2R*	1.78730658	0.00395378
ASB11	2.33668974	1.91E-08	*CHRNB2*	1.77159074	0.0007942
GPR83	2.26736462	0.00625465	*TERT*	1.75944547	0.0163133
SBK3	2.25693442	6.36E-05	*KLHL38*	1.72663464	0.03748131

Table 4. 20. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with at-requirement MHA.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
MMP27	-6.0315498	2.29E-05	*HTR3A*	-4.0154485	0.02848144
MLANA	-5.929532	0.01675698	*ALB*	-3.9871319	4.42E-05
DCDC2	-5.5173397	0.00027613	*RASL10B*	-3.9177659	0.00170372
MMP13	-5.3747406	0.01166894	*MAP7D2*	-3.8313215	0.00589263
CCL19	-5.321126	1.75E-07	*MDK*	-3.8275727	0.00629931
MYL10	-5.2760815	9.26E-05	*PHOX2B*	-3.7691574	0.02072625
KCNS2	-5.0357162	0.00919248	*BAALC*	-3.7366625	6.02E-08
VEPH1	-4.8956935	0.02220898	*DSG2*	-3.6057693	0.00512795
CALB2	-4.8394932	0.00059493	*MYBPC1*	-3.595325	0.02496916
NELL1	-4.7910472	0.03124703	*PHGDH*	-3.5670719	8.71E-06
LHFPL3	-4.7838562	0.0324376	*UPK1B*	-3.5001902	0.02141619
TPH2	-4.7484041	0.01750613	*SYNDIG1L*	-3.4485589	0.00125166
RSPO4	-4.7179854	0.01507802	*MASP1*	-3.3886444	0.00111572
GDPD4	-4.6357419	0.00075129	*TCF21*	-3.3776542	0.018694
BCAN	-4.5379684	0.000511	*FAM3B*	-3.3740613	0.00259099
HTR4	-4.4724471	0.01692694	*JCHAIN*	-3.367621	1.43E-11
CPLX1	-4.381143	0.02075556	*STMN2*	-3.3573374	0.0002391
ZP1	-4.3072296	1.85E-08	*LVRN*	-3.354644	0.00789761
CTHRC1	-4.2801444	0.00273309	*BRINP2*	-3.3079534	0.01094982
UTS2B	-4.2173604	0.0416979	*EPSTI1*	-3.2958054	2.50E-07
MYL3	-4.1318736	0.00147248	*NMU*	-3.2896776	0.01354321
FAM26F	-4.1258151	0.00017793	*MCOLN2*	-3.2814616	0.00395378
CRH	-4.1120895	0.00380144	*IRX6*	-3.2805105	0.04019662
F2RL1	-4.0231129	0.00036993	*SLC6A17*	-3.1978486	0.01211101
PRLH	-4.0176443	0.01613048	*CACNG7*	-3.1647876	0.00056445

Table 4. 21. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the finisher phase whose diets were supplemented with at-requirement MHA.

KEGG Term	Associated Genes
Upregulated	
Ribosome	*FAU, MRPL12, MRPL17, MRPL18, MRPL2, MRPL24, MRPL27, MRPL9, MRPS11, MRPS15, MRPS18A, MRPS18C, MRPS2, MRPS21, RPL12, RPL23A, RPL30, RPL32, RPL37A, RPL38, RPL3L, RPL7A, RPL8, RPS11, RPS12, RPS19, RPS20, RPS26, RPS27, RPS27L, RPS29, RPSAP58, UBA52*
Autophagy	*ATG101, ATG14, ATG16L2, ATG2A, ATG9A, BCL2L1, BNIP3, IRS2, LAMP1, MAPK8, MTMR3, NRBF2, PIK3CA, PIK3R4, PRKACB, RPTOR, SMCR8, STK11, SUPT20H, TRAF6, TSC2, WDR41, WIPI2*
Ubiquitin mediated proteolysis	*ANAPC10, ANAPC5, BTRC, CDC26, CUL3, DET1, KEAP1, NEDD4, NHLRC1, PIAS1, STUB1, TRAF6, UBA3, UBE2D2, UBE2E3, UBE2G1, UBE2H, UBE2Q1, UBE2S, UBE3B, UBE4B, UBOX5*
RNA transport	*CASC3, CLNS1A, DDX20, EIF2B2, EIF3A, EIF3H, EIF5, GEMIN2, GEMIN5, GEMIN8, NUP155, NUP88, NUP98, RBM8A, SUMO3, UPF1*
Spliceosome	*CCDC12, CDC40, CWC15, DDX23, DHX8, EFTUD2, PPIL1, PRPF3, PRPF40A, PRPF8, RBM8A, SNRNP200, SNRNP27, SNRPA1, SNU13, SRSF10*
Downregulated	
Focal adhesion	*ACTN1, CAPN2, CAV1, CDC42, CHAD, COL1A2, COL6A1, COL6A2, COL6A3, CTNNB1, FLNB, FN1, FYN, HGF, ITGA4, ITGA6, ITGA8, ITGB1, MAPK10, MET, MYL10, MYLK, PAK5, PARVG, PIK3CB, PIK3CD, PPP1CB, PPP1R12A, PRKCB, RAC2, RAP1B, RHOA, ROCK1, SHC2, THBS2, THBS4, TLN1, TNC, VAV2, VAV3, ZYX*
Regulation of actin cytoskeleton	*ACTN1, ARHGEF12, ARHGEF6, ARPC1B, ARPC2, ARPC3, ARPC5, BDKRB1, BDKRB2, CDC42, CFL2, CHRM4, CXCL12, CXCR4, CYFIP1, DIAPH3, FN1, IQGAP2, ITGA4, ITGA6, ITGA8, ITGB1, ITGB2, LPAR5, MRAS, MYL10, MYLK, NCKAP1, PAK5, PFN2, PIK3CB, PIK3CD, PIP4K2A, PIP5K1B, PPP1CB, PPP1R12A, RAC2, RDX, RHOA, ROCK1, SCIN, TMSB4X, VAV2, VAV3*
Endocytosis	*ARAP2, ARF5, ARFGAP3, ARPC1B, ARPC2, ARPC3, ARPC5, ASAP1, ASAP2, BIN1, CAV1, CCR5, CDC42, CHMP2B, CHMP4B, CXCR4, CYTH1, CYTH4, DNAJC6, IL2RG, PDCD6IP, PIP5K1B, RAB11B, RAB11FIP4, RAB31, RHOA, SH3GL1, SNX1, SNX3, SNX32, USP8, WIPF1*
Neuroactive ligand-receptor interaction	*ADORA3, APLNR, BDKRB1, BDKRB2, C3, C3AR1, CALCA, CCKBR, CHRM4, CRH, EDNRA, F2RL1, F2RL2, GABBR2, GABRA5, GRIA3, GZMA, HTR2B, HTR4, LOC107051813, LPAR3, NMU, P2RX7, P2RY13, P2RY6, P2RY8, PENK, PRLH, PTGER2*
Influenza A	*APAF1, BID, BLB1, CASP3, CDK6, CHUK, CIITA, DMB2, FADD, FAS, FDPS, IFIH1, IFNAR2, IRF7, KPNA2, MYD88, NLRX1, PIK3CB, PIK3CD, PRKCB, RAB11B, RSAD2, SLC25A5, STAT1, TLR3, TLR4, TLR7, TNFRSF1A, TNFSF10, TRIM25*

Table 4. 22. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with above-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
U11	4.09633978	0.00148885	*APOD*	1.79811238	1.72E-08
SNORA12	4.09410344	0.00077532	*METTL7A*	1.78033345	0.00000596
PVALB	3.47719169	2.93E-18	*GIF*	1.77101254	0.00144915
SYPL1	3.4644302	4.54E-12	*KCNN1*	1.74479306	0.0000137
PPIL6	3.44905872	0.00630375	*BBOF1*	1.7167985	6.51E-08
CA12	3.14783561	0.00044147	*ASPA*	1.71376195	0.00028941
GPM6A	3.04610814	0.00509339	*SYNPR*	1.70401809	0.02082476
CNTN2	2.91993744	0.020149	*KLF9*	1.68165311	0.0000274
CXR	2.83685156	0.01137382	*DIRAS2*	1.68066632	0.00293618
BRSK2	2.80558627	0.00146388	*SLC35F3*	1.67435579	0.02212714
CXCR5	2.72317612	0.00098757	*ST6GALNAC1*	1.65856167	0.00048044
GPR182	2.59701042	0.00032886	*ACTL6B*	1.65644165	0.04683434
ZBTB16	2.4046554	0.00062635	*ANKRD55*	1.58410652	0.02304681
CLDN3	2.38558584	0.00987345	*CD9*	1.57991656	0.01831249
MFSD2B	2.24122717	0.00958879	*CDHR2*	1.57757927	0.00378814
MAP3K15	2.10564402	0.0000182	*NETO2*	1.5758789	0.0000801
FKBP5	2.09018113	0.00460003	*MAL*	1.57091225	0.04040521
MPZ	2.05546481	0.00092465	*HPSE2*	1.57037721	0.00048044
GDAP1	2.04973648	0.04717202	*GMEB2*	1.56596178	8.91E-11
SPACA9	2.02701585	0.04796244	*TINAG*	1.56253293	0.00000213
VIT	1.98584476	0.01769899	*ZNF365*	1.53897664	1.81E-11
DRP2	1.96824051	0.00842699	*PRTG*	1.52598008	0.0033784
ATP8B3	1.90534794	0.00024731	*C7*	1.52308712	0.0000132
SLC7A4	1.88930386	0.00328239	*RND1*	1.52161388	0.00000678
PLCH1	1.80538035	0.00185407	*MYO7B*	1.51054304	0.0000711

Table 4. 23. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with above-requirement DLM.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
MMP9	-4.1667962	0.00063224	*PSTPIP2*	-2.3399453	0.00074073
NMU	-3.473353	0.03283151	*NUGGC*	-2.3337506	0.00000678
GAP43	-3.4163272	0.0000663	*TNFSF11*	-2.3171177	0.01121933
CCK	-3.2670659	0.00719362	*AMBP*	-2.3141821	0.04377783
CNTNAP5	-3.1317936	0.02538752	*MMP7*	-2.3117721	0.00294104
PCDH15	-3.0511029	0.00112798	*WNT16*	-2.3012981	0.00266073
OASL	-3.0267198	0.0000981	*SERPINB2*	-2.2910283	0.04458666
POU2AF1	-3.0084734	0.02405581	*CCL19*	-2.2907314	0.00017732
IRX6	-3.0021146	0.02067821	*LRTM2*	-2.2866278	0.03412156
MMP27	-3.0016881	0.00095556	*NOV*	-2.2486516	0.00547035
ZP1	-2.983582	0.0000395	*SMPX*	-2.2109584	6.39E-10
PHGDH	-2.9758186	0.00198527	*TSPAN10*	-2.1994094	0.03115999
ASNS	-2.8422948	7.91E-11	*CYP11A1*	-2.195336	0.0000103
JCHAIN	-2.7325391	0.00037836	*SLC2A9*	-2.1753101	0.00239983
TGM4	-2.6620471	0.00091153	*SYNDIG1*	-2.1665138	0.03584048
C3	-2.6358939	0.0000886	*SLC7A1*	-2.1643403	0.0000464
STMN2	-2.6254746	0.0000102	*HPGDS*	-2.1437588	3.04E-08
IL20RA	-2.5530272	0.00017267	*RNF157*	-2.1317274	0.02692558
LMX1A	-2.5067496	0.00156007	*C1QB*	-2.1131686	3.52E-08
IL1R2	-2.5029817	0.04474789	*COL22A1*	-2.1110532	0.00907117
KIF26B	-2.4905069	0.04271237	*XDH*	-2.0967784	0.0085816
ARC	-2.4395992	0.04564934	*MT-CO1*	-2.0859747	5.07E-17
RSAD2	-2.3690069	0.00272392	*CXorf21*	-2.0594808	0.02173829
EPSTI1	-2.3624585	3.44E-07	*STARD10*	-2.0448752	0.0127243
DCSTAMP	-2.3447858	0.00083963	*THBS4*	-2.036158	0.00000932

Table 4. 24. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the finisher phase whose diets were supplemented with above-requirement DLM.

KEGG Term	Associated Genes
Upregulated	
Ribosome	*MRPL10, MRPL12, MRPL17, MRPL2, MRPL24, MRPL3, MRPL9, MRPS11, MRPS18A, MRPS2, MRPS21, RPL12, RPL21, RPL23A, RPL30, RPL34, RPL37, RPL37A, RPL38, RPL5, RPL7, RPL7A, RPL8, RPS12, RPS19, RPS20, RPS26, RPS27, RPS29, UBA52*
Autophagy	*ATG16L2, ATG2A, ATG9A, BNIP3, LAMP1, NRBF2, PIK3CA, PIK3R4, PRKACB, PTEN, RAB1B, RPTOR, RRAGC, RRAS, SH3GLB1, SUPT20H, TSC2, UVRAG, WDR41, WIPI2*
RNA transport	*CASC3, CLNS1A, DDX20, EIF2B2, EIF3A, EIF3B, EIF3G, EIF3H, EIF4E, EIF4E2, EIF4G2, EIF5, GEMIN2, GEMIN5, NUP155, NUP54, NUP88, NUP98, PABPC1, RANGAP1, RBM8A, SUMO3, UBE2I, UPF1, XPOT*
Ubiquitin mediated proteolysis	*ANAPC10, ANAPC2, BTRC, CDC16, CUL3, CUL4B, DET1, ITCH, KEAP1, NHLRC1, PIAS1, PIAS4, RFWD2, STUB1, UBA3, UBE2D2, UBE2E3, UBE2G1, UBE2I, UBE2Q1, UBE2S, UBE3B, UBE4B*
Wnt signaling pathway	*AXIN1, BTRC, CACYBP, CAMK2D, CBY1, CREBBP, CSNK1A1, CSNK1E, CSNK2A2, DVL1, FZD2, GSK3A, LOC101748851, NLK, PRKACB, PRKCA, PSEN1, ROR1, WNT9A*
Downregulated	
Focal adhesion	*CAPN2, CAV1, CDC42, CHAD, COL1A2, COL6A1, COL6A2, COL6A3, CTNNB1, EGF, FLNB, FN1, FYN, ITGA11, ITGA4, ITGB1, ITGB5, MET, PARVG, PDGFB, PDGFC, PDGFD, PDGFRB, PIK3CB, PIK3CD, PPP1CB, PRKCB, RAC2, RAP1B, ROCK1, SHC2, THBS4, TLN1, VAV3, ZYX*
Regulation of actin cytoskeleton	*ARHGEF12, ARPC1B, ARPC5, CDC42, CFL2, CXCR4, CYFIP1, DIAPH3, EGF, FGF10, FN1, INSRR, IQGAP2, ITGA11, ITGA4, ITGB1, ITGB2, ITGB5, LIMK1, LPAR1, LPAR4, NCKAP1L, PDGFB, PDGFC, PDGFD, PDGFRB, PIK3CB, PIK3CD, PPP1CB, RAC2, RDX, ROCK1, TMSB4X, VAV3*
Herpes simplex virus 1 infection	*APAF1, BF2, BLB1, C3, CARD9, CD74, DMB2, EIF2AK2, EIF2AK3, EIF2AK4, FAS, IFIH1, IRF7, PIK3CB, PIK3CD, PPP1CB, SRSF5, STAT1, SYK, TAP1, TLR2A, TLR3, TNFRSF1A, TRAF5*
Neuroactive ligand-receptor interaction	*ADRA2A, APLNR, C3, C3AR1, CCK, CRH, EDNRA, FSHR, GCG, GRIK4, GZMA, HRH4, HTR2B, HTR7, LOC107051813, LPAR1, LPAR4, NMU, NPY2R, P2RX7, P2RY13, P2RY6, P2RY8, RXFP1, VIPR2*
Influenza A	*APAF1, BLB1, CDK6, CIITA, DMB2, EIF2AK2, FAS, FDPS, IFIH1, IRF7, KPNA2, NLRX1, PIK3CB, PIK3CD, PRKCB, RSAD2, SLC25A5, STAT1, TLR3, TLR4, TLR7, TNFRSF1A, TNFSF10, TRIM25*

