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Identification of Molecular Signatures of Environmental and Nutritional Stress Responses in Poultry

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Identification of Molecular Signatures of Environmental and Nutritional Stress Responses in Poultry

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by

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ABSTRACT

Poultry production plays an important role in the food supply and security for billions of people. Poultry meat is considered the most efficient, nutritious source of protein, without religious taboos, and is relatively inexpensive. However, the poultry industry is facing substantial challenges. The global environmental temperature has experienced dramatic changes in the last few years. Modern broilers are not well adapted to environmental challenges (heat and cold stress), which have resulted in heavy economic loss in the poultry industry worldwide. In addition, the use of feed restriction regimens in management of breeders as well as the transportation of young chicks could induce additional metabolic disorders. Although seminal managerial thermo-conditioning, nutritional, and genetic efforts have been applied to partially alleviate these negative effects, poultry productivity still declines due to the adverse effects of these stressors and limited fundamental knowledge. There is, therefore, a critical need for mechanistic understanding of poultry response to these stressors which may help for the subsequent development of potential mechanism-based strategies to improve bird well-being, and thereby enhance poultry productivity and sustainability.

The present study aimed to identify new molecular signatures that are involved in environmental and nutritional stress responses in poultry. We found that chronic mild cold conditioning (CMCC) improves growth performance in young chicks via modulation of Adenosine mono phosphate- activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) pathways. In addition, we identified heat shock protein A9 (HSPA9) or glucose regulated protein (GRP75), orexin (ORX) and its related receptors (ORXR1 and 2), and Dicer 1 as new molecular markers involved in stress response in poultry. HSPA9/GRP75 was identified to be expressed differently in various tissues of Jungle fowl and quail, as well as avian cell lines and was affected by heat stress. Orexin and its related receptors are altered by heat and

oxidative stress in avian muscle both *in vivo* and *in vitro*. The ribonuclease type III, Dicer 1 which plays a key role in microRNA biogenesis, is regulated by nutritional stress (feed deprivation) in quail and chicken livers, and in immortalized chicken embryo liver (CEL-im) and neuroblastoma (SH-SY5Y) cell lines.

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DEDICATION

This edition of the Doctoral dissertation: “Identification of molecular signatures of environmental and nutritional stress responses in poultry” is dedicated to all master’s and doctoral students at the University of Arkansas.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PUBLISHED PAPERS

All of Chapters 3 and 4 come from the published papers:

NGUYEN, P. GREENE, E. ISHOLA, P. HUFF, G. DONOGHUE, A. BOTTJE, W. & DRIDI, S. (2015). Chronic Mild Cold Conditioning Modulates the Expression of Hypothalamic Neuropeptide and Intermediary Metabolic-Related Genes and Improves Growth Performances in Young Chicks. *PLoS ONE* **10**(11), e0142319.

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1. INTRODUCTION

Poultry production plays an important role in the food supply and food security for billions of people. Due to the high food demand of a rapidly growing human population, it is projected that poultry meat and egg consumption will increase by 73% by 2050 worldwide (Alexandratos & Bruinsma 2012). Poultry meat is considered an efficient nutritious source of protein, without religious taboos, and is relatively inexpensive (Hoffman & Falvo 2005; Hahn 2015). For instance, in early 2015, one pound of chicken meat was around $1 compared to $6.41 per pound for beef in the U.S. (Hahn 2015). However, the poultry industry is facing substantial challenges including: meeting this high global food demand, prevalence of metabolic disorders (white striping, woody breast, bacterial chondronecrosis with osteomyelitis) and the need to adapt to the environmental changes and pressures on natural resources due to climate change (Yegani 2011). Indeed, large and widespread heat waves have occurred in the last few years, and are projected to increase in the next decades. Modern broilers are not well adapted to environmental challenges, mainly heat stress, because they lack sweat glands and have a high metabolic activity as they are selected for high growth rate (Cahaner & Leenstra 1992; Havenstein et al. 1994; De Jong et al. 2002; Soleimani et al. 2011). Heat stress has resulted in heavy economic loss to the U.S. poultry, which is estimated to average $128 to $165 million per year (St-Pierre et al. 2003).

Conversely, several regions in the globe have witnessed extreme low winter temperatures and high precipitations (Walthall et al. 2012; Pachauri et al. 2014). This also has a strong negative effect on poultry production. In fact, a snow storm in China during 2008 has resulted in 19.55 million poultry deaths and an economic loss of about 14.5 million US dollars (Chen et al. 2014).

In addition, the use of feed restriction regimens in the management of breeders (Siegel 1980; Jayaprakash et al. 2016) as well as the transportation of young chicks could induce additional metabolic stress. This stress could affect growth performance and the immune system of birds leading to several metabolic disorders (Das et al. 2011). Therefore, there is a critical need to identify mechanism – based strategies to alleviate the adverse effects of environmental stress, improve animal welfare, and poultry production sustainability.

Although managerial (thermo-conditioning), nutritional, and genetic research work has been conducted to partially alleviate these negative effects; poultry productivity still declines during hot or cold weather. The above mentioned approaches have been applied with limited fundamental knowledge.

This study aimed to identify new molecular signatures involved in environmental and nutritional stress responses in poultry. In the chronic mild cold conditioning (CMCC) study (Chapter 3), it was found that CMCC may regulate AMPK-mTOR pathway in a tissue specific manner and this pathway controls cellular fatty acid utilization to enhance growth performance of young chicks. We have also identified new potential molecular signatures that play key roles in the control of energy homeostasis and stress in avian species. HSPA9/GRP75 was identified to be expressed differently in various tissues of Jungle fowl and quail; as well as avian cell types and this gene was affected by heat stress. Moreover, we found that heat and oxidative stress alter the expression of ORX and its related receptors in avian muscle both *in vivo* and *in vitro*. Dicer 1, a ribonuclease type III, vitally involves in microRNA biogenesis, is regulated by feed deprivation in quail and chicken livers, immortalized chicken embryo liver (CEL-im) and neuroblastoma (SH-SY5Y) cell lines, which indicate its potential role as an energy sensor.