Table 4. 25. Top 50 upregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with above-requirement MHA.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
PVALB	5.07293081	8.76E-34	*SNORA12*	2.26610785	0.02085708
U11	4.19468697	0.00103766	*ST6GALNAC1*	2.2542815	0.00000326
SYPL1	4.12775945	4.94E-09	*METTL7A*	2.24752938	5.06E-25
SRRM3	4.0012586	0.00879073	*ADPRHL1*	2.24629409	0.00000254
FAT2	3.96322669	0.00313967	*ACE*	2.23483178	4.79E-10
CHRNB3	3.8813035	0.00802341	*CHRND*	2.2312812	6.62E-31
B3GALT5	3.7274831	0.01854125	*KCNN1*	2.19023378	1.76E-37
ZBTB16	3.31309236	0.00050853	*APOD*	2.16660565	1.12E-29
KERA	3.23299868	0.00155094	*COL4A3*	2.14341139	0.0000161
IL17B	3.21472521	0.00522483	*SLC35F3*	2.05648663	0.00158212
PRTG	3.20064603	3.04E-12	*WBP1L*	2.05329518	5.45E-11
TTC34	2.9585818	0.03983118	*SLC30A2*	2.0462246	0.00580424
FKBP5	2.82813637	0.01453697	*HGD*	2.02284847	0.00429099
FAM162B	2.79287599	0.02257834	*CHRNA1*	2.01792683	1.26E-26
ANKRD55	2.72214912	0.0000765	*ADMP*	2.01629783	0.0120365
SYNPR	2.67124575	0.012608	*ATP8B3*	1.98542866	1E-09
NXNL1	2.66228524	0.01154392	*ALDH1A1*	1.97927218	4.11E-07
CXR	2.51765777	0.00255047	*GDAP1*	1.97634562	0.00829358
UNC5C	2.44613067	0.00482516	*SLC47A2*	1.96851022	0.0000234
KLHL38	2.40304042	0.00010913	*IRS2*	1.96367979	0.0000219
DRC3	2.3859508	0.02301521	*ATP2C2*	1.90771394	0.0000381
STMN3	2.37562447	0.02165359	*ABCA4*	1.9053494	0.03312803
CA12	2.34717862	0.00166874	*RND1*	1.89767809	0.0000343
POU3F2	2.31730987	0.00876282	*PLCH1*	1.87194337	0.00491768
GPM6A	2.3167567	0.00023799	*C9orf172*	1.85395472	0.00871743

Table 4. 26. Top 50 downregulated genes in *Pectoralis major* of chickens reared at 35°C during the finisher phase whose diets were supplemented with above-requirement MHA.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
CCK	-6.5848633	0.0000119	*EPYC*	-2.7722338	0.04986938
SPIRE2	-5.2267627	0.00047074	*TGM4*	-2.7696899	9.07E-07
SMPD3	-4.659866	0.00773612	*GUCA1B*	-2.7321032	0.01011014
OSTN	-3.9993713	0.00904992	*CA2*	-2.7186872	2.05E-36
MMP7	-3.8698321	0.00029133	*CD180*	-2.7179145	1.74E-07
PDE6H	-3.8085366	0.03262696	*MPEG1*	-2.7109749	9.24E-19
DCSTAMP	-3.7815003	3.94E-07	*IL20RA*	-2.7076557	0.000031
USP35	-3.6926165	0.00014386	*P2RY4*	-2.7015635	0.01414866
HIST1H4J	-3.6832449	0.0131377	*SBK2*	-2.6609481	6.4E-16
GZMA	-3.5330462	1.7E-08	*CAMK1G*	-2.6553328	0.00162632
LIPI	-3.45392	0.0177396	*CCL4*	-2.6479174	3.47E-07
MMP27	-3.3671113	0.00052086	*IL1R2*	-2.6146777	0.00390122
LRIT1	-3.2968928	0.00731888	*CCL19*	-2.58792	0.00013849
ZP1	-3.1730174	0.00077287	*TNFSF8*	-2.576822	0.03448953
C10orf107	-3.1006283	0.04092481	*C1QB*	-2.5531407	1.16E-25
MYO5C	-3.071466	0.03875507	*RNF157*	-2.5174284	0.01846304
NUGGC	-3.0295913	2.14E-08	*JCHAIN*	-2.5108821	3.58E-16
STMN2	-3.0268775	0.00000137	*RGS1*	-2.5060793	0.00000531
IL18RAP	-2.9805301	0.00432922	*TNIP3*	-2.4893577	0.0356659
GABRB2	-2.9113956	0.0026919	*MTERF2*	-2.433003	5.15E-11
C14orf37	-2.8797829	0.00000241	*TNFSF11*	-2.4310669	0.01165047
SNORA75	-2.8614363	0.04770433	*CSMD1*	-2.4300774	4.96E-07
OASL	-2.8577799	0.0000121	*BAALC*	-2.41538	0.00058824
SLITRK1	-2.8381764	0.02044487	*IL18R1*	-2.3974995	4E-09
EPSTI1	-2.774706	3.13E-11	*LAMB3*	-2.3594819	0.04317913

Table 4. 27. Top KEGG pathways for differentially expressed genes of chickens reared at 35°C during the finisher phase whose diets were supplemented with above-requirement MHA.

KEGG Term	Associated Genes
Upregulated	
Ribosome	*FAU, MRPL12, MRPL13, MRPL16, MRPL17, MRPL18, MRPL2, MRPL24, MRPL3, MRPL9, MRPS10, MRPS11, MRPS18C, MRPS2, MRPS21, RPL12, RPL14, RPL21, RPL22, RPL23, RPL23A, RPL30, RPL31, RPL32, RPL34, RPL35, RPL36, RPL37, RPL37A, RPL38, RPL3L, RPL5, RPL6, RPL7A, RPL8, RPS12, RPS17, RPS19, RPS2, RPS20, RPS21, RPS26, RPS27, RPS29, RPSAP58, RSL24D1, UBA52*
RNA transport	*CASC3, CLNS1A, EIF3A, EIF3B, EIF3D, EIF3E, EIF3G, EIF3H, EIF4E, EIF4E2, EIF4G2, EIF4G3, EIF5, GEMIN2, GEMIN5, NUP153, NUP155, NUP54, NUP88, NUP98, PABPC1, PYM1, RANGAP1, RBM8A, SUMO3, TGS1, THOC5, TPR, TRNT1, UBE2I, UPF1, XPO1, XPO5, XPOT*
Spliceosome	*BUD31, CCDC12, CDC40, CDC5L, CHERP, CWC15, DDX23, DHX38, DHX8, EFTUD2, PPIH, PPIL1, PRPF3, PRPF40A, PRPF6, PRPF8, PUF60, RBM8A, SF3A2, SF3B4, SMNDC1, SNRNP200, SNRNP27, SNRPA1, SNRPC, SNRPN, SNU13, SNW1, SRSF10, TCERG1, U2AF1*
Wnt signaling pathway	*APC, AXIN1, BTRC, CACYBP, CBY1, CREBBP, CSNK1A1, CSNK1E, CSNK2A2, CTBP1, DVL1, DVL2, EP300, FBXW11, FZD1, FZD2, FZD7, GSK3A, LOC101748851, LRP6, MAPK8, MAPK9, NOTUM, PPARD, PRKACB, PRKCA, ROR1, RYK, WNT5B, WNT9A*
Ubiquitin mediated proteolysis	*ANAPC10, ANAPC2, BTRC, CDC26, CUL3, CUL4B, DET1, FBXW11, KEAP1, NEDD4, PIAS1, PIAS4, SAE1, STUB1, TRAF6, UBA3, UBE2D1, UBE2D2, UBE2E3, UBE2G1, UBE2H, UBE2I, UBE2J2, UBE2O, UBE2Q1, UBE2S, UBE3B, UBE4B, UBR5, WWP2*
Downregulated	
Focal adhesion	*ACTG1, ACTN1, CAPN2, CAV1, CAV2, CAV3, CDC42, CHAD, COL1A2, COL4A1, COL6A1, COL6A2, COL6A3, CTNNB1, FLNB, FLT4, FN1, FYN, HGF, ITGA1, ITGA2, ITGA4, ITGA6, ITGA7, ITGB1, ITGB5, LAMB3, MET, MYLK, PARVG, PDGFA, PDGFB, PDGFC, PDGFRB, PIK3CD, PPP1CB, PPP1R12A, PRKCB, PTK2, RAC2, RAP1B, ROCK1, ROCK2, THBS4, TLN1, VAV3, ZYX*
Regulation of actin cytoskeleton	*ACTG1, ACTN1, ARHGEF12, ARHGEF6, ARPC1B, ARPC5, CDC42, CFL2, CXCR4, CYFIP1, FGFR2, FN1, IQGAP2, ITGA1, ITGA2, ITGA4, ITGA6, ITGA7, ITGB1, ITGB2, ITGB5, LIMK1, LPAR4, MYLK, NCKAP1, NCKAP1L, PDGFA, PDGFB, PDGFC, PDGFRB, PIK3CD, PPP1CB, PPP1R12A, PTK2, RAC2, ROCK1, ROCK2, SCIN, TIAM1, TMSB4X, VAV3*
Calcium signaling pathway	*ADCY7, ATP2B1, CACNA1E, CACNA1H, CAMK1G, CXCR4, EDNRA, GNA14, GNAQ, HRH2, HTR2B, HTR7, MYLK, NOS1, NOS2, P2RX7, PDE1C, PDGFRB, PHKA1, PHKB, PHKG1, PLCB2, PLCD1, PPP3CA, PPP3CB, PRKCB, PTAFR, PTK2B, RYR3, SLC8A1, TRDN*
Cytokine-cytokine receptor interaction	*ACKR4, CCL19, CCL4, CCL5, CCR5, CSF1R, CSF3R, CX3CR1, CXCR4, IFNAR1, IFNAR2, IL12RB2, IL15, IL16, IL18R1, IL18RAP, IL1R2, IL1RAP, IL20RA, IL21R, IL2RG, IL31RA, IL6ST, LIF, LIFR, OSMR, TNFRSF1A, TNFSF11, TNFSF8, XCR1*
Herpes simplex virus 1 infection	*BLB1, C3, CARD9, CASP3, CCL5, CD74, CHUK, CYCS, DMB2, EIF2AK3, EIF2AK4, FADD, IFIH1, IFNAR1, IFNAR2, IRF7, PIK3CD, PPP1CB, SOCS3, SRSF5, STAT1, SYK, TAP1, TLR2A, TLR3, TNFRSF1A, TRAF5*

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CHAPTER 5

THERMAL CONDITIONING DOES NOT AID BROILER CHICKEN PERFORMANCE, YET DIETARY METHIONINE ENACTS CHANGES TO THE *PECTORALIS MAJOR* TRANSCRIPTOME AFTER PROLONGED THERMAL CHALLENGE

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ABSTRACT

Thermal conditioning exposes developing birds to a hot environment in hope of the bird developing mechanisms to better cope with an environment similar to the conditioning environment later in life. This method has mixed results when applied to broiler production, with some studies showing improved performance while others showing no performance benefit. In addition to thermal conditioning, increasing methionine in the diet of heat stressed broilers has been beneficial in negating the deleterious effects of heat stress. By using RNA-sequencing, this study aimed to identify the transcriptome response of heat stressed broilers who underwent thermal conditioning and were fed different sources and levels of methionine. This study used a 2x2x2 design consisting of environmental treatment (conditioned or non-conditioned), dietary methionine source (D,L-methionine or D,L-2-hydroxy-(4-methylthio) butanoic acid), and dietary methionine inclusion level (at- or above-requirement). Thermal conditioning lasted from days 7 to 10 post-hatch at 35°C. Broiler in both conditioned and non-conditioned environments were subjected to elevated temperatures (35°C) from days 22 to 35. Tissue from the *Pectoralis major* was collected for RNA-sequencing from broilers in all 8 treatment groups at day 35. Performance results showed thermal conditioning did not benefit broiler performance under heat stress. Thermally conditioned broilers fed at-requirement DLM performed most similar to non-conditioned broilers. Transcriptome analysis revealed focal adhesions and the actin cytoskeleton are most effected by thermal conditioning, possibly through the protective effects of heat shock proteins. Supplementing at-requirement MHA differentially regulated the most genes in thermally conditioned broilers, including the upregulation of proteolysis and the upregulation of genes which respond to oxidative stress. When supplemented with at-requirement DLM, thermally conditioned broilers downregulated genes related to oxidative stress and phagocytosis. These results show

methionine source and inclusion level in a broiler's diet does affect the molecular response when they are thermally conditioned, even when performance tells otherwise.

INTRODUCTION

A significant stressor in the poultry industry is heat. Heat stress is induced when the amount of energy produced by the bird is greater than the energy exiting the bird. The imbalance creates a pooling of energy within the bird in the form of heat (Lucas and Marcos 2013). Upon experiencing a larger heat load, blood flow is diverted from the viscera to the periphery to facilitate heat exchange, cooling the bird. This diversion of blood flow during heat stress leads to hypoxia and nutrient deficiency in various tissues including the liver, muscle, and intestines (Hall et al. 1999). Numerous studies have determined heat stress is detrimental to broiler performance (Hurwitz et al. 1980; Attia et al. 2011; Sohail et al. 2012; Awad et al. 2018; Goo et al. 2019; Lin Law et al. 2019). Additionally, heat stress inhibits proper immune function (Trout and Mashaly 1994; Bartlett and Smith 2003; Borges et al. 2004; Mashaly et al. 2004). The deleterious effects of heat stress on the bird's immune system as well as the depression in broiler performance cause significant economic loss to poultry producers (St-Pierre et al. 2003). It is in the interest of poultry producers to therefore minimize the deleterious effects of heat stress through various practices, including thermal conditioning.

Thermal conditioning exposes pre- and postnatal animals to environmental temperatures outside their thermoneutral zone in hope of developing certain adaptations to better suit a similar environment seen later in life (Yahav and McMurtry 2001). When fruit flies were exposed to heat stress at an early age, they outperformed and showed better survivability than fruit flies which had not been thermally conditioned when exposed to heat stress later in life. Additionally, fruit flies which were thermally conditioned closer to the heat stress challenge showed improved performance than fruit flies thermally conditioned further from the heat stress challenge. The authors hypothesize protein manipulation is prolonged, thus the early exposure to heat stress

produces proteins which benefit the animal when challenged later in life (Krebs and Loeschcke 1994). This hypothesis was validated by Vinoth et al. (2015), who found embryonic thermal conditioning enacts numerically higher protein expression of heat shock protein (HSP) 90 and significantly higher protein expression of HSP70, HSP60, and ubiquitin in the liver of broilers at 42 days of age.

In broilers, thermal conditioning has been shown to improve performance and mortality when challenged with heat stress later in life (Yahav and Hurwitz 1996; Yahav and Plavnik 1999; Meteyake et al. 2020). However, Inês Müller Fernandes et al. (2013) has shown thermal conditioning did not improve performance or mortality of broilers challenged with heat stress at 35 days of age. Because the mechanisms behind thermal conditioning are not fully understood, it is difficult to interpret these varying performance results. Much molecular research on thermal conditioning in poultry has focused around HSPs and the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-thyroid axes. However, there may be other mechanisms which greatly contribute to a bird's performance under heat stress when thermally conditioned.

Nutritional intervention is more widely studied in mitigating the effects of heat stress on poultry. Under thermoneutral temperatures, NRC (1994) recommends broiler diets contain 0.50%, 0.38%, and 0.30% methionine for starter, grower, and finisher phases, respectively. However, under suboptimal conditions, particularly heat stress, these recommendations may not be adequate to fulfill the bird's requirement for methionine to mitigate performance loss and impaired immunity. Previous research has shown increasing only essential amino acids, including methionine, benefit broilers subjected to heat stress (Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005). Ojano-Dirain and Waldroup (2002) have shown that under moderately hot temperatures (26.7ºC) methionine and lysine requirements for broilers are more than requirements

suggested by NRC (1994). Suganya et al. (2015) recommends a 1-2% reduction in crude protein with a 5-10% increase of critical amino acids under heat stress temperatures. In chronically heat stressed broilers, the doubling of methionine inclusion in the diet increased weight gain (Balnave et al. 1999). Soares et al. (2020) reared broilers under cyclical heat stress while feeding diets differing in protein content during 22-42 days of age. They found weight gain and FCR were improved in heat stressed broilers supplemented with 220 g/kg protein compared to those supplemented with 160 g/kg protein.

Today, synthetic methionine is the primary source of methionine in broiler diets. Synthetic methionine can be supplemented in various forms, including D-, L-, or DL-Methionine, or as a methionine analogue. Only L amino acids are biologically available, therefore the conversion of the D enantiomorph or methionine analogue must occur for proper utilization (Dibner and Knight 1984). Results vary regarding broiler performance in relation to methionine source. Some research shows under heat stress conditions, broilers supplemented with DL-2-hydroxy-(4-methylthio)butanoic acid (MHA) have better performance and lower mortality than broilers supplemented with DL-methionine (DLM) (Swick and Pierson 1988). Studies have additionally shown DLM improves broiler performance while MHA does not (Balnave and Oliva 1990). However, it has been shown methionine supplement source has no effect on broiler performance under both thermoneutral conditions or heat stress conditions (Ribeiro et al. 2001).

Recently, Lee et al. (2021) studied the transcriptome of heat stressed broilers fed either D-methionine or L-methionine. Their work found gene expression in both the liver and ileum differed between broilers fed D-methionine and L-methionine, even though performance was not different between those same birds. Transcriptomics can be a powerful tool in studying molecular changes to birds under heat stress and has been used in several recent studies (Coble et al. 2014; Lan et al.

2016; Jun-Mo et al. 2017; Hubbard et al. 2019). To our knowledge, no transcriptomic study has investigated the gene expression of thermally conditioned broilers. The objective of this study is to identify the molecular processes which occur in the breast muscle of thermally conditioned broilers under heat stress. Additionally, this study aimed to identify how methionine source and inclusion level affect the transcriptome of heat stressed broilers when combined with thermal conditioning.

MATERIALS AND METHODS

Experimental design

This study consisted of 8 groups in a 2x2x2 factorial design comprised of environmental treatment (thermally conditioned or non-thermally conditioned), dietary methionine source (DLM or MHA), and dietary inclusion of methionine (100% or 140% of requirement). At hatch, chicks were placed on pine shaving-covered floor pens (0.072 m^2/bird) with ambient temperature set to 32^oC. Each group consisted of 6 replicate pens with 20 birds per replicate. Ambient temperature was gradually reduced over the next 7 days in accordance with industry standards. On day 7, environmental treatment was initiated, and thermally conditioned broilers were subjected to environmental temperature of 35^oC. On day 10, thermal conditioning was ended, and all broilers were housed at 25^oC. Thermal challenge was initiated on day 22, and all broilers were subjected to an environmental temperature of 35^oC for 24-hours a day until day 35 (outlined in Figure 5.1).

Feed and water were provided *ad libitum*. Broiler body weight and feed weight were taken on days 0, 10, 21, and 35. The 4 unique diets were formulated for broiler starter (day 0-10), grower (day 11-21), and finisher (22-35) phases from a common basal diet (Table 3.1). For each phase, diets containing 100% and 140% of Cobb500 methionine requirement were formulated using DLM. Then, diets containing MHA were formulated to remain equimolar to 100% DLM and 140%

DLM, respectively. Starter diets contained 22% crude protein (CP) and 12.4 MJ apparent metabolizable energy (AME). The 100% DLM and 140% DLM starter diets contained 0.653% and 0.792% methionine and 0.997% and 1.131% total sulfur amino acids (TSAA), respectively. Grower diets for 100% DLM and 140% DLM contained 20% CP and 12.8 MJ AME with 100% DLM and 140% DLM diets containing 0.573 and 0.669% methionine, and 0.880 and 0.975% TSAA, respectively. Finisher diets 100% DLM and 140% DLM contained 18% CP and 12.8 MJ AME. The 100% DLM and 140% DLM finisher diets contained 0.511 and 0.630% methionine, and 0.880 and 0.918% TSAA, respectively (Table 3.2).

Replicate total pen body weight and feed weight were measured at days 0, 10, 21, and 35. Blood was additionally drawn from 5 randomly selected birds per group at days 10, 21, and 35. At day 35, 5 birds per group were randomly selected for tissue sampling and euthanized. Tissue from the *Pectoralis major* were collected, then flash frozen in liquid nitrogen and stored at -80°C.

Hormone assays

Blood from sampled birds was allowed to coagulate, then spun down and serum collected, and stored at -30°C. Serum samples from all groups at days 10, 21, and 35 were assayed for triiodothyronine (T_3), thyroxine (T_4), and corticosterone (CORT). Both T_3 and T_4 levels were measured using radioimmunoassay (MP Biomedicals, Santa Ana, CA). CORT was measured using ELISA (Cayman, Ann Arbor, MI). Samples in all hormone assays were run in duplicate and performed in accordance with the manufacturer's protocol.

RNA preparation and RNA-sequencing

Sampled *Pectoralis major* tissue was coarsely ground in liquid nitrogen and further broken down via lysing matrix tubes (MP Biomedicals, Santa Ana, CA). Total RNA was extracted using TRIzol reagents (Invitrogen, Carlsbad, CA), then cleaned with RNeasy Mini Kit (Qiagen,

Valencia, CA, USA). The RNA was treated with RNase-Free DNase (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. Purified RNA was resuspended in RNase-free water. RNA concentration and purity was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm an $OD_{260/280}$ ratio greater than 1.9. cDNA libraries were prepared with 4 µg total RNA using TruSeq RNA sample Preparation Kit to obtain cDNA fragments of 229 bp average size, or 355 bp including adapter sequences. All cDNA libraries were sequenced with 150 bp paired-end read chemistry using the Illumina HiSeq 2000 system.

Analysis of RNA-sequencing data

FastQC was used to identify the quality of raw reads. Low-quality bases and adapter sequences were trimmed using Trimmomatic v.0.36 (Bolger et al. 2014) and Flexbar v.2.4 (Dodt et al. 2012). Reads were aligned to the chicken reference genome (*Gallus gallus* 4.0, version 81, Ensembl) using STAR aligner v.2.5.2b (Dobin et al. 2013). Hit counts were counted with featureCounts v.1.5.2 (Liao et al. 2014), using only unique reads moving forward. DESeq2 (Anders and Huber 2010; Love et al. 2014) was used for differential gene expression analysis. For pairwise analysis, four comparisons were devised: non-conditioned broilers fed 100% DLM versus conditioned broilers fed 100% DLM (100DLM), non-conditioned broilers fed 100% MHA versus conditioned broilers fed 100% MHA (100MHA), non-conditioned broilers fed 140% DLM versus conditioned broilers fed 140% DLM (140DLM), non-conditioned broilers fed 140% MHA versus conditioned broilers fed 140% MHA (140MHA). Counts were normalized using the transcripts per million method. Normalized counts were subjected to $\log_2$ fold change based on the comparisons outlined using the Wald test to generate p-values. An adjusted p-value for false discovery rate (FDR) was generated using the Benjamini-Hochberg method (Benjamini and

Hochberg 1995). Genes were considered differentially expressed when the FDR ≤ 0.05 and fold change ≥ 1.2 or ≤ -1.2.

The ClueGO Plugin v2.5.4 (Bindea et al. 2009) was used for gene ontology (GO) within Cytoscape v3.7.2. Only genes considered to be differentially expressed were used for analysis. The majority of analysis was done using Kyoto Encyclopedia of Genes and Genomes (KEGG) terms, however molecular and biological GO pathways/networks were used, when stated.

Quantitative reverse transcription-PCR verification of RNA-sequencing data

Aliquots from all RNA samples used for RNA-sequencing were retained and used for RT-qPCR validation of genes. Nine differentially expressed genes identified by RNA-sequencing were used for validation. These included *GLO1*, *TGM4*, *PAG1*, *PLK3*, *ROCK2*, *CA2*, *ETNK1*, *ATF4*, and *NCF2* (Table 5.1). cDNA was synthesized from two micrograms of total RNA using high-capacity cDNA Reverse Transcription Kit in accordance with the manufacture's protocol (Applied Biosystems, Foster City, CA) and using a thermocycler (Eppendorf, Hauppage, NY) with the following settings: 10 min at 25°C, 120 min at 37°C, five min at 85°C and final cycle at 4°C. Newly synthesized cDNA was stored at -20°C until further use. cDNA was diluted so two ng would be used for each reaction during RT-qPCR, additionally using 0.3 µL of forward primer (10 µM), 0.3 µL of reverse primer (10 µM), 8.4 µL of RNase-DNase-free water, and 10 µL of SYBR Green Master Mix (Applied Biosystems, Carlsbald, CA). Each biological sample was run in triplicate for each gene. Conditions for RT-qPCR were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds using a StepOnePlus (Applied Biosystems, Carlsbald, CA). Chicken *β-actin* was used as an endogenous control and differential expression was measured using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001), with relative expression comparisons identical to the comparisons used for RNA-sequencing.

Statistical analysis

Due to high biological variance of measured T_3, T_4, and CORT levels, outliers were eliminated for each hormone assay using Lund's test in SAS (SAS Institute, Cary, North Carolina). Statistical analysis of T_3, T_4, CORT, body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) results for each day were determined using the GLMMIX procedure in SAS (SAS Institute, Cary, North Carolina). All tests were performed with a 5% level of significance. Environment, methionine source, and methionine level were considered as main effects. Least square means was used to compare effects using PDIFF in SAS when found to be significant. Probability values were generated using analysis of variance for main and interaction effects. For validation of differentially expressed genes (DEGs), linear regression using PROC REG (SAS Institute, Cary, North Carolina) tested the strength of RNA-sequencing $\log_2$ fold change predicting RT-qPCR $\log_2$ fold change.

RESULTS

Thermal conditioning did not improve the performance of heat stressed broilers. During the conditioning period, conditioned broilers had numerically lower BWG than three of the 4 dietary treatments of non-conditioned broilers (Table 5.2). Feeding above-requirement MHA resulted in the lowest BWG during days 0 to 10, regardless of conditioning status. Non-conditioned broilers fed DLM had the numerically highest BWG during days 0 to 10 and were significantly higher than thermally conditioned broilers fed at-requirement DLM and at- and above-requirement MHA. From days 11 to 22, BWG was lower in thermally conditioned broilers (Table 5.3). Non-conditioned broilers had larger BWG during the finisher phase than conditioned broilers (Table 5.4). Only conditioned broilers fed at-requirement DLM did not have significantly lower BWG

compared to non-conditioned broilers during the final two weeks. Interestingly, FCR did not significantly differ between any dietary or environmental factors during the trial.

Increasing DLM inclusion in the diet significantly reduced circulating CORT in thermally conditioned broilers at day 10 (Table 5.2). Thermally conditioned broilers in 100DLM and 100MHA showed numerically increased CORT at day 10 when compared to their non-conditioned counterparts. After 11 days of husbandry at a thermoneutral temperature, all 8 groups had similar circulating CORT levels (Table 5.3). At day 35, main affect test showed not all group CORT level means are equal, however multiple comparisons did not show difference between any two groups (Table 5.4). After thermal challenge, thermally conditioned broilers in 140DLM and 140MHA did have numerically higher CORT levels than any other group.