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2. LITERATURE REVIEW

2.1 INTRODUCTION

Poultry meat is highly regarded worldwide, highly nutritious, and relatively inexpensive. However, poultry production is easily affected by different challenges such as environmental and nutritional stress. Within just a few decades, climate change has led to extreme weather globally; and these changes are predicted to be aggravated over the next decades (Hansen et al. 2012; Pachauri et al. 2014). In addition, broiler (meat-type) chickens have been genetically selected for high growth rate and; in turn, have a high metabolic rate, making them more sensitive to environmental and nutritional stressors (Cahaner & Leenstra 1992; Havenstein et al. 1994; De Jong et al. 2002; Soleimani et al. 2011b). Heat stress results in a series of changes from behavior to physiological and neuro-endocrinological alterations in birds (Kadim et al. 2008). Early research in poultry exposed to acute cold stress has clearly shown suppression in development, survival, and egg production (Sagher 1975; Renwick et al. 1985; Sahin et al. 2003).

Nutritional stress during transportation or during feeding programs (especially for breeders) may have additional metabolic stress (Siegel 1980). Indeed, several studies have shown that short-term fasting (one or two days) and long-term feed restriction induce: changes in growth rates, reproduction, metabolic blood index, physiological behavior, and the immune system (Yu & Robinson 1992; Gebhardt-Henrich & Marks 1995). Interestingly, the results from these studies are very controversial in showing the advantages and disadvantages of feed restriction in bird performance (Yu & Robinson 1992; Gebhardt-Henrich & Marks 1995). Stress in birds, including both thermal and nutritional stress, have resulted in several health issues; which have repeatedly affected the poultry industry. These stressors affect the expression of heat shock proteins (HSPs) and heat shock factors (HSFs) (Morimoto et al. 1994; Björk &

Sistonen 2010). Recently, HSPA9/GRP75, orexin, and its related receptors orexin receptor 1 (ORXR1) and orexin receptor 2 (ORXR2); as well as Dicer 1 have been reported to play important roles in the stress responses in mammals (Kaul et al. 2007; Wiesen & Tomasi 2009; Berridge et al. 2010). In this review, the impacts of thermal and nutritional stress on poultry growth and reproductive performance will be summarized. In addition, the current understanding of the underlying molecular mechanisms involved in the stress response in different species, particularly mammals; and how these compare with avian will be addressed. The long term goal is to identify mechanism- based strategies to alleviate these adverse effects and to improve poultry production sustainability.

2.2 STRESS DEFINITION

"Stress" is an extensively used term in many fields from humans to many other species, including plants. In order to study stress in animals, researchers have defined this term in different ways. In general, understanding "stress" starts with the term "homeostasis". It is stated that "Homeostasis is the stability of physiological systems that are essential to life", including "pH, body temperature, glucose levels, and oxygen tension (those aspects of physiology that "keep us alive")" (Blas 2015).

"Stress" has three meanings: "(1) the stimuli that challenge homeostasis (i.e., stressors, perturbations), (2) the emergency responses to perturbations (stress response), or (3) the chronic state of imbalance that follows over-activation of the adrenocortical axis (pathology, chronic stress)." (Blas 2015). This dissertation focused mainly on environmental (heat and cold) stress, and nutritional deprivation: two of the most challenging stressors in poultry.

2.3 EFFECTS OF ENVIRONMENTAL AND NUTRITIONAL STRESSES IN POULTRY

Regulation of bird body temperature follows the definition of three zones: "a zone of body cooling, a zone of evaporative regulation, and a zone of comfort" (Yahav 2015). The zone of comfort, the "thermoneutral zone", is comfortable for birds (55° to 75°F) and they control body temperature mainly by conduction. In the zone of body cooling, birds reduce heat loss by vasoconstriction, and piloerection of feathers, while they cool by vasodilation, followed by evaporation in the evaporative regulation zone (over 80°F). The last two conditions result in cold or heat stress in birds, and depends on the tolerant ability of the animals from extreme conditions. Heat and cold stress will affect the productive performance of birds (Yahav 2015; Anderson 2007). Figure 1 shows how birds regulate body temperature as environmental temperature changes (Anderson 2007).

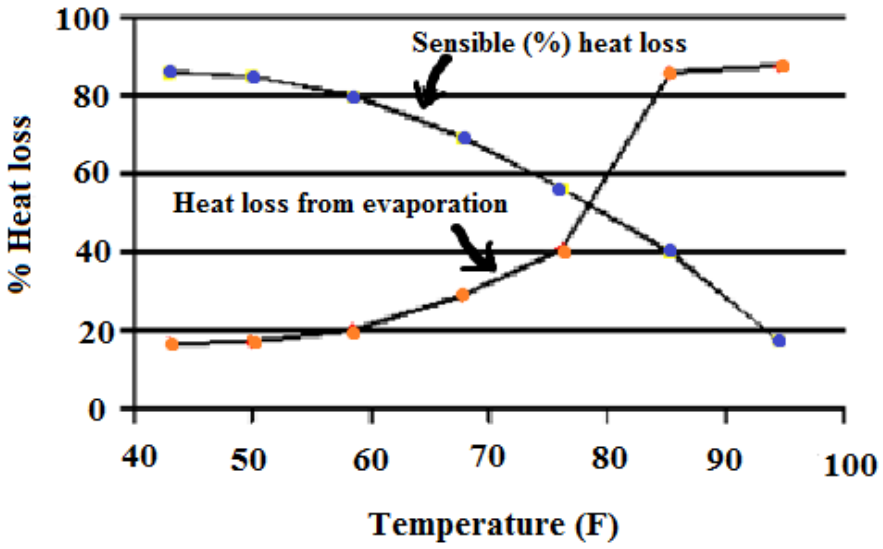

Figure 1. Heat loss method in birds as temperature changes (adapted from Anderson 2007)