Thermal conditioning additionally affected circulating T_3 levels. All thermally conditioned broilers had reduced T_3 levels at day 10, while those in 100MHA, 140DLM, and 140MHA had significantly lower T_3 levels than their non-conditioned counterparts (Table 5.2). At days 21 and 35, however, circulating T_3 levels were not different between the 8 groups (Table 5.3 and 5.4). Likewise, circulating T_4 levels were similar between all 8 groups at days 10, 21, and 35 (Table 5.2, 5.3, and 5.4).

Transcriptome

Sequence alignment and mapping

A total of 1.33 billion raw reads were obtained from the 40 samples (8 groups x 5 replicate birds) subjected to RNA-sequencing, averaging 33.1 million raw reads per bird (range of 25.3 to 43.6 million). About 89.3% of raw reads mapped to the chicken reference genome (*Gallus gallus* 4.0, version 81, Ensembl), with approximately 84.4% of raw reads being uniquely mapped. A total of 14 data-points were selected for validation via RT-qPCR based on significance, comparison,

and sampling time. Regression analysis revealed strong correlation ($p < 0.0001$, $R^2 = 0.8726$) between the log2 fold change of significantly DEGs determined by RNA-sequencing and RT-qPCR (Figure 5.2).

Differential gene expression of thermally conditioned broilers supplemented with at-requirement DLM (100DLM)

A total of 176 genes were differentially expressed between thermally conditioned and non-conditioned broilers fed at-requirement DLM. Of these, 17 genes were upregulated, and 159 genes were downregulated (Figure 5.3). The most upregulated genes include *GREM1*, *STXBP5L*, *GRB14*, *NSG1*, and *SDK1* (Table 5.5) while the most downregulated genes include *TGM4*, *MMP7*, *RSPO4*, *PTX3*, and *FGG* (Table 5.6). Enrichment analysis of KEGG pathways reveled phagosome, ECM-receptor interaction, and Jak-STAT signaling pathway to be most associated with negatively expressed genes in thermally conditioned broilers (Table 5.7). Both *TGM4* and *FGG* are associated with an inflammatory response and coagulation, while *PTX3* is associated with reactive oxygen species metabolic process. According to GO molecular function, *GREM1* is associated with activation of protein kinase activity and *STXBP5L* with regulation of hydrolase activity.

Differential gene expression of thermally conditioned broilers supplemented with at-requirement MHA (100MHA)

Supplementing at-requirement MHA resulted in 415 differentially expressed genes in thermally conditioned broilers in comparison to non-conditioned broilers. Of those, 244 genes were upregulated, and 171 genes were downregulated (Figure 5.3). The genes *FMO4*, *RHOBTB1*, *PAK5*, *TMEM79*, an *SBK2* were most upregulated (Table 5.8) while the most downregulated genes were *PMP2*, *C1QL2*, *SBK3*, *PDK4*, and *MMP9* (Table 5.9). GO KEGG analysis shows thermal conditioning upregulates the most genes associated with aminoacyl-tRNA biosynthesis, regulation

of actin cytoskeleton, and oxidative phosphorylation when broilers are fed at-requirement MHA. Those broilers also downregulate genes associated with focal adhesion, glycerophospholipid metabolism, and ECM-receptor interaction (Table 5.10). No other comparison differentially regulated genes related to oxidative phosphorylation or aminoacyl-tRNA biosynthesis. Additionally, conditioned broilers in 100MHA were the only ones to upregulate more genes related to the actin cytoskeleton (Figure 5.4).

Differential gene expression of thermally conditioned broilers supplemented with above-requirement DLM (140DLM)

A total of 128 genes were differentially expressed between thermally conditioned and non-conditioned broilers fed above-requirement DLM. Of those, 43 genes were upregulated while 85 genes were downregulated (Figure 5.3). The most upregulated genes in thermally conditioned broilers were *GADD45B*, *NOXRED1*, *ADPRHL1*, *CEBPB*, and *PM20D2* (Table 5.11) while the most downregulated genes were *ATP6V1G3*, *EMILIN3*, *FGFR2*, *SFRP2*, *FBN3* (Table 5.12). According to GO KEGG pathway analysis, the terms Wnt signaling pathway, regulation of actin cytoskeleton, inositol phosphate metabolism, glycerophospholipid metabolism, and phosphatidylinositol signaling system were associated with downregulated genes (Table 5.13).

Differential gene expression of thermally conditioned broilers supplemented with above-requirement MHA (140MHA)

When supplemented with above-requirement MHA, thermal conditioned broilers upregulated three genes while downregulating 40 genes when heat stressed in comparison to newly stressed broilers (Figure 5.3). The genes *CILP*, *CWF19L2*, and *RNASEH2B*, were upregulated in thermally conditioned broilers while the genes *BRK-3*, *PAG1*, *PTPN21*, *NPR3*, and *MYLK4* were the most downregulated (Table 5.14). KEGG pathway enrichment showed the downregulated

genes *ACTN4*, *ITGA1*, *ITGAV*, *MYLK4*, *ROCK2* are all related to focal adhesion and regulation of actin cytoskeleton while *RNASEH2B* is related to DNA replication.

DISCUSSION

The concept of thermal conditioning is centered around molding the mechanisms of an organism to better regulate temperature during a time of development. At an immature age, mechanisms within an organism are still developing and establishing themselves (Modrey and Nichelmann 1992). By exposing an organism to a challenging environmental condition, mechanisms and systems may establish parameters based those conditions, better suiting the organism for further challenge. In practice, thermal conditioning has proven effective in curving the deleterious effects of heat stress on broiler performance (De Basilio et al. 2001; Yahav and McMurtry 2001). At times, this does come with the caveat of reduced performance during conditioning (Yahav and Hurwitz 1996; Yahav et al. 1997). Thermal conditioning relies on the bird to increase feed intake and weight gain to ultimately overcome the reduced growth during conditioning. Compensatory growth does not always recover the performance loss during conditioning, particularly when conditioning is extended past one day. Additionally, performance under chronic heat stress later in life is not effected by thermal conditioning, explained by Ncho et al. (2021). Their work examined 17 thermal conditioning studies on broilers and determined thermal conditioning appears to benefit broiler performance under acute heat stress. There is, however, large variation in these results that appears to be dependent on age when thermally conditioned, duration and temperature of conditioning, and the age and duration of thermal challenge.

In the current study, thermal conditioning did not improve performance of broilers subjected to heat stress. Conditioning time for this study lasted from days 7 to 10 post-hatch, 24

hours per day. This resulted in reduced feed intake and subsequent reduced BWG of conditioned broilers during brood and conditioning periods (Table 4.1). The reduced performance coincides with a numeric decrease of triiodothyronine (T3) levels in all thermally conditioned groups (Table YB). Yahav and Plavnik (1999) saw similar effect where a 24-hour thermal conditioning period at 5 days of age resulted in lower body weight and lower plasma T3 levels than non-conditioned birds. Those birds were able to recover the body weight deficit and out-performed non-conditioned broilers during a 6-hour thermal challenge. In the current study, thermally conditioned broilers never recovered body weight during the compensatory growth period. Coincidently, thermal conditioning did not improve the body weight of broilers after the 14-day thermal challenge. Circulating T3 levels did not differ between thermally conditioned and non-conditioned broilers after the thermal challenge, possibly due to the longevity of heat stress. Interestingly, only conditioned broilers fed at-requirement DLM did not have significantly lower total BWG than their non-conditioned counterparts. However, broilers fed at-requirement DLM performed the worst out of all the non-conditioned groups, which aided in the similarity between conditioned and non-conditioned broilers fed at-requirement DLM.

Oxidative status

While broilers in the 100DLM comparison performed similarly over the 35-day study, thermally conditioned broilers in 100MHA had significantly lower BWG then their non-conditioned counterparts. GO revels broilers in 100MHA differentially expressed genes related to oxidative phosphorylation and response to oxidative stress, while no DEGs in 100DLM are associated with these pathways. Under heat stress, the production of reactive oxygen species (ROS) is significantly increased (Brooks et al. 1971; El-Orabi et al. 2011). The primary source of ROS results from mitochondrial dysfunction. During normal function, the electron donors NADH

and $FADH_2$ donate electrons to the electron transport chain (ETC) which pass the electron through a series of complexes which move protons from the mitochondrial matrix to the inner mitochondrial membrane. This creates an electrochemical gradient that is used by adenosine triphosphate (ATP) synthase to synthesize ATP from adenosine diphosphate and inorganic phosphate. The electron used to generate this gradient is properly utilized to reduce O_2 to H_2O (Lehninger et al. 2005). Under dysfunction, this proper reduction of O_2 does not occur and ROS are instead formed. In humans, there are 11 known sites along the ETC to generate ROS (Monti et al. 2001; Lin et al. 2002; Zhao et al. 2006; Brand 2016). In poultry, it appears complexes I and III most contribute to ROS formation (Iqbal et al. 2001). Under non-stressful conditions, 1% to 4% of useful oxygen is converted to superoxide by electron leakage through complexes I and III (Davies 1995; Jastroch et al. 2010). These amounts of ROS are controlled by the antioxidant defense of the cells (Pamplona and Costantini 2011), however it is known under heat stress the amount of ROS can increase to uncontrollable levels. Zhang et al. (2019) has shown heat stress downregulates genes associated with oxidative phosphorylation and the ETC in poultry. This downregulation and reduced activity of complexes leads to greater generation of superoxide and ROS (Emelyanova et al. 2016).

Exposure to cyclical heat stress increases mitochondria content in muscle cells as well as increased expression of complexes I and V in the ETC (Liu and Brooks 2012; Tamura et al. 2014). Thermal condition additionally induces the expression of HSPs (Vinoth et al. 2015; Vinoth et al. 2018), and increased abundance of mitochondria and function has been associated with increased HSP72 expression (Henstridge et al. 2014). Additionally, Madkour et al. (2021) found 6 hours of thermal conditioning downregulated the genes *NOX4*, *GR*, *SOD*, *SOD2*, and *CAT*, indicating reduced ROS activity. Based on these findings, it is expected thermal conditioning in the current

study would elicit differential regulation of genes related to the ETC and antioxidant function. Indeed, thermal conditioning did upregulate 8 genes associated with oxidative phosphorylation in heat stressed broilers fed at-requirement MHA. However, these broilers show signs of increased oxidative stress compared to non-conditioned broilers. Microsomal glutathione S-transferase 3 is highly expressed during times of oxidative stress (Aniya and Anders 1989). The gene *MGST3* codes for this enzyme in chickens, and its upregulation in 100MHA may indicate increased oxidative stress in thermally conditioned broilers. Furthermore, the upregulation of genes which respond to oxidative stress (*ATF4*, *FBXW7*, *LRRK2*, and *PARK7*) indicate thermal conditioning may have increased the production of ROS in broilers fed at-requirement MHA. During the grower and finisher phases of the current study, thermally conditioned broilers ate significantly less feed than non-conditioned broilers when both were fed at-requirement MHA. In poultry, heat stress and subsequent reduced feed intake is believed to lead to greater ROS generation (Del Vesco and Gasparino 2013). Glutathione transferases, catalase, and superoxide dismutase are more likely to be upregulated during dietary restriction (Walsh et al. 2014), so the upregulation of *MGST3* and genes related to oxidative stress may be related to reduced feed intake of broilers in 100MHA. The increased response to ROS may not only be due to reduced feed intake, however. Heat stress significantly alters amino acid and nutrient uptake (Habashy et al. 2017), and MHA has shown to be less efficient at producing GSH than DLM (Wang et al. 2019). Therefore, the combined effect of limited feed intake, reduced nutrient uptake, and possible inefficiencies of MHA could lead to increased ROS generation and oxidative damage in thermally conditioned broilers fed at-requirement MHA.

Although feed intake was significantly reduced in all thermally conditioned broilers, heat stress does not appear to increase the response to ROS in 100DLM, 140DLM, or 140MHA.

Increasing methionine supplementation can increase the flux through the ETC (Tripodi et al. 2018), and work has shown one-carbon metabolism plays a role in mitochondrial function (Lozoya et al. 2018). The higher availability of methionine to broilers supplemented with above-requirement MHA and those fed DLM may have assisted in proper function of the mitochondria. The bioavailability of methionine to broilers fed MHA is lower than DLM (Lemme et al. 2002), so it may be the already impaired thermally conditioned broilers in 100MHA suffered greater mitochondrial dysfunction upon thermal challenge.

The immune system is also responsible for ROS production and oxidative stress. Activation of the immune and proinflammatory systems is primary done by nuclear factor-kappaB (NF-κB) (Kawai and Akira 2007). During times of hyperthermia, the activation and translocation of NF-κB is increased (Pogliaghi et al. 1995), triggering an inflammatory response (Lambert et al. 2002; Andreasen et al. 2008). Circulating CORT is also increased under heat stress (Xu et al. 2018). CORT is involved in the suppression of an inflammatory response by inhibiting the translocation of NF-κB (Dong et al. 2018). Both the activation of an inflammatory response and the actions of CORT appear to contradict immune response in birds under stress. Liu et al. (2018) explains preexposure to CORT in mice can alter the immune response to further immune challenge in non-classical ways. A part of immune activation is increased activity of phagocytes and the phagosome (Underhill et al. 1999). NADPH oxidase generates superoxide which serves as the main weapon of phagosomes to combat pathogens upon immune activation (Nauseef 2007). The gene *NCF2* codes for a subunit of NOX2, a NADPH oxidase, and is downregulated in thermally conditioned broilers in 100DLM. In addition, *BACH1*, whose expression is correlated with macrophage activity and tissue injury (Patsalos et al. 2019), was downregulated in thermally conditioned broilers. The additional negative expression of *ATP6V0D2*, *RAB7B*, *TCIRG1*, *THBS1*, *TUBAL3*, and *TUBB6*

indicate the phagosome is downregulated in 100DLM, possibly resulting from reduced oxidative stress and immune activation.

The peroxisome-proliferator-activated receptors (PPAR) signaling pathway responds to oxidative stress and activates transcription factors involving in lipid and glucose metabolism, cell differentiation, and inflammatory response (Kliewer et al. 2001). Lipid oxidation increases PPAR signaling (Ricote et al. 1998; Staels et al. 1998) and the action of PPAR directs the transcription of endogenous antioxidants (Lebovitz et al. 1996; Xu et al. 2012). PPAR signaling has shown to be an important regulator of phagocytosis (Han and Ravichandran 2011), so the downregulation of genes related to the phagosome agree with the downregulation of *ANGPTL4*, *APOA5*, and *FABP4* in 100DLM. The gene *ANGPTL4* is related to lipid and glucose metabolism and is stimulated by glucocorticoids under hypoxic conditions (Koliwad et al. 2009). Thermal conditioning is thought to reduce CORT levels in broilers (Hiroshi et al. 2014) and Lan et al. (2016) saw chickens with greater heat resistance downregulated the genes *ANGPTL4* and *GADD45* in the liver. Although thermally conditioned broilers in 100DLM did not display significantly lower CORT levels at day 35, the transcriptome indicates some benefit of thermal conditioning.