2.3.1 COLD STRESS

Cold stress has been raising concerns in bird health and production; especially since climate change has resulted in extreme low temperature and precipitation in many regions worldwide (Sagher 1975; Renwick et al. 1985; Pachauri et al. 2014). Acute cold stress lowers the muscle development of young chicks, reduces body weight, and breast and thigh muscle yield. It also alters the content of protein, fat, potassium, magnesium, calcium and sodium in these muscles (Sagher 1975). Cold brooding temperature (26.7^{o}C), as compared with 32.2^{o}C of the control group caused higher mortality in 1-14 day old chicks (Renwick et al. 1985). Long term cold increased heat production and triiodothyronine concentrations in chicks (Collin et al. 2003). More thermogenic responses to acute cold exposure were also found in different tissues of neonatal chicks: such as higher plasma corticosterone levels, oxygen consumption, heat production, and mitochondrial substrate oxidation enzyme activities (Mujahid 2010). Cold exposure also influenced the antioxidant activity in the thymus, spleen, and bursa of Fabricius tissues and causing oxidative stress in chicks; however, the effects are not the same between acute and chronic cold stress. Acute cold stress decreased the activity of total anti-oxidation capacity (T-AOC), but chronic cold stress increased it (Zhao et al. 2014). In another study, cold stress up-regulated the gene expression of cytokines; which may suggest the stimulation of innate and adaptive immune systems by cold stress (Hangalapura et al. 2006). It has also been shown that innate immunity reacted quickly with acute cold stress and adaptive immunity responded with chronic cold stress in chickens (Hangalapura et al. 2003). A cold environment affects egg laying birds as well. The cold decreased the plasma insulin concentration and increased the corticosterone level (Şahin et al. 2001). The heterophil-to-lymphocyte ratio is significantly elevated when laying hens were exposed to cold (0 to 10^{o}C). Eight days under 4^{o}C

conditions significantly increased skeletal muscle weight, cytochrome oxidase and lactate dehydrogenase activities of 7 day old chicks (Ijiri et al. 2009). It was found that cold exposure on early-aged birds (2d) at 20^{o}C for 6 h can improve weight gain and feed efficiency (Shahir et al. 2012). It also has been found that chronic mild cold stress improved growth performance of young chicks; a suggestion of environmental adaptation (Nguyen et al. 2015).

2.3.2 HEAT STRESS

A series of changes from behavior thermoregulation to body systemic alteration was observed in heat stress studies in birds. The thermoneutral zone of a bird decreases from 35^{o}C at hatch to about 24^{o}C at 4 weeks old (Teeter & Belay 1996). Birds raised in an open-sided house at 37^{o}C and humidity level of 50-60% showed signs of panting and wing lifting, elevation of body temperature, lower feed consumption, an higher feed conversion ratio, and lowered body weight gain (Kadim et al. 2008). In addition to the decrease in both feed intake and body weight gain, heat stress affects the body composition of broilers causing a lower breast yield, increased abdominal, and intermuscular fat deposition; and this impact of heat stress depends on the poultry breeds (Lu et al. 2007). Further studies have investigated negative impacts of heat exposure on the digestive system of poultry. Chen and colleagues found that challenging birds at 40^{o}C for 2 h everyday suppressed the activities of digestive enzymes, glutathione and D-xylose absorption ability, levels of antibody IgA, lymphocytes, and other immune function in the intestines (Chen et al. 2014). Moreover, a hot environment (30^{o}C) decreased broiler jejunum weight and length; but increased hexose-transport capacity of the intestine (Garriga et al. 2006). In addition, chronic heat stress elevated the excretion of N, Ca, P, Zn, Fe, and Cr and reduced their retention rates in Japanese quail (Sahin et al. 2003). Physiological changes in poultry have also been observed in response to acute heat stress. Specifically, the increase of plasma

corticosterone level and lowering of plasma thyroid hormones in commercial broilers (Garriga et al. 2006; Soleimani et al. 2011b) and the increase of glucocorticoid receptor expression in young quail (Soleimani et al. 2011a). Heat stress reduces sodium, potassium, partial CO_2 pressure, and lymphocytes in blood (Borges et al. 2004). Furthermore, heat exposure affects the immune system in chickens by causing the reduction of IFN-γ and IL-2 and the increase of TNF-α, IL-4 (Xu et al. 2014) and the heterophil:lymphocyte ratio (Soleimani et al. 2011b). Several studies showed the induction of oxidative stress and oxidative damage by heat stress in birds through the increase of mitochondrial reactive oxygen species (ROS) production (Mujahid et al. 2005; Azad et al. 2010; Xu et al. 2014).

In egg production, heat stress has a significant harmful impact on: body weight, and feed consumption of laying hens at peak production, egg weight, shell weight, shell thickness, and gravity (Bollengier-Lee et al. 1998; Mashaly et al. 2004; Ciftci et al. 2005; Youssef et al. 2013). Heat stress suppresses immune function by inhibiting total white blood cell counts and antibody level; thereby increasing mortality in laying hens (Mashaly et al. 2004). Plasma calcium and phosphorous concentrations were reduced by heat stress in 53 to 61 week old laying hens. However, the total lipids and cholesterol of these birds were increased during summer (Youssef et al. 2013). Acute heat stress (38^{o}C) did not change plasma glucose, uric acid, creatinine, electrolytes, or and alkaline phosphatase in layers (Koelkebeck & Odom 1995).