Proteolysis and translation

The generation of ROS seems to be largest in thermally conditioned broilers fed at-requirement MHA, so it is unsurprising to find 100MHA upregulated 15 genes associated with regulation of protein catabolic process. In an oxidative environment, amino acids become oxidized, damaging proteins and inducing proteolysis (Zhang et al. 2013). The catabolism of proteins is primarily performed by the 26s proteosome and the ubiquitin proteasome pathway (UPP) (Baumeister et al. 1998; Li et al. 2003). Warm temperatures decrease protein synthesis and induce

proteolysis (Temim et al. 2000; Boussaid-Om Ezzine et al. 2010), however the improved performance and weight gain of thermally conditioned broilers is thought to be the result of more adapt HSP expression (Vinoth et al. 2015; Vinoth et al. 2018) or metabolic shift (Yahav and Plavnik 1999; De Basilio et al. 2001; Yahav and McMurtry 2001). Still, thermal conditioning does not always enact protection against proteolysis (Loyau et al. 2014). In the current study, thermal conditioning did not attenuate protein catabolic processes when broilers were exposed to heat stress. In fact, the upregulation of the genes *FAM192A*, *FBXW7*, *HSP90AB1*, *LRRK2*, *PARK7*, *PDCL3*, *PSMC2*, *RDX*, *SIRT2*, *USP14*, and *USP25* indicate the combination of at-requirement MHA supplementation and thermal conditioning increases proteolysis compared to newly challenged broilers. In production animals, MHA has shown to be less efficacious for performance compared to DLM (Balnave and Oliva 1990; Shoveller et al. 2010). Yet this study shows thermally conditioned broilers in 100MHA had similar performance loss when heat stressed to those in 100DLM, 140DLM, and 140MHA, despite increased proteolysis. The upregulation of 22 genes associated with translation could explain why 100MHA did not show significantly less BWG.

The upregulation of genes associated with translation and proteolysis indicate 100MHA has higher protein turnover than any other comparison. Eukaryotic translation initiation factor 3 subunit J (EIF3J) regulates the translation of mRNA specific to cell proliferation (Lee et al. 2015). The gene coding for EIF3J was upregulated in 100MHA, indicating thermal conditioning enacted some anabolic process during thermal challenge. Recent work has shown EIF3J expression is increased in thermally conditioned broilers (Darae and Kwan Seob 2021), similar to what was seen in 100MHA. The additional upregulation of the genes *AARS*, *CARS*, *DARS*, *EPRS*, *GATB*, *NARS2*, *QARS*, *SARS*, *TARS*, and *TRMT13* in 100MHA seem to confirm protein synthesis is reduced in newly challenged broilers fed at-requirement MHA.

The actin cytoskeleton

Increased translation of proteins also coincides with increased regulation of the actin cytoskeleton in 100MHA. The cytoskeleton of a cell is comprised of microtubules, intermediate filaments, and microfilaments. Actin is the primary protein of microfilaments and makes up a large portion of protein in skeletal muscles (Anderson 1976; Murakami and Uchida 1985). During myogenesis, actin plays a crucial role in cell fusion. Various actin-binding proteins interact with actin filaments to regulate actin growth, organization, and signaling which contributing to the growth and structure of a cell. When stressed, HSPs bind to actin in an attempt to mitigate damage to the cellular structure (Archana et al. 2017). Small HSPs (sHSPs) are primarily responsible for this action, responding to stress, cell growth, apoptosis, and signal transduction (Yin et al. 2019). Under oxidative stress, ROS commonly attack cysteine and methionine residues in actin monomers. Three methionine residues and one cysteine residue are fully exposed to the cytoplasm while 4 other methionine residues are additionally vulnerable to oxidation (Dalle-Donne et al. 2002; Dalle-Donne et al. 2003). When challenged with heat stress or oxidative stress, cells overexpressing HSP27 show higher stability of actin and more rapid recovery (Lavoie et al. 1993a; Lavoie et al. 1993b; Huot et al. 1996). Additionally, HSP25 expression is elevated during the first phases of myogenesis (Dubińska-Magiera et al. 2014; Thakur et al. 2019), indicating protection of actin filament assembly and organization during the development of muscular tissue. Thermal conditioning induces the expression of HSPs (Vinoth et al. 2015; Vinoth et al. 2018), which can improve actin proliferation in skeletal muscle (Halevy et al. 2001; Ouchi et al. 2021).

The upregulation of the genes *ACTN2*, *CDC42*, *CFL2*, *FHOD1*, *MYO18A*, *PFN2*, *TMOD4*, and *TNNT3* in 100MHA indicate actin filament reorganization is increased in thermally conditioned broilers. This upregulation of actin proliferation coincides with increased tRNA

synthesis in thermally conditioned broilers, agreeing with previous conditioning studies (Halevy et al. 2001; Ouchi et al. 2021). This was only seen in broilers supplemented with at-requirement MHA, however. Thermally conditioned broilers supplemented with above-requirement methionine downregulated genes related to the actin cytoskeleton compared to newly stressed broilers. The gene *ROCK2* was similarly downregulated in both comparisons, but not differentially regulated in 100DLM or 100MHA. Rho-associated coiled-coil-containing protein kinase (ROCK) 1 and 2 are in the family of RhoA small guanosine triphosphateases (GTPases), which interact with actin to control focal adhesion and actin stress fibers (Amano et al. 1997). ROCK2 has been shown to stabilize the cytoskeleton (Moore et al. 2004; Shi et al. 2013), so its upregulation in non-conditioned broilers in 140DLM and 140MHA indicates increasing methionine in the diet along with thermal conditioning may ease actin destabilization. As mentioned, actin contains 7 methionine residues highly susceptible to oxidation (Dalle-Donne et al. 2002; Dalle-Donne et al. 2003). So, methionine's requirement for actin synthesis in addition to the antioxidant benefit of methionine could explain why increasing methionine in the diet would result in improved protection of the cytoskeleton. Combine this with the protective effects of sHSPs and it is possible the combination of thermal conditioning and increased inclusion of methionine benefits the cytoskeleton of myocytes.

Cell cycle

ROCK2 not only plays a role in actin stabilization, but it is also involved with progression of the cellular cycle (Kümper et al. 2016). When experiencing hypoxia, *in vitro* expression of ROCK2 is associated with apoptosis and the regulation of the Wnt4 and β-catenin (Luo et al. 2019). Low-density lipoprotein receptor-related protein 6 (LRP6) is an essential receptor for Wnt signal transduction (MacDonald et al. 2009), so the downregulation of the genes *LRP6*, *ROCK2*,

GPC4, *PLCB1*, and *SFRP2* in 140DLM indicates non-conditioned broilers increase Wnt signaling and apoptosis when heat stressed. Similarly, 100DLM downregulated the genes *MMP7*, *MYC*, and *SFRP4*, all related to Wnt signaling. The genes *BUB1* and *GADD45B*, associated with the cell cycle, were additionally downregulated in 100DLM. Growth arrest and DNA damage inducible 45 beta, coded by *GADD45B*, is a part of the response to DNA damage (Liu et al. 2009). Diets deficient in sulfur amino acids induce the expression of *GADD45B* (Rao et al. 2001), and its expression is linked with oxidative damage (Barger et al. 2012). Oxidative damage to DNA induces cell arrest and degradation by the phagosome (Clopton and Saltman 1995). The downregulation of genes associated with phagocytosis and reduced expression of *GADD45B* in 100DLM suggest thermal conditioning reduces apoptosis by diminishing DNA oxidation.

CONCLUSION

Thermal conditioning aims to prepare the bird for heat stress, improving survivability and performance. The current study shows thermal conditioning did not improve broiler performance when subjected to chronic heat stress. Reduced metabolic rate is thought to contribute to a bird better managing heat stress, and thermally conditioned broilers did numerically reduce circulating T3 after the thermal conditioning period. However, circulating T3 was not lower in those broilers during the compensatory growth period or thermal challenge, possibly contributing to the lack of improved performance. RNA-sequencing did reveal thermal conditioning enacts DGE, in addition to transcriptome changes between dietary treatments. Studies have shown MHA to be less efficacious in combating ROS, and this study shows an increased response to oxidative stress, as well as increased regulation of the ETC in thermally conditioned broilers fed at-requirement MHA. In contrast, broilers fed at-requirement DLM reduced regulation of the phagosome when thermally conditioned, possibly reducing oxidative stress. 100MHA was the only comparison to increase

proteolysis, while also increasing translation of proteins. This protein turnover could be a result of actin reorganization, also upregulated in 100MHA. Increased inclusion of methionine in the diet of thermally conditioned broilers reduced the expression of *ROCK2*, whose expression is associated with hypoxia. Together, these results suggest thermal conditioning does not aid the performance of chronically stressed broilers. Methionine source, on the other hand, does elicit DGE of various metabolic processes in the breast muscle of broilers. These differences should be considered when defining the effect of thermally conditioning and heat stress in broilers, and not be solely reliant on performance.

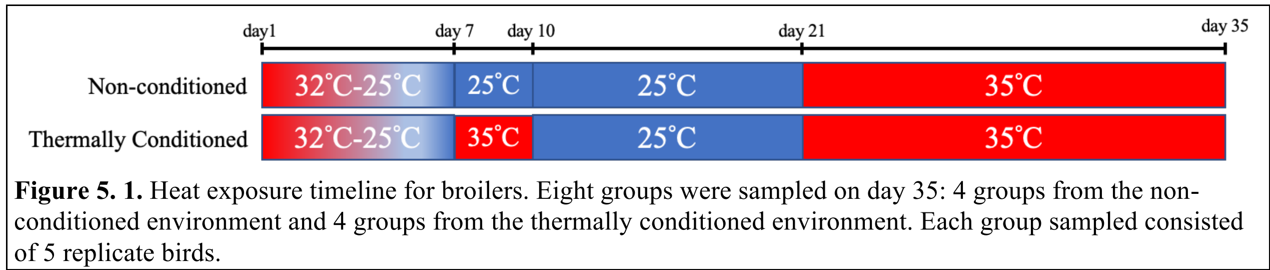

Figure 5. 1. Heat exposure timeline for broilers. Eight groups were sampled on day 35: 4 groups from the non-conditioned environment and 4 groups from the thermally conditioned environment. Each group sampled consisted of 5 replicate birds.

Table 5. 1. Forward and reverse primers used for RT-qPCR validation of RNA-sequencing.

Gene	Forward Primer	Reverse Primer
TGM4	5′ GTCAAGGTACTTTCCAGTGTGGC 3’	5′ GCGTTCACTTCCGCGTACA 3’
PLK3	5′ AAGGCCAAGAAGGGCTCTCT 3’	5′ CGGCAGATGGACGTCTTCA 3’
PAG1	5′ CGCCGTCACCACCATATCTT 3’	5′ GCATCAGCATGTCCTGGAACC 3’
GLO1	5′ CCGAGGATTTGGGCACATT 3’	5′ ATGCTCCTTGCTGAAGCTCAT 3’
ROCK2	5′ ACACTGAAAACCCAGGCTGT 3’	5′ TCCCATAGGCTCCACAGGAA 3’
CA2	5′ TGACCCTACTGGACTGCTGC 3’	5′ TGACAGTGATGGGCTCCTTC 3’
ETNK1	5′ ACTCTGGATACAACTACCTGGC 3’	5′ AAGAAGTGGGAAGCCAGTGC 3’
ATF4	5′ CACCCCAACACAGTCCTACC 3’	5′ ATAGGGTTTGGACCGCACAG 3’
NCF2	5′ TCTTTGCTTGCGAGGTGGT 3’	5′ TTTCTGGTGTCTTGGGCCTG 3’
β-actin	5′ AGACATCAGGGTGTGATGGTTGGT 3’	5′ TCCCAGTTGGTGACAATACCGTGT 3’

Table 5. 2. Effect of conditioned status, methionine source, and dietary inclusion of methionine on broiler chicken body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), circulating triiodothyronine (T_3), thyroxine (T_4), and corticosterone (CORT) during the starter phase.

Environment[1]	Source[2]	Level[3]	BWG (g)	FI (g)	FCR	T_3 (ng/ml)	T_4 (ng/ml)	CORT (pg/ml)
NC	DLM	100	174.3^{a}	134.8^{a}	0.77	2.18^{a}	8.31	7,610.8^{ab}
NC	DLM	140	170.8^{a}	125.6^{ab}	0.74	2.32^{a}	9.53	2,300.4^{ab}
NC	MHA	100	164.9^{ab}	130.8^{ab}	0.79	2.23^{a}	9.8	3,938.8^{ab}
NC	MHA	140	151.9^{c}	114.9^{b}	0.76	2.36^{a}	8.53	6,338.7^{ab}
TC	DLM	100	157.1b^{c}	114.6^{b}	0.73	1.70^{ab}	8.48	9,579.4^{a}
TC	DLM	140	162.5^{abc}	123.4^{ab}	0.76	1.73^{ab}	7.66	1,775.2^{b}
TC	MHA	100	156.1^{bc}	122.9^{ab}	0.79	1.31^{b}	9.06	5,257.2^{ab}
TC	MHA	140	154.7^{bc}	116.5^{b}	0.75	1.27^{b}	8.33	3,565.3^{ab}
SEM			2.8	3.8	0.02	0.16	1.39	1,634.1
Main effect of environment								
NC			165.5^{a}	126.5^{a}	0.76	2.27^{a}	9.04	5,047.2
TC			157.6^{b}	119.3^{b}	0.76	1.50^{b}	8.38	5,044.3
SEM			1.4	1.9	0.01	0.09	0.7	842.2
Main effect of methionine source								
DLM			166.2^{a}	124.6	0.75	1.98	8.5	5,316.4
MHA			156.9^{b}	121.3	0.77	1.79	8.93	4,775.1
SEM			1.4	1.9	0.01	0.09	0.7	842.2
Main effect of methionine level								
100			163.1	125.8^{a}	0.77	1.85	8.91	6,596.5^{a}
140			160.0	120.1^{b}	0.75	1.92	8.51	3,494.9^{b}
SEM			1.4	1.9	0.01	0.09	0.7	842.2
Probability								
Model			<0.0001	0.003	0.3388	0.0003	0.9731	0.0304
Environment			0.0002	0.0114	0.6042	<0.0001	0.5191	0.9981
Source			<0.0001	0.2253	0.1241	0.1445	0.6739	0.6527
Level			0.1203	0.0412	0.2254	0.625	0.6988	0.0142
Environment x Source			0.0168	0.1446	0.8883	0.0803	0.8535	0.5475
Environment x Level			0.125	0.0145	0.2422	0.5903	0.7154	0.1771
Source x Level			0.0434	0.0482	0.2994	0.8846	0.5586	0.0069
Environment x Source x Level			0.7461	0.4292	0.2386	0.8978	0.5325	0.7396

[1]NC = non-conditioned; TC = thermally conditioned
[2]DLM = DL-methionine; MHA = D,L-2-hydroxy-(4-methylthio) butanoic acid
[3]100 = at-requirement methionine; 140 = above-requirement methionine
Mean values within a row with no common superscript differ significantly ($p<0.05$)

Table 5. 3. Effect of conditioned status, methionine source, and dietary inclusion of methionine on broiler chicken body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), circulating triiodothyronine (T_3), thyroxine (T_4), and corticosterone (CORT) during the grower phase.

Environment[1]	Source[2]	Level[3]	BWG (g)	FI (g)	FCR	T_3 (ng/ml)	T_4 (ng/ml)	CORT (pg/ml)
NC	DLM	100	608.45^{ab}	927.5^{a}	1.50	2.53	8.01	5,224.5
NC	DLM	140	639.9^{a}	896.9^{a}	1.40	2.20	5.73	5,700.6
NC	MHA	100	597.5^{abc}	901.7^{a}	1.52	1.88	6.95	3,870.7
NC	MHA	140	600.4^{abc}	883.1^{ab}	1.50	2.68	7.28	6,819.0
TC	DLM	100	522.8^{bc}	824.9^{ab}	1.58	1.95	7.70	5,544.1
TC	DLM	140	551.9^{abc}	823.6^{ab}	1.49	2.32	7.37	3,604.7
TC	MHA	100	537.4^{bc}	785.9^{b}	1.47	2.14	7.11	6,308.2
TC	MHA	140	513.9^{c}	848.5^{ab}	1.65	1.84	7.66	4,939.7
SEM			20.2	24.4	0.07	0.18	0.93	1,071.5
Main effect of environment								
NC			611.6^{a}	902.3^{a}	1.49	2.32	6.99	5,403.7
TC			531.5^{b}	820.7^{b}	1.55	2.06	7.50	5,099.2
SEM			10.1	12.2	0.03	0.09	0.47	493.9
Main effect of methionine source								
DLM			580.7	868.2	1.50	2.24	7.20	5,018.5
MHA			562.3	854.8	1.54	2.14	7.25	5,484.4
SEM			10.1	12.2	0.03	0.09	0.47	493.9
Main effect of methionine level								
100			566.5	860.0	1.53	2.12	7.44	5,236.9
140			576.5	863.0	1.51	2.26	7.01	5,266.0
SEM			10.1	12.2	0.03	0.09	0.47	493.9
				Probability				
Model			0.0003	0.0024	0.3045	0.0228	0.7951	0.2876
Environment			<0.0001	<0.0001	0.2376	0.0543	0.4974	0.6706
Source			0.2027	0.4407	0.5239	0.39	0.9475	0.5161
Level			0.4867	0.8611	0.7866	0.3014	0.5288	0.9675
Environment x Source			0.6364	0.7124	0.8771	0.8013	0.7746	0.417
Environment x Level			0.6184	0.1166	0.1938	0.4521	0.4299	0.0244
Source x Level			0.1623	0.2777	0.0435	0.3939	0.2069	0.2919
Environment x Source x Level			0.6762	0.4557	0.4301	0.0017	0.5312	0.5077

[1]NC = non-conditioned; TC = thermally conditioned
[2]DLM = DL-methionine; MHA = D,L-2-hydroxy-(4-methylthio) butanoic acid
[3]100 = at-requirement methionine; 140 = above-requirement methionine
Mean values within a row with no common superscript differ significantly ($p<0.05$)

Table 5. 4. Effect of conditioned status, methionine source, and dietary inclusion of methionine on broiler chicken body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), circulating triiodothyronine (T_3), thyroxine (T_4), and corticosterone (CORT) during the finisher phase.

Environment[1]	Source[2]	Level[3]	BWG (g)	FI (g)	FCR	T_3 (ng/ml)	T_4 (ng/ml)	CORT (pg/ml)
NC	DLM	100	838.6^{ab}	1,747.5^{a}	2.12	0.82	6.33	3,978.9
NC	DLM	140	926.1^{a}	1,873.7^{a}	2.02	0.77	5.51	1,783.2
NC	MHA	100	966.9^{a}	1,848.6^{a}	1.95	0.83	4.36	2,511.1
NC	MHA	140	922.3^{a}	1,780.3^{a}	1.96	0.94	5.63	2,539.6
TC	DLM	100	721.4^{abc}	1,374.1^{b}	1.93	0.71	5.14	2,478.1
TC	DLM	140	557.7^{c}	1,208.7^{b}	2.25	0.82	5.31	4,463.9
TC	MHA	100	656.5^{bc}	1,329.1^{b}	2.10	0.98	5.53	1,780.7
TC	MHA	140	598.5^{bc}	1,221.7^{b}	2.11	0.81	5.62	4,163.5
SEM			55.60	44.7	0.12	0.1	1.0	650.0
Main effect of environment								
NC			913.5^{a}	1812.5^{a}	2.01	0.84	5.46	2,703.2
TC			633.5^{b}	1283.4^{b}	2.10	0.83	5.4	3,221.6
SEM			27.8	22.4	0.06	0.05	0.48	324.9
Main effect of methionine source								
DLM			760.9	1,550.9	2.08	0.78	5.57	3,176.0
MHA			786.0	1,544.9	2.03	0.89	5.28	2,748.7
SEM			27.8	22.4	0.06	0.05	0.48	324.9
Main effect of methionine level								
100			795.8	1,574.8	2.02	0.83	5.34	2,687.2
140			751.1	1,521.1	2.08	0.83	5.52	3,237.5
SEM			27.8	22.4	0.06	0.05	0.48	324.9
			Probability					
Model			<0.0001	<0.0001	0.5523	0.6076	0.9483	0.0273
Environment			<0.0001	<0.0001	0.3335	0.8686	0.9273	0.2752
Source			0.5273	0.8493	0.5358	0.1347	0.6734	0.367
Level			0.2624	0.0972	0.4694	0.9879	0.7933	0.2473
Environment x Source			0.3507	0.7556	0.4292	0.7924	0.3528	0.8791
Environment x Level			0.1003	0.0126	0.2296	0.6181	0.9426	0.0014
Source x Level			0.8676	0.2871	0.5438	0.6785	0.4647	0.1703
Environment x Source x Level			0.1387	0.0528	0.2164	0.137	0.4296	0.3353

[1]NC = non-conditioned; TC = thermally conditioned
[2]DLM = DL-methionine; MHA = D,L-2-hydroxy-(4-methylthio) butanoic acid
[3]100 = at-requirement methionine; 140 = above-requirement methionine
Mean values within a row with no common superscript differ significantly ($p<0.05$)

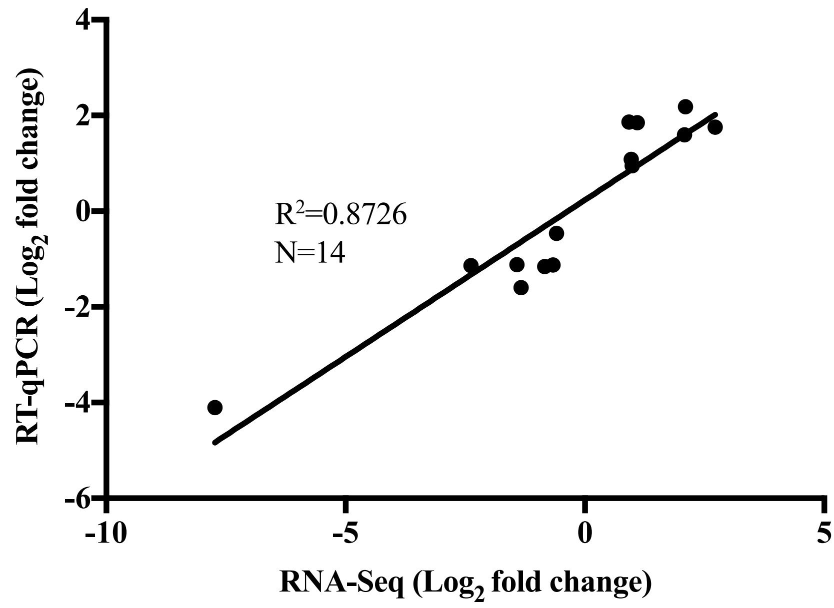

Figure 5. 2. Regression analysis of the log_2 fold change values between RNA-sequencing and RT-qPCR.

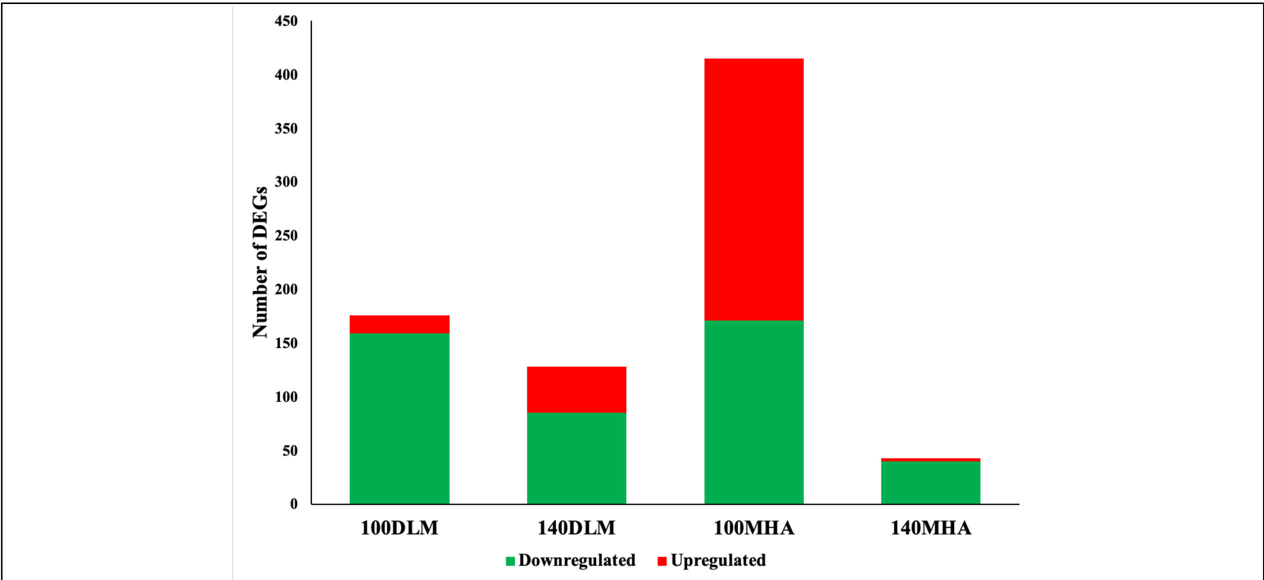

Figure 5. 3. Number of differentially expressed genes (DEGs) in the *Pectoralis major* of thermally conditioned chickens subjected to heat stress and supplemented with either at- (100) or above-requirement (140) levels of either D,L-methionine (DLM) or 2-hydroxy-4-methylthio-butanoic acid (MHA) at 35 days of age.

Table 5. 5. Upregulated genes in *Pectoralis major* of chickens whose diets were supplemented with at-requirement DLM and thermally conditioned at 35 days of age.

Gene	Log_2 fold change	Adjusted P-value
GREM1	2.71928829	5.45E-05
STXBP5L	2.05906875	0.02073339
GRB14	1.6704062	0.03627375
NSG1	1.47566549	0.02238713
SDK1	1.30331044	0.01852838
ATOH8	1.23963103	0.00833923
METTL21C	1.21374537	0.03672185
HABP4	1.12859289	0.02601149
GLO1	1.09709429	0.04127034
TESK2	0.96293634	0.02396193
GOLM1	0.90595756	0.03129007
GART	0.89532082	0.04365311
PALMD	0.77539093	0.02462019
ACADSB	0.6279929	0.04776518
CYP2U1	0.59848878	0.04608398
SYNCRIP	0.51999946	0.03438731
SAMD4A	0.48794684	0.0211449

Table 5. 6. Top 50 downregulated genes in *Pectoralis major* of chickens whose diets were supplemented with at-requirement DLM and thermally conditioned at 35 days of age.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
TGM4	-7.7304347	0.00111979	*GGCL1*	-3.5365079	0.03674852
MMP7	-6.6943209	0.01852838	*Agrp*	-3.5250354	0.04290762
RSPO4	-6.493767	0.02601149	*F2RL1*	-3.4863445	0.01096904
PTX3	-6.1939555	0.00833923	*SPIRE2*	-3.4488678	0.03506702
FGG	-5.9851931	0.01777006	*cPAD2*	-3.4466881	0.0211449
CSTA	-5.8198374	0.00111979	*ANGPTL4*	-3.4108399	0.01694293
HTR3A	-5.4035374	0.02911684	*HEPHL1*	-3.3547395	0.03627375
APOA4	-4.9621659	0.00833923	*ARL4C*	-3.3032037	0.02462019
SERPINB2	-4.7563341	0.01918715	*HPX*	-3.2458956	0.02238713
ATP6V0D2	-4.5850652	0.01470439	*CRYBB3*	-3.23981	0.03672185
MAP7	-4.5329195	0.00833923	*PCSK1*	-3.2311901	0.02974043
VRTN	-4.5220299	0.00833923	*DUSP5*	-3.2105323	0.01777006
TNC	-4.5133349	0.00320648	*CSF3R*	-3.1934068	0.03835863
IL20RA	-4.3218347	0.02974043	*DUSP4*	-3.1379837	0.02393602
NCF2	-4.2218987	0.01096904	*PROSER2*	-3.1007418	0.02462019
APOA5	-4.1920014	0.01096904	*VDR*	-3.0958398	0.01852838
DCSTAMP	-4.1035382	0.03674852	*FOSL2*	-3.0816375	0.01658626
MSX1	-4.0879767	0.02230047	*CRH*	-3.0808522	0.02102906
KIF14	-4.0483653	0.00111979	*SLC17A9*	-3.0125049	0.01954172
LGALS3	-3.8869604	0.01852838	*ETV4*	-2.9356091	0.01852838
TNFRSF6B	-3.8337613	0.03129007	*NR4A3*	-2.9173278	0.01777006
UNC13A	-3.6798135	0.01997461	*GADD45B*	-2.8947509	0.01096904
RRAD	-3.6727695	0.0206599	*CHST11*	-2.8343914	0.02601149
ADAMTS4	-3.64298	0.01852838	*MMP9*	-2.8225399	0.04791323
SAXO1	-3.5550138	0.01777006	*ELF3*	-2.7800023	0.03672185

Table 5. 7. Top KEGG pathways for differentially expressed genes of chickens whose diets were supplemented with at-requirement DLM and thermally conditioned at 35 days of age.