2.3.3 NUTRITIONAL STRESS

The poultry industry has applied several feed restriction regimens in breeders due to their hyperphagy and risk of fat deposition and reproductive decline (Siegel 1980). Studies utilizing short-term fasting and long-term feed deprivation have been conducted to observe changes in growth rates, reproduction, chemical blood indices, physiological behavior, and the immune

system. Feed restriction causes both behavioral and nutritional stress in birds (De Jong et al. 2002). A previous study showed that fasting for two to four days caused a decrease in body weights of 6-week old chickens, along with decreases in liver and kidney weights. However, refeeding four days after four days of fasting recovered the body weights of birds. This study also showed that fasting induced hepatic and renal gluconeogenic enzyme activities such as fructose-1,6-diphosphatase and glucose-6-phosphatase, and refeeding returned these activities to basal levels (Shen & Mistry 1979). Fasting for 48 h significantly increased low-density lipoprotein (LDL) and high-density lipoprotein (HDL) and decreased the binding of LDL to hepatic membranes; this suggests the catabolism of LDL from the circulation (Loo et al. 1990). A study of short-term fasting in adult Japanese quail showed that 12, 24 and 48 h of fasting reduced the hepatic glucose concentration in both male and female birds, plasma total lipids in females, and lipids in the livers of both sexes. This indicated that fasting has an impact on lipid metabolism in adult quail (Lamošová et al. 2004). Feed restriction enhanced innate immunity and increased plasma corticosterone (CORT) levels in chickens; however, CORT level change may be line dependent (Hangalapura et al. 2005). Fasting and refeeding alters the plasma and intrahepatic T3 and T4 levels in four-week old chickens (Reyns et al. 2002). Other research showed decreased egg quality with feed restriction. Feed deprivation for 24 h reduced the egg shell quality in hens (Cason & Britton 1986). Long term-feed restriction (70% of *ad libitum* fed control group) for 44 d at random and 28 d in high growth rate quail lines resulted in significant decreased body weight with compensatory gains two weeks after restriction was ended; egg hatchability was not affected, but mortality rate doubled (Gebhardt-Henrich & Marks 1995).

In a separate study, 3 wks of feed restriction early in life did not cause a difference in the mortality rate of quail. Similarly, this study observed compensatory growth within one week

after feed restriction (85 or 70% of feed a*d libitum*) ended and the restriction did not affect reproductive parameters at a later period (Hassan et al. 2003). A skip-a-day feeding program has been shown to decrease body weights while improving reproduction through egg production, fertility, and hatchability in chickens. This study also showed that the response to restricted feeding was different among genotypes (Proudfoot 1979). These controversial reports raise the question of how feed deprivation affects the avian body and further studies on molecular mechanism will be discussed in the following section.

2.4 MOLECULAR MECHANISM INVOLVED IN ENVIRONMENTAL AND NUTRITIONAL STRESS RESPONSES IN POULTRY

2.4.1 HEAT SHOCK PROTEINS AND HEAT SHOCK FACTORS

a. Heat shock proteins

Heat shock proteins (HSPs) are highly conserved molecular chaperones recognized for their roles as stress response proteins that assist in: protein folding and unfolding, assembly of multi protein complexes, transport and sorting of proteins into subcellular compartments, minimization of protein aggregation, and protection of cells against apoptosis (Morimoto et al. 1994; Li & Srivastava 2001). Therefore, they are important in protecting and repairing cells and tissues (Zhao et al. 2013). There are various heat shock protein families that have been discovered and named based on their molecular weights including: HSP110, HSP90, HSP70, HSP60, small molecule HSPs, and ubiquitin (Morimoto et al. 1994; Zhang et al. 2013). HSP104 and HSP70 have a close functional relationship in thermotolerance; in that the loss of either protein is compensated by the high expression of the other (Sanchez et al. 1993). HSP60 is a mitochondrial chaperone protein required for the protein-folding system in the mitochondrial matrix (Cheng et al. 1989; Deocaris et al. 2006). The two families of HSP70 and HSP60 have the

roles of preventing newly synthesized polypeptides from aggregation and assist with their folding (Hartl et al. 1994). Conversely, HSP90 binds to client proteins such as steroid hormone receptors and transcription factors to regulate them in their native state confirmations (as reviewed by Wegele et al. 2004).

b. Heat shock factors

Heat shock factors (HSFs) are transcription factors that regulate the gene expression of HSPs induced by cellular stress (Björk & Sistonen 2010). HSFs are structurally and functionally conserved regulators throughout animal species; they bind to the heat shock response element (HSE) in the promoter region and regulate the transcription of different genes including HSPs (Sakurai & Enoki 2010). Moreover, their involvement is in sensing cell growth control, differentiation signals, and normal physiological processes (Morano & Thiele 1999). In mammalian species, four members of the HSF family have been identified: HSF1, HSF2, HSF3, and HSF4 (Fujimoto & Nakai 2010). While HSF1 and avian HSF (called HSF3) play essential regulatory roles in the heat stress response, the roles of HSF2 and HSF4 are more prominent as developmental factors (Nakai 1999; Björk & Sistonen 2010). It was found that HSP70 may inhibit HSF1 in human cells when they interact with each other (Abravaya et al. 1992). HSP90 is also a repressor of HSF1, as the reduction of HSP90 protein level significantly activates HSF1 protein expression. HSP90 and HSF1 form a complex under non-stress conditions and dissociate when cells are stressed (Zou et al. 1998). These suggest a negative feedback mechanism between HSF1 and HSP70 or HSP90 under non-stress and stress condition (Abravaya et al. 1992; Morimoto 1998; Zou et al. 1998; Björk & Sistonen 2010). In avian species, Nakai and Norimoto found that the DNA binding domain of chicken HSF3 (cHSF3) has 61% homology to that of chicken HSF1. However, HSF3 DNA binding activity could not be induced by heat shock as the

DNA binding activity of HSF1 was. HSF3 is co-expressed with HSF1 and HSF2 during development in several tissues and may regulate the transcription of heat shock genes in erythrocytes (Nakai & Morimoto 1993).

2.4.2 REGULATION OF HSPs AND HSFs BY ENVIRONMENTAL STRESS IN AVIAN SPECIES

a. Heat stress

It has been shown that HSPs are present in different cell types and tissues in avian species, and have a molecular weight ranging from 22 to 108kD (Givisiez et al. 2001). A novel small heat shock protein with a molecular weight of 29kD called HSP29 was found in broiler chicken heart and lung tissue after three hours of heat exposure. This novel protein was suggested to be specific for later protection during heat stress (Einat et al. 1996). Acute heat stress at 37^{o}C caused the highest protein expression of HSP27, HSP70, and HSP90 four hours after heat exposure and HSP29 after 3 h of heat exposure in 6 week old broiler hearts and lungs (Einat et al. 1996). Zang and colleagues found that HSP70 and HSF3 gene expression is tissue- and species-specific under heat stress (Zhang et al. 2014). Chronic cyclic heat stress up-regulates the gene expression of HSP70, but down-regulates HSP90 in the heart and liver of broiler breeder chickens. Furthermore, acute heat treatment induced HSF 2, 3, 4, and HSP70 in chicken livers. This study also showed that muscle has less protection by HSPs compared with liver and heart (Xie et al. 2014). Fifteen days of high temperature challenge (37^{o}C) to 42 day old female black-bone chickens increased the mRNA levels HSP27, HSP70, and HSP90 in the bursa and spleen; but HSP27 and HSP90 gene expression were decreased in the thymus (Liu et al. 2014). This again indicates the tissue-specific regulation of heat shock proteins. In testes of Taiwanese roosters exposed to acute heat stress (4 h); the significant alteration of HSP25, HSP70, and

HSP90 gene expression suggested an important role of HSPs in chicken male reproduction under heat stress (Wang et al. 2013). However, chronic heat stress (4 weeks) did not affect the gene expressions of HSP70 and HSP90 in 4-week old broiler livers (Rimoldi et al. 2015). Chronic heat conditioning of daily 1 hour exposure to 41^{o}C increased the protein expressions of HSP70 and HSP90 in peripheral leukocytes of both broiler chickens and turkey poults (Wang & Edens 1998). This suggests that the expression of heat shock proteins varies upon the duration and severity of heat stress. Effect of heat challenge during embryogenesis and post-hatch on mRNA levels of HSP90, HSP60 and HSF1 in the brain, heart and muscle tissues showed significant enhancement of these gene expressions in early heat exposure and suggest an improving thermo-tolerance ability of young chicks (Al-Zghoul et al. 2015).

b. Cold stress

In quail, acute cold stress (4^{o}C for 30 minutes) significantly up-regulated protein expression of HSP70 in heart tissues along with the decline of core body temperature (Hoekstra et al. 1998). Similarly, acute and chronic cold stress exposure (12^{o}C) in 15 d old chicks up-regulated both gene and protein expression of HSP70, HSP60, HSP27 in heart, spleen, thymus, and bursa. However, these conditions down-regulated gene and protein expression of HSP90 in heart tissues but not in spleen, thymus, and bursa (Zhao et al. 2013; Zhao et al. 2014). This indicates the protective functions of HSPs in chicken heart and immune organs. Cold stressed chicken embryos (32^{o}C) showed an increase in HSP70 protein expression in hearts at embryonic day (ED) 16 and breast muscle at ED 19 compared with controls. This study reveals the age-dependent regulation of HSP70 protein expression in chicken embryos (Leandro et al. 2004). Furthermore, Nguyen and colleagues observed changes in gene expression of HSPs and HSFs in chronic mild cold conditioned chicks where the temperature was started at 26.7^{o}C and gradually

decreased to 19.7^{o}C by the end of first week. Gene expression of HSF1, 2, 3, and 4 in bird hypothalamus, liver and leg muscles; hepatic HSP70 and HSP27 were significantly induced compared to the control groups. Chronic mild cold conditioning significantly up-regulated HSF1 mRNA level in chick leg muscles (Nguyen et al. 2015). In conclusion, HSPs and HSFs are protective molecules and their gene and protein expression is differentially regulated by duration and type of stressor and in a tissue and species specific manner.

2.4.3 EXPRESSION OF METABOLIC GENES IN NUTRITIONAL STRESS

Feed deprivation and/or feed restriction affects the energy homeostasis of chickens and have been studied at the molecular level in numerous tissues. Fasting for 2 d affected the Liver kinase B1 (LKB1)/AMPK pathway in the chicken hypothalamus by increasing phosphorylated-AMPKα:total AMPKα and phosphorylated-LKB1:total LKB1 ratios; however, re-feeding restored these ratios to normal levels (Song et al. 2012). This study showed that orexingenic neuropeptide gene expression (neuropeptide Y,NPY), agouti-related peptide (AgRP), melanin concentrating hormone (MCH), melanocortin receptor 4 (MC4R), and prepro-orexins were significantly up-regulated; conversely, some lipogenic genes (fatty acid synthase-FAS and Sterol regulatory element-binding protein - SREBP-1) were down-regulated in the hypothalamus of chickens (Song et al. 2012). Zhou and colleagues found that NPY protein content in the broiler hypothalamus played an essential role in energy homeostasis through increase of its content during fasting but returned to basal levels after refeeding (Zhou et al. 2005). In the liver of 4-wk old chicken subjected to 16 or 48 h of fasting; genes involved in gluconeogenesis, ketogenesis, and peroxisomal fatty acid beta-oxidation were significantly increased. Genes involved in the synthesis of fatty acids and cholesterols were markedly down-regulated (Désert et al. 2008). Also in this study, the down-regulation of SREBP1 and SREBP2, known transcriptions factors of lipid

metabolism and cholesterol related genes, was observed (Désert et al. 2008). Skip-a-day feeding for 14 d post-hatch altered triglyceride (TG), high density lipoprotein cholesterol (HDLC), lipoprotein lipase (LPL), and total cholesterol (TC) concentrations in chick serum at day 14. This treatment also altered mitochondria morphology and function, possibly affecting the expression of lipogenic genes such as ACC-α (Acetyl-CoA carboxylase α), CPT-1 (carnitine palmitoyltransferase I), and SREBP-1 (Yang et al. 2010). Due to genetic selection, fasting has different effects on ubiquitin-proteasome system in the skeletal muscle of broiler and layer chickens. In the muscle of 14-d broiler and layer chicks, 24 h fasting significantly up-regulated the expression of genes involved in proteolysis such as forkhead box class O (FOXO) 1, muscle RING finger-1 (MuRF-1) and atrogin-1. However, atrogin-1 and FOXO3 mRNA levels were significantly higher in layer muscle than those in broiler muscle; and this may be due to fast growth rate in broilers (Saneyasu et al. 2015). In 7d chicks, 24-h feed deprivation altered the following expressions of LKB1, fatty acid synthase (FAS), AMPK alpha 2 subunit (AMPKα2), and carnitine palmitoyltransferase 1 (CPT1), suggesting that the AMPK/FAS pathway may play a role in energy metabolism in chicken muscle. In addition, this study reported the increase of phosphorylated mTOR after refeeding (Hu et al. 2016).