KEGG Term	Associated Genes
Downregulated	
Phagosome	*ATP6V0D2, NCF2, RAB7B, TCIRG1, THBS1, TUBAL3, TUBB6*
ECM-receptor interaction	*ITGA3, SDC4, THBS1, TNC*
Jak-STAT signaling pathway	*CSF3R, IL13RA2, IL20RA, MYC*
Purine metabolism	*AK4, GDA, POLR3A*
Focal adhesion	*ITGA3, THBS1, TLN1, TNC*

Table 5. 8. Top 50 upregulated genes in *Pectoralis major* of chickens whose diets were supplemented with at-requirement MHA and thermally conditioned at 35 days of age.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
FMO4	1.70330417	0.03956349	*ND5*	0.8168887	0.00462154
RHOBTB1	1.55550801	0.01747478	*MT-ND4L*	0.78122162	0.00657414
PAK5	1.4187549	0.04261705	*ST3GAL6*	0.7806729	0.01470842
TMEM79	1.38122971	0.01105391	*SCG5*	0.77468803	0.03695358
SBK2	1.35484578	0.01207915	*ZDHHC16*	0.77449154	0.02439797
CA2	1.31570021	0.00634354	*MT-CYB*	0.76310903	0.00220409
DOK5	1.28078556	0.00747803	*IDH3G*	0.7532141	0.00220409
MME	1.26932416	0.01091964	*SERGEF*	0.74888743	0.02138284
MT-ND2	1.22645311	0.00803972	*MT-ND4*	0.72654384	0.01245715
LPAR6	1.2088409	0.02751111	*COQ9*	0.72027841	0.02904692
C15orf59	1.11557555	0.03272859	*ECHDC2*	0.71501006	0.01245715
DUSP26	1.10501679	0.04066217	*RNF141*	0.71135248	5.83E-05
SPTBN5	1.03666705	0.00015669	*DJ-1*	0.70755782	0.02138284
NRGN	1.0243035	5.16E-07	*CTNNA3*	0.69864674	0.02368933
MT-ND3	1.01572723	0.00476519	*TFG*	0.69538357	0.03884168
HSP90AB1	1.01398831	0.00541221	*BCKDHB*	0.69140853	0.00298253
CXXC5	1.01311129	0.0003926	*DCAF17*	0.68903584	0.03185482
FBXO9	0.98449644	0.00015669	*MSRB3*	0.68851932	0.01393973
GLO1	0.96645898	0.00476519	*MTRF1*	0.6884994	5.83E-05
SLC35G2	0.96333889	0.00532027	*NAP1L4*	0.68227576	0.00641645
DNAJB5	0.9449043	0.01487004	*SORD*	0.68172245	0.00534553
ND1	0.94323569	0.04140062	*ATOH8*	0.67768241	0.03074179
MSS51	0.91336449	0.00575931	*HSDL2*	0.67302407	0.04981729
SLC16A3	0.9025677	0.04981729	*PDGFRL*	0.67197978	0.03782934
MT-ATP8	0.8574101	0.00056909	*TNNT3*	0.66945591	0.00657414

Table 5. 9. Top 50 downregulated genes in *Pectoralis major* of chickens whose diets were supplemented with at-requirement MHA and thermally conditioned at 35 days of age.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
PMP2	-4.3486973	0.02061112	*MMRN1*	-1.4524366	0.00541221
C1QL2	-4.2719418	0.03782934	*MST1R*	-1.4325643	0.00977406
SBK3	-3.7142895	1.31E-15	*RFESD*	-1.4297024	0.02061112
PDK4	-3.5932274	0.00295948	*GCGR*	-1.4289041	0.01390252
MMP9	-2.9074817	0.00718559	*CES1*	-1.4267811	0.00534553
HBAA	-2.8722421	0.02127224	*SYPL1*	-1.3925912	0.03074179
NXNL1	-2.6039245	0.00044781	*THSD4*	-1.3396277	0.01096719
MFSD2B	-2.501697	0.02609807	*ADGRD1*	-1.3017284	0.00018038
U1	-2.4041168	0.00603467	*DZIP1*	-1.2993031	0.00032619
GDAP1	-2.3877359	0.02148727	*MFSD7*	-1.2638333	0.03438476
H2B-VII	-2.2443586	0.01860289	*TTLL1*	-1.2599527	0.02974708
TRIM27.1	-2.2013985	0.00834997	*DUSP15*	-1.2551703	0.01568883
DUSP5	-2.1063419	0.02314485	*PEX11A*	-1.2518708	0.04065655
TERT	-2.0193022	0.03438476	*AGPAT2*	-1.2481182	0.00316459
C7	-1.9392053	0.00570546	*KAZALD1*	-1.239962	0.03015121
SLC1A6	-1.8799294	0.01213067	*PTGS2*	-1.2366348	0.01053751
SLC4A1	-1.7998638	0.01390339	*SLC27A1*	-1.2350485	0.00052733
NETO2	-1.7734115	0.00467842	*PTCHD3*	-1.2085653	0.01369972
GADD45B	-1.6639437	0.00060805	*FABP4*	-1.2075698	0.0362652
PTN	-1.6291323	0.00763312	*CIDEA*	-1.1795911	0.0018704
MPZ	-1.5931763	0.03043248	*RELN*	-1.1319495	0.00020109
EGR1	-1.57478	0.03046245	*SLC45A4*	-1.1276431	0.00476519
ACKR4	-1.498532	0.00555889	*TUBB1*	-1.124849	5.83E-05
IGDCC3	-1.4794738	0.03358442	*PRKAR2B*	-1.1204156	0.04574586
DGKQ	-1.4746781	0.02191651	*CEBPB*	-1.0796338	0.03150941

Table 5. 10. Top KEGG pathways for differentially expressed genes of chickens whose diets were supplemented with at-requirement MHA and thermally conditioned at 35 days of age.

KEGG Term	Associated Genes
Upregulated	
Aminoacyl-tRNA biosynthesis	*AARS, CARS, DARS, EPRS, GATB, NARS2, QARS, SARS, TARS*
Regulation of actin cytoskeleton	*ACTN2, CDC42, CFL2, PFN2, PIP4K2A, RDX*
Oxidative phosphorylation	*ATP5I, COX3, ND1, ND5, NDUFA12, NDUFB3, NDUFV2, UQCRC2*
Calcium signaling pathway	*CALM, PHKA1, PHKG1, VDAC1*
GnRH signaling pathway	*ATF4, CALM, CDC42*
Downregulated	
Focal adhesion	*COL4A5, COL4A6, COMP, ITGAV, ITGB4, PDGFRA, RELN*
Glycerophospholipid metabolism	*AGPAT2, CDS2, DGKE, DGKQ, ETNK1, PLA2G15*
ECM-receptor interaction	*COL4A5, COL4A6, COMP, ITGAV, ITGB4, RELN*
AGE-RAGE signaling pathway in diabetic complications	*COL4A5, COL4A6, EGR1, JAK2, THBD*
Phosphatidylinositol signaling system	*CDS2, DGKE, DGKQ*

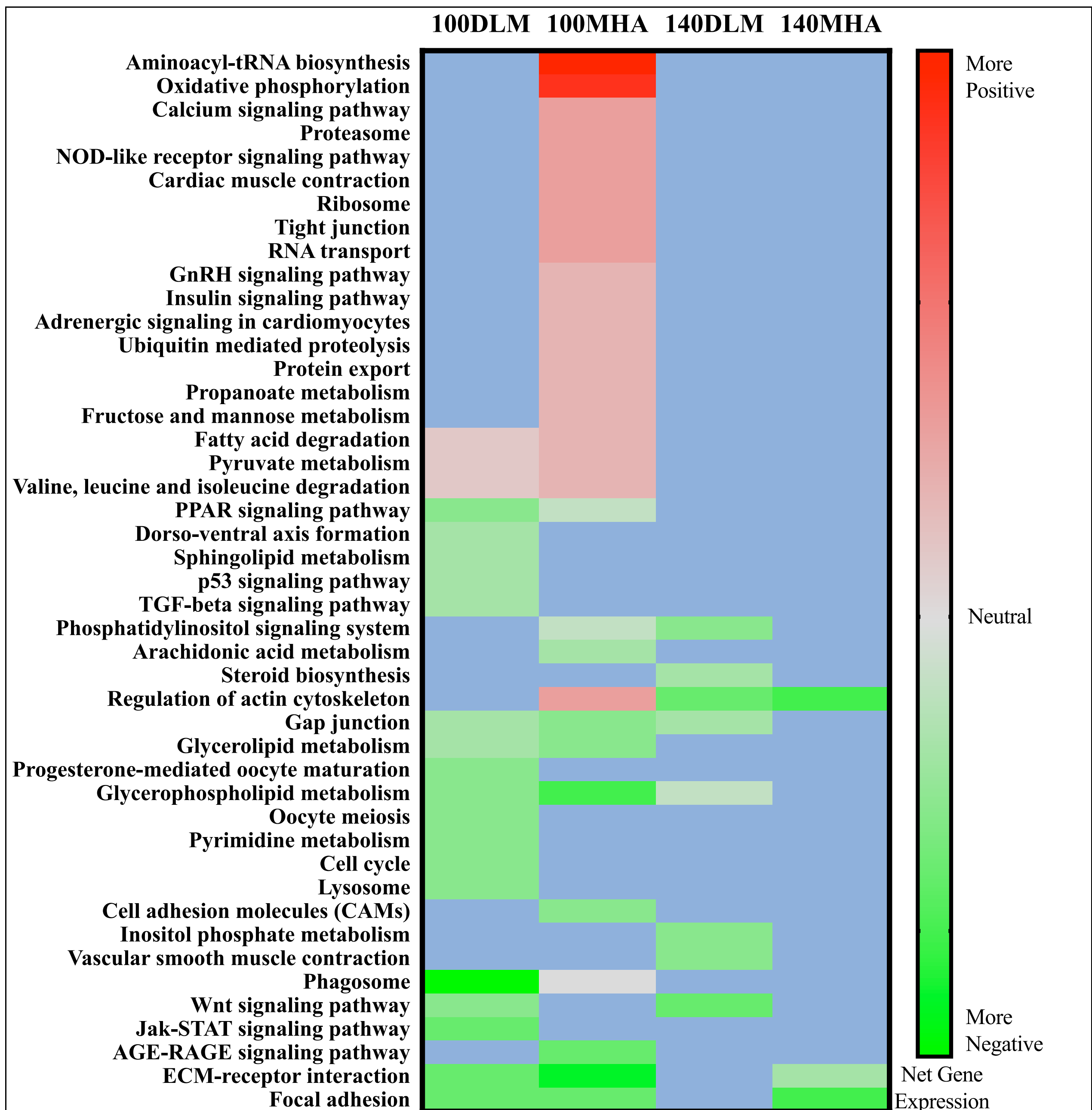

Figure 5. 4. Net gene expression of KEGG terms in the *Pectoralis major* of thermally conditioned broiler chickens reared at 35°C during the finisher phase. For each individual KEGG term, the number of positively (+1) and negatively (-1) differentially expressed genes were added to obtain net expression for thermally conditioned broilers fed at-requirement D,L-methionine (100DLM), at-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (100MHA), above-requirement D,L-methionine (140DLM), and above-requirement D,L-2-hydroxy-(4-methylthio) butanoic acid (140MHA). Blue fill indicates non-enriched pathways.

Table 5. 11. Upregulated genes in *Pectoralis major* of chickens whose diets were supplemented with above-requirement DLM and thermally conditioned at 35 days of age.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
GADD45B	2.80191135	0.01347702	*YPEL5*	0.840778	0.01719892
NOXRED1	2.21976844	0.0222223	*CUBN*	0.82148819	0.00285498
ADPRHL1	2.10328663	0.00499472	*U6*	0.78383062	0.03987256
CEBPB	1.78535063	0.00499472	*TP63*	0.7709323	0.04001461
PM20D2	1.68921794	0.01128091	*RPIA*	0.71811262	0.03814785
DDIT4	1.68736486	0.01258666	*NFE2L1*	0.71299792	0.04001461
ALPK1	1.50701831	0.03546503	*BTG2*	0.69999459	0.03546503
NFIL3	1.49811194	0.01500522	*TMEM201*	0.68962497	0.01586767
7SK	1.30856928	0.00959179	*KAZALD1*	0.67855384	0.04216666
PLA2G15	1.30179626	0.02219097	*SPECC1*	0.6768934	0.02281366
UCP3	1.26038153	0.04001461	*SMAD1*	0.60928828	0.04210887
TMEM100	1.23198557	0.0222223	*ASB5*	0.58418136	0.04001461
STK17A	1.14669634	0.0222223	*FERMT2*	0.55727335	0.00074974
PPARD	1.11601448	0.03329922	*SETD2*	0.53016949	0.04301459
SLC22A23	1.0967577	0.00013995	*AKAP9*	0.51807839	0.04900824
FOXO3	1.05318901	0.00923588	*RABGGTB*	0.51707012	0.04434123
SPSB1	1.01859995	0.03987256	*ASCC1*	0.49936863	0.02219097
POMK	1.00511707	0.02241828	*C10orf76*	0.48078932	0.0222223
NSG2	0.93985214	0.04025717	*TAOK1*	0.3760856	0.04210887
PLPPR5	0.91816015	0.01565682	*MBTPS1*	0.35893323	0.03015315
UBXN10	0.88890684	0.04471666	*EIF3A*	0.33674235	0.0222223
KIAA1549	0.88808838	0.03422731			