2.5 HSPA9/GRP75

2.5.1 HSPA9/GRP75 ROLE AND WORKING MECHANISM

GRP75/mortalin/mtHSP70/HSPA9 (here after referred to as HSPA9) was first identified in the cytosolic fraction of mouse embryonic fibroblasts; it has a molecular weight of 75 kDa and is in heat shock protein 70 family. It was initially considered to be involved in cellular mortality and cell growth (Wadhwa et al. 1993; Starenki et al. 2015). Its presence was reported first in mitochondria; later it was documented in endoplasmic reticulum, cytoplasmic vesicles, and cell

cytosol (as reviewed by Ran et al. 2000; Wadhwa et al. 2002). HSPA9 assists in mitochondrial protein import by forming an import motor complex associated with the mitochondrial membrane to transport the unfolded targeted protein and help refold this protein when it is in the matrix (Voos & Röttgers 2002). HSPA9 is also considered a guardian, since it has multiple roles in intracellular trafficking, the endocytic pathway in mammalian cells, stress and immune responses, and prevention of apoptosis (Kaul et al. 2007; Wittrup et al. 2010; Yang et al. 2011). *In vitro* and *in vivo* studies by Mizukoshi and colleagues reported that HSPA9 may be involved in intracellular trafficking by forming physical interactions with fibroblast growth factor-1 (FGF-1), a factor involved in cell growth and differentiation (Mizukoshi et al. 1999). In cancer research, HSPA9 has been studied extensively. It has been found that the up-regulation of HSPA9 in different cells and tissues of human and mouse significantly promotes tumorigenesis (Wadhwa et al. 2006; Flachbartova & Kovacech 2013; Starenki et al. 2015).

2.5.2 HSPA9/GRP75 AND STRESS

Mitsumoto and colleagues reported that accumulation of HSPA9 in mitochondria was stimulated by hydrogen peroxide (H_2O_2) induced oxidative stress in human endothelial cells (Mitsumoto et al. 2002). HSPA9 overexpression reduced the accumulation of ROS in glucose-deprived PC12 cells (rat pheochromocytoma cell) (Liu et al. 2005). Further findings in glucose deprivation stress in cells indicate that HSPA9 overexpression inhibited apoptosis possibly via activating RAC-alpha serine/threonine-protein kinase (AKT), which has anti-apoptotic effects in cell (Yang et al. 2011). In a study of amino acid deprivation stress, HSPA9 protein content was increased two-fold after 5-10 hours of amino acid free culture in Chinese Hamster Ovary cells (CHO). In addition, this study showed that the induction of HSPA9 protein was variable following different types of amino acid deprivation and that protein induction was not positively

correlated with gene expression (Heal & McGivan 1997). Over-expression of HSPA9 was reported to prevent rat liver injury and in the normal human liver cell line (HL-7702) from oxidative stress. Treatment with H_2O_2 caused oxidative stress, high cytochrome c protein expression, and a rapid increase of ROS in control cells; but these effects were lowered in over-expressed HSPA9 cells. Tail vein injection of HSPA9-overexpressing vector helped alleviate serum aspartate aminotransferase, alanine transaminase, and cytochrome c protein expression in rats. This data suggests the protective ability of HSPA9 in oxidative stressed rat livers (E et al. 2013). Human Parkinson disease research observed the involvement of HSPA9 in pathways such as: mitochondrial function, proteasomal function, and oxidative stress (Jin et al. 2006).

Numerous studies of HSPA9 have been conducted in human and rodents. These studies discovered multiple roles of HSPA9 in diseases and under stress conditions; both of which are high concern in poultry. Therefore, the role of GRP75 in poultry, particularly as it relates to stressors such as heat and cold exposure, needs to be elucidated.

2.6 OREXIN SYSTEM

2.6.1 PHYSIOLOGICAL ROLES OF OREXIN (ORX)

Orexins (orexin A and orexin B) or hypocretins (hypocretin 1 and hypocretin 2) were first identified in and around the lateral and posterior hypothalamus of rat brain (de Lecea et al. 1998; Sakurai et al. 1998). These neuropeptides bind to two G-protein couple receptors, also in the brain, named orexin receptor 1 (ORXR1) and orexin receptor 2 (ORXR2) (Sakurai et al. 1998; Trivedi et al. 1998). In mammals, it is widely recognized that orexins and their receptors are involved in the regulation of feed intake (Sakurai et al. 1998; Haynes et al. 2000), glucose homeostasis (Tsuneki et al. 2010), drinking behavior (Kunii et al. 1999), sleeping and wakefulness (Ohno & Sakurai 2008), and cardiovascular and respiratory functions in rodents (Li

& Nattie 2014). Recently, orexin was also shown to participate in arousal and stress regulation (Berridge et al. 2010). Studies showed the increase of ORX mRNA level in lateral hypothalamic area (LHA) of rats under immobilization stress and cold stress (Ida et al. 2000). Nevertheless, Furlong and colleagues found that it is not involved in all forms of stress by comparing different stress conditions related to arousal (Furlong et al. 2009).

2.6.2 AVIAN ORX SYSTEM AND STRESS

ORX and its receptors have since been found in many species including birds (as reviewed by (Wong et al. 2011). In avian species, orexin and its receptors are distributed in brain regions such as the hypothalamus, anterior median eminence, basal hypothalamus, posterior median eminence, the pituitary gland, the adrenal gland, gut and the testis/ovary (Ohkubo et al. 2003; Miranda et al. 2013; Arcamone et al. 2014). In 2015, orexin and its receptors were identified in numerous chicken tissues and characterized in chicken muscle and a quail muscle cell line (QM7) (Lassiter et al. 2015). In poultry, the roles of orexins have not been well established but do not appear to be the same as in mammals. For instance, no change in orexin mRNA level was found in the hypothalamus of 24 hour feed-deprived female chickens, indicating that it did not stimulate feed intake (Ohkubo et al. 2002), but it may be involved in the sleep wake cycle and have neuroendocrine functions in chickens (Ohkubo et al. 2003). However, Miranda and colleagues found that hypothalamic orexins did not control the sleep-wake cycle in chickens, but orexin mRNA level was upregulated when feed was long term restricted in broilers; suggesting its role in energy homeostasis (Miranda et al. 2013). In peripheral tissues, ORX system gene and protein expression were observed to be significantly down-regulated in heat stressed quail livers and LMH (leghorn male hepatoma) cells (Greene et al. 2016). In addition, oxidative stress caused by H_2O_2 treatment reduced ORX protein level; but significantly

increased its mRNA level in a dose-dependent manner. Taken together, this shows the regulation of the ORX system and suggests its role as a molecular signature in the responses to heat and oxidative stress in avian livers (Greene et al. 2016). Interestingly, QM7 cells have been shown to secrete orexin; which suggests possible autocrine, paracrine, and/or endocrine roles for orexins. This study also revealed the role of the ORX system in the mitochondrial network by affecting mitochondrial dynamics (Lassiter et al. 2015). Since there is no information on ORX and its receptors regulation in stressed avian muscle, their roles were examined in quail leg muscle and the QM7 cell line under heat and oxidative stress in chapter 5.

2.7 DICER - RIBONUCLEASE III ENZYME

2.7.1 DICER ROLE AND WORKING MECHANISM

Dicer, a ribonuclease III enzyme, plays a key role in microRNA (miRNA) biogenesis. Dicer cleaves single-stranded pre-miRNAs into miRNAs or double-stranded RNA (ds RNAs) into siRNAs (Bernstein et al. 2001; Kurzynska-Kokorniak et al. 2015). miRNAs play several important roles in growth, death, stress, glucose, lipid metabolism and metabolic diseases (Ambros 2003; Lynn 2009; Fernández-Hernando et al. 2013). In *Drosophila melanogaster*, two types of Dicer have been identified called Dicer 1 and Dicer 2 (Kurzynska-Kokorniak et al. 2015). While Dicer 1 involved in the processing of miRNA, Dicer 2 is necessary for siRNA biogenesis (Lee et al. 2004). However, a study on the evolution of the Dicer gene showed that only Dicer 1 homologs spanned in vertebrates, not Dicer 2 (Mukherjee et al. 2013).

Several studies have shown different and important roles of Dicer 1 in mammals such as in cell viability and metabolism. Bernstein and colleagues observed an early lethality in the development of mouse embryos due to stem cell depletion caused by Dicer 1 loss (Bernstein et al. 2003). Dicer 1 disruption in mouse hepatocytes resulted in steatosis and glycogen depletion in

the cells; leading to the impairment of lipid and glucose metabolism in mutant mice (Sekine et al. 2009). Knockdown of Dicer 1 inhibited lipid accumulation and adipocyte differentiation in 3T3-L1 cells by suppressing gene expression of PPARγ (Peroxisome proliferator-activated receptor gamma), C/EBPα (CCAAT-enhancer-binding protein α), and FAS (Fujimoto et al. 2012). High glucose exposure for 24 hours in human pancreatic islet cells resulted in the significant up-regulation of abundant proteins including Dicer 1, indicating the role of Dicer 1 in glucose regulation (Schrimpe-Rutledge et al. 2012). In mouse skeletal muscle, inactivated Dicer 1 decreased muscle miRNAs and suppressed muscle development by abnormal myofiber morphology (O'Rourke et al. 2007).

2.7.2 DICER IN HOMEOSTASIS AND STRESS

Since Dicer was found to be essentially involved in cell viability and metabolism, the roles of Dicer in homeostasis and stress were also revealed in other studies. In *Drosophila*, Dicer 2 has functions in affecting metabolic gene expression, lifespan, and defense response to multiple stress conditions (oxidation, starvation, and cold) (Lim et al. 2011). Acute heat stress in yeast (*Schizosaccharomyces pombe*) caused an accumulation of unfolded Dicer 1 in the cytoplasm and co-localized Hsp104 helps to disaggregate Dicer 1 protein suggesting a negative feedback mechanism between Dicer 1 and Hsp104 (Oberti et al. 2015). Other studies showed that Dicer 1 is involved in cellular stress response. Different stressors (reactive oxygen species, Ras oncogene and phorbol esters) inhibited the protein expression of Dicer 1 in many cell types including JAR trophoblast cells and IMR-90 cells (Wiesen & Tomasi 2009). Dicer declines with age in the adipose tissue of mice and worms (*Caenorhabditis elegans*), but its expression can be restored by caloric restriction (Mori et al. 2012). In addition, Dicer 1 knockdown in mice resulted in a hypersensitive response to oxidative stress and reduction of stress tolerance in *C. elegans*,

but its overexpression assisted intestine against stress (Mori et al. 2012). Furthermore, Dicer 1 is an energy sensor as its mRNA expression is regulated by nutrient availability. Fasting upregulated Dicer 1 gene expression in mouse hypothalamus. In the same study, Dicer 1 deletion in POMC-expressing cells of mice alters energy homeostasis in mutant mice such as an increase of body weight, hyperphagia, plasma leptin, blood triglycerides concentration, adiposity, and defects in glucose homeostasis (Schneeberger et al. 2013).