Table 5. 12. Top downregulated genes in *Pectoralis major* of chickens whose diets were supplemented with above-requirement DLM and thermally conditioned at 35 days of age.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
ATP6V1G3	-2.5052683	1.53E-07	*NMRK2*	-1.021534	0.00401846
EMILIN3	-2.2903425	8.12E-05	*CNTNAP1*	-1.0185205	0.00698055
FGFR2	-2.0437922	0.00816456	*FDPS*	-1.0180951	0.0222223
SFRP2	-1.6766012	0.02549355	*THSD4*	-1.0081126	0.00285498
FBN3	-1.5746818	0.00408976	*ITGA9*	-1.0023799	0.0127794
FBLN7	-1.5721152	1.08E-08	*PTPN21*	-0.9984465	0.00868534
Fgf16	-1.5591629	0.00157313	*PLXDC1*	-0.9653639	0.01128091
BRK-3	-1.548426	1.08E-08	*ITGB1BP2*	-0.9627223	0.00055512
GATM	-1.5039064	0.01125353	*STARD4*	-0.9432936	0.02825985
COL21A1	-1.434227	0.03859242	*PLCB1*	-0.9416323	0.01565682
LSS	-1.4226525	0.01300207	*SIRT5*	-0.9343712	0.00757028
GFRA2	-1.3776984	0.03460248	*C1QTNF2*	-0.898844	0.01892513
FNDC1	-1.3719667	0.04301459	*INPP4B*	-0.8947158	0.00831292
PAG1	-1.3323468	6.83E-06	*SC5D*	-0.8877518	0.02681587
COL14A1	-1.3238044	0.03669257	*CLIP4*	-0.8869759	0.03987256
RGCC	-1.2751325	0.04471666	*KLHL23*	-0.8644658	0.04471666
COL28A1	-1.2168731	0.04210887	*ABI3BP*	-0.8597442	0.02580065
GLS	-1.1918758	8.12E-05	*XYLT1*	-0.8503272	0.02557409
PXDN	-1.1552524	0.00959179	*NPR3*	-0.8397053	0.03814785
SLC7A5	-1.1112804	0.01378064	*FAM129A*	-0.827732	0.03814785
SLC4A7	-1.1111847	0.00012049	*PITPNM3*	-0.8267534	0.02773505
ACOX2	-1.1070921	0.00053684	*PTK2*	-0.7904679	0.00706882
CA2	-1.0906416	0.0222223	*HMCN1*	-0.78609	0.03159456
SYNE3	-1.077065	0.00959179	*ZCCHC24*	-0.7713962	8.12E-05
NT5DC1	-1.0428737	0.00959179	*DENND1B*	-0.7522573	7.50E-06

Table 5. 13. Top KEGG pathways for differentially expressed genes of chickens whose diets were supplemented with above-requirement DLM and thermally conditioned at 35 days of age.

KEGG Term	Associated Genes
	Downregulated
Wnt signaling pathway	*GPC4, LRP6, PLCB1, ROCK2, SFRP2*
Regulation of actin cytoskeleton	*FGFR2, ITGA9, PTK2, ROCK2*
Inositol phosphate metabolism	*INPP4A, INPP4B, PLCB1*
Glycerophospholipid metabolism	*ETNK1, GPCPD1*
Phosphatidylinositol signaling system	*INPP4A, INPP4B, PLCB1*

Table 5. 14. Top differentially expressed genes in *Pectoralis major* of chickens whose diets were supplemented with above-requirement MHA and thermally conditioned at 35 days of age.

Gene	Log_2 fold change	Adjusted P-value	Gene	Log_2 fold change	Adjusted P-value
		Upregulated			
CILP	0.76312227	5.53E-05	*RNASEH2B*	0.52958533	0.0288819
CWF19L2	0.54550047	0.01803583			
		Downregulated			
BRK-3	-1.693114	2.02E-19	*JAK2*	-0.7606646	1.05E-05
PAG1	-1.388323	0.01209018	*PHIP*	-0.7247296	0.00216954
PTPN21	-1.3374869	9.30E-07	*DENND4A*	-0.7216725	0.0069075
NPR3	-1.3044043	0.00693955	*DICER1*	-0.7058029	0.02680945
MYLK4	-1.212804	1.05E-05	*ZBTB34*	-0.6740764	0.00291551
ZCCHC24	-1.1508063	3.92E-09	*CELF1*	-0.6645165	0.00707265
LNPEP	-1.1446573	0.00707265	*CLEC16A*	-0.6499887	0.00662339
DENND1B	-1.1056477	1.61E-06	*USP53*	-0.6432836	0.00783515
FRS2	-1.0742321	0.00627053	*LMBRD2*	-0.6312146	0.0302227
BBX	-0.9826035	4.44E-06	*ATP7A*	-0.6253054	8.69E-05
THSD4	-0.9803783	0.01515779	*TRIP11*	-0.6205756	0.00024072
ETNK1	-0.9478266	0.00707265	*SH3PXD2A*	-0.5994891	0.00442674
ITGAV	-0.9200457	0.02498924	*FRMD5*	-0.5728091	0.00925227
LRP6	-0.8457636	0.00044458	*AKAP11*	-0.5701206	0.02418766
QSER1	-0.8384667	4.68E-09	*RC3H2*	-0.5452722	0.01020738
FOXN3	-0.7945691	0.00442674	*SUOX*	-0.5121093	0.00014505
ITGA1	-0.7900638	0.00464211	*DHX29*	-0.5107451	0.0207594
ROCK2	-0.7773456	0.02680945	*AFF4*	-0.3981141	0.000144
PTPRB	-0.7683828	0.00055322	*UBR2*	-0.3891487	0.01632918
ZBED4	-0.761911	0.00442674	*ACTN4*	-0.3519141	0.02680945

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CHAPTER 6

DISCUSSION

Heat continues to be a major environmental stressor and causes great economic loss to poultry producers (St-Pierre et al. 2003) through high bird mortality and reduced performance (Hurwitz et al. 1980; Attia et al. 2011; Sohail et al. 2012; Awad et al. 2018; Goo et al. 2019; Lin Law et al. 2019). Methionine is thought to benefit a bird succumb to heat stress, as research shows improved performance of broilers when dietary methionine is increased (Ojano-Dirain and Waldroup 2002; Corzo et al. 2003; Gonzalez-Esquerra and Leeson 2005). Today, methionine is included in poultry diets through either DL-methionine (DLM) or as a methionine analogue (MHA). Results vary regarding broiler performance in relation to methionine source (Swick and Pierson 1988; Balnave and Oliva 1990). Recently, Lee et al. (2021) studied the transcriptome of heat stressed broilers fed either D-methionine or L-methionine. Gene expression differed between broilers fed different sources of methionine, indicating methionine source has an impact on molecular processes of a broiler.

The first objective of this study was to investigate heat stress's effect on the transcriptome of broilers fed either DLM or MHA at either requirement or above-requirement levels. The *Pectoralis major* was sampled after broilers were raised at 35°C for 3, 14, and 28 days. Overall, it seems heat stress has the largest effect on the mitochondria and electron transport, aerobic respiration, the cell cycle, the actin cytoskeleton, and proteolysis in broiler breast muscle. Heat stress deprives tissue of oxygen, creating a hypoxic environment (Hall et al. 1999). This results in mitochondrial dysfunction (Downs and Heckathorn 1998; Monti et al. 2001; Zhao et al. 2006), generating superoxide and other reactive oxygen species (ROS). This work shows heat stress

induced downregulation of genes related to oxidative phosphorylation, possibly contributing to increased oxidative stress. Increasing DLM inclusion in the diet of heat stressed broilers decreased the number of differentially expressed genes related to the ETC after 28 days of heat stress. In response to the possible reduction of ROS, broilers fed above-requirement DLM showed a significant reduction in differential expression of genes related to ubiquitin mediated proteolysis compared to broilers fed at-requirement DLM. The ubiquitin proteasome pathway (UPP) is activated under oxidative stress (Medicherla and Goldberg 2008; Lee et al. 2010; Manohar et al. 2019). The slight improvement in performance of broilers fed above-requirement methionine could be attributed to this, as well as reduced apoptosis seen in those same broilers.

Interestingly, genes regulating the actin cytoskeleton were upregulated by heat stress at a younger age, while heat stress for 28 days downregulated genes related to the actin cytoskeleton. Again, increasing methionine inclusion resulted in fewer differentially expressed genes. This led to the development of our second objective, which investigated transcriptome differences between broilers subjected to heat stress during just one phase of production: the starter, grower, or finisher phase. Indeed, regulation of the actin cytoskeleton was positively expressed when broilers are subjected to heat stress during the grower phase. Actin is highly susceptible to oxidative stress (Dalle-Donne et al. 2002; Dalle-Donne et al. 2003), and the reorganization and polymerization of actin could indicate younger broilers are more adapt to dealing with heat stress. Indeed, high environmental temperatures do not have a profound effect on the performance of young chicks (Inês Müller Fernandes et al. 2013). This could be why the UPP is not highly influenced by heat stress during the starter or grower phases.

Because broilers have a higher tolerance for heat stress at a younger age, early-age thermal conditioning has been studied to better acclimate poultry to hot environments. By exposing an

organism to a challenging environmental condition, molecular mechanisms and systems may establish parameters based those conditions, better suiting the organism for further challenge. (Modrey and Nichelmann 1992). Thermal conditioning has proven effective in curving the deleterious effects of heat stress on broiler performance (De Basilio et al. 2001; Yahav and McMurtry 2001), however this is highly dependent on age and longevity of thermal challenge (Ncho et al. 2021). For the final objective of this work, we used RNA-sequencing to investigate the molecular response to thermal conditioning in the *Pectoralis major*. Results from the study show thermally conditioning broilers from ages 7 to 10 days post-hatch did not improve broiler performance when subjected to heat stress from days 21 to 35 post-hatch. The transcriptome shows very few genes were differentially regulated between thermally conditioned broilers and non-conditioned broilers after both were subjected to the thermal challenge. This indicates thermal conditioning does not elicit strong molecular change, possibly because the length of thermal challenge was prolonged. Dietary methionine did, however, bring about some differential regulation. Thermally conditioned broilers fed at-requirement MHA upregulated both protein anabolic and catabolic processes compared to non-conditioned broilers. Additionally, actin regulation was positively regulated, which agrees with increased translation of proteins in those broilers (Halevy et al. 2001; Ouchi et al. 2021). MHA has shown to be more beneficial in defending against oxidative stress and apoptosis (Pan et al. 2016; Pan et al. 2017). It could be the benefit of MHA in combination with thermal conditioning creates a more favorable environment for protein synthesis, however this did not translate to improved performance.

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CHAPTER 7

CONCLUSION

The health, wellbeing, and growth of broilers in hot environments is an important topic, as heat stress contributes significant economic loss to poultry producers. The preference for a broiler to rest and limit nutrient intake is detrimental to growth, while the stress initiated by high environmental temperatures harms cellular processes and ultimately the health of the bird. Through nutritional and environmental intervention, producers hope to limit performance loss and improve the health of the bird. This work consistently showed prolonged heat stress greatly reduces the growth of broilers. High environmental temperatures during the starter phase of broiler production did not impact performance, however a hot environment limited feed intake and growth of broilers during the grower and finisher phases. Based on our work, it seems heat stress induced protein degradation via ubiquitin mediated proteolysis in the breast muscle, possibly in response to damage done by ROS or to enable the survival and function of more critical cells and processes. Mitochondria are responsible for the majority of ROS production, and heat stress downregulated genes associated with the ETC. After experiencing a hot environment from days 7 to 21 post-hatch, broilers fed above-requirement DLM showed the most profound downregulation of genes related to oxidative phosphorylation. However, the downregulation of the ETC may be to limit ATP production, reducing the heat load of the bird.

Energy metabolism is somewhat inefficient, and energy is released as heat during both ATP synthesis and catabolism. By reducing cellular respiration, the bird reduces heat production. Interestingly, energy metabolism in broilers fed MHA was less effected by heat stress during the grower phase. After constant heat stress from days 7 to 35 post-hatch, regulation of cellular

respiration was more similarly downregulated, regardless of diet. The apparent reduction of ATP synthesis also influenced cellular growth and development, as regulation of the cellular cycle was greatly downregulated. Genes associated with proteolysis were mostly upregulated by heat stress during this time, supporting limited broiler weight gain seen in heat stressed groups.

One of the more prominent proteins in skeletal muscle is actin. Age at which heat stress is experienced greatly influenced how the actin cytoskeleton is regulated. At a younger age, heat stress likely induced actin reorganization and possibly strengthened the cytoskeleton. This is evident by the general upregulation of genes related to the actin cytoskeleton in all dietary treatments when subjected to heat stress. Dietary methionine inclusion additionally influenced actin and protein turnover during the grower phase. Protein synthesis was less influenced when broilers were fed above-requirement methionine, resulting in slightly improved weight gain. Methionine metabolism is additionally benefited by increased dietary methionine; however, this effect was only seen in broilers during the grower phase. Increasing dietary methionine in finisher diets did not benefit weight gain, the actin cytoskeleton, or protein turnover of heat stressed broilers.

One method thought to curb the detrimental effects of heat stress is thermal conditioning. In this work, thermal conditioning did not aid broiler performance when heat stressed from days 21 to 35 post-hatch. In fact, thermal conditioning in this work harmed broilers, hampering their performance when reared in both thermoneutral and hot environments. After thermal conditioning from days 7 to 10 post-hatch, broiler weight gain and feed intake were numerically reduced during the compensatory growth period. A notable to exception to this, however, was seen when broilers were fed above-requirement DLM. Circulating CORT, T3, and T4 levels were not affected by thermal conditioning in those broilers, possibly aiding weight gain during the starter and grower

phases. The positive effects of supplementing above-requirement DLM when thermally conditioned did not carry over when thermal challenge was initiated on day 22, however. At the conclusion of thermal challenge on day 35, all thermally conditioned broilers showed poorer weight gain than non-conditioned broilers. The breast muscle transcriptome after thermal challenge revealed thermal conditioning had the greatest molecular effect when broilers were fed at-requirement MHA. Eight genes related to oxidative phosphorylation were upregulated in 100MHA, indicating thermal conditioning aided mitochondrial function. In addition, it seems protein synthesis is reduced in non-conditioned broilers fed MHA, again indicating some benefit of thermal conditioning.

This work provides insight into the molecular response broiler breast muscle has to heat stress. Though broiler performance indicated dietary methionine source and inclusion level gave little benefit under heat stress, the transcriptome tells us a different story. Dietary methionine did influence a myriad of cellular processes in stressed birds, including oxidative phosphorylation, aerobic respiration, regulation of the actin cytoskeleton, and proteolysis. These processes are critical to skeletal muscle function, however further work should investigate other tissue. Much of nutrient metabolism is performed in the liver and intestine. Future studies should examine dietary methionine and heat stress's effect on these tissues to gain a more complete picture. Still, this work shows broiler performance should not be the only parameters tested in heat stress and nutrition studies.