Dicer 1 has been studied extensively in human and mammals, but there is a paucity of research regarding Dicer 1 in avian species. Tian and co-authors reported that chicken Dicer 1 gene is located on chromosome 5 at 1040 cRs and is consistent with human gene location. They also found that Dicer 1 genes of chicken and human have an 87% homology (Tian et al. 2007). A broad gene expression of Dicer 1 was observed in multiple tissues of chick embryos (Carraco et al. 2014). Since, Dicer 1 is involved in energy homeostasis, metabolism, and stress, a study of nutritional stress in avian species was conducted to gain a further understanding of the regulation of Dicer 1 in feed restricted poultry.

2.8 OBJECTIVES

The review above has shown that environmental and nutritional stresses may adversely affect poultry health, wellbeing and production. There are several studies conducted to observe and understand how and why these stressors affect avian species. However, the molecular mechanism underlying poultry stress response are not thoroughly understood. Several molecular signatures such as HSPA9, ORX, and Dicer 1 involved in stress response have been extensively studied in human and mammals, but not in avian species. My research hypothesis is that these molecular markers (HSPA9, ORX and its receptors and Dicer 1) may also play important roles in avian energy homeostasis under environmental and nutritional stresses which can be used for

subsequent development of strategies to improve poultry thermo-tolerances and production sustainability. The objectives of the research are:

- To increase the understanding of the molecular mechanism of cold stress in young chicks
- Define the role of HSPA9 in avian species, especially in metabolic tissues under heat stress
- Determine the regulation of ORX system in heat stressed avian muscle tissues and cells
- Determine the regulation of DICER1 expression in avian species under nutritional stress.
- Decipher the molecular mechanism of DICER in brain cell model under nutritional stress.

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CHAPTER 3

Chronic Mild Cold Conditioning Modulates the Expression of Hypothalamic Neuropeptide and Intermediary Metabolic-Related Genes and Improves Growth Performances in Young Chicks

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3.1. Abstract

Background

Low environmental temperatures are among the most challenging stressors in poultry industries. Although landmark studies using acute severe cold exposure have been conducted, still the molecular mechanisms underlying cold-stress responses in birds are not completely defined. In the present study we determine the effect of chronic mild cold conditioning (CMCC) on growth performances and on the expression of key metabolic-related genes in three metabolically important tissues: brain (main site for feed intake control), liver (main site for lipogenesis) and muscle (main site for thermogenesis).

Methods

80 one-day old male broiler chicks were divided into two weight-matched groups and maintained in two different temperature floor pen rooms (40 birds/room). The temperature of control room was 32°C, while the cold room temperature started at 26.7°C and gradually reduced every day (1°C/day) to reach 19.7°C at the seventh day of the experiment. At day 7, growth performances were recorded (from all birds) and blood samples and tissues were collected (n = 10). The rest of birds were maintained at the same standard environmental condition for two more weeks and growth performances were measured.

Results

Although feed intake remained unchanged, body weight gain was significantly increased in CMCC compared to the control chicks resulting in a significant low feed conversion ratio (FCR). Circulating cholesterol and creatine kinase levels were higher in CMCC chicks compared to the control group ($P<0.05$). CMCC significantly decreased the expression of both the hypothalamic orexigenic neuropeptide Y (NPY) and anorexigenic cocaine and amphetamine

regulated transcript (CART) in chick brain which may explain the similar feed intake between the two groups. Compared to the control condition, CMCC increased the mRNA abundance of AMPKα1/α2 and decreased mTOR gene expression ($P<0.05$), the master energy and nutrient sensors, respectively. It also significantly decreased the expression of fatty acid synthase (FAS) gene in chick brain compared to the control. Although their roles are still unknown in avian species, adiponectin (Adpn) and its related receptors (AdipoR1 and 2) were down regulated in the brain of CMCC compared to control chicks ($P<0.05$). In the liver, CMCC significantly down regulated the expression of lipogenic genes namely FAS, acetyl-CoA carboxylase alpha (ACCα) and malic enzyme (ME) and their related transcription factors sterol regulatory element binding protein 1/2 (SREBP-1 and 2).

Hepatic mTOR mRNA levels and phosphorylated mTOR at Ser2448 were down regulated ($P<0.05$), however phosphorylated ACCαSer79 (inactivation) was up regulated ($P<0.05$) in CMCC compared to control chicks, indicating that CMCC switch hepatic catabolism on and inhibits hepatic lipogenesis. In the muscle however, CMCC significantly up regulated the expression of carnitine palmitoyl transferase 1 (CPT-1) gene and the mRNA and phosphorylated protein levels of mTOR compared to the control chicks, indicating that CMCC enhanced muscle fatty acid β-oxidation.

Conclusions

In conclusion, this is the first report indicating that CMCC may regulate AMPK-mTOR expression in a tissue specific manner and identifying AMPK-mTOR as a potential molecular signature that controls cellular fatty acid utilization (inhibition of hepatic lipogenesis and induction of muscle fatty acid β-oxidation) to enhance growth performance during mild cold acclimation.

3.2. Introduction

Global warming can lead to extreme weather in various portions of the globe so that some regions have extreme snowfall and others have increased intense and frequent heat waves [1]. These climate changes are predicted to continue to rise [2]. Environmental stressors (cold and heat) are already affecting animals, insects and crops [3]. In expanding worldwide broiler (meat-type chicken) production, the effects of cold stress on growth performances are controversial depending on the stress severity, the exposure period, and the age of the chicken. Indeed, sudden severe decrease of environmental temperature has been reported to negatively affect growth performance (feed efficiency, meat yield, ascites, mortality) [4] and result in serious annual economic losses to the livestock and poultry industries [5]. On the other hand, conditioning exposure of chickens at an early age to moderate decreased environmental temperature has been reported to have a long-lasting effect and improve the ability of the chicken to acclimatize and to cope better with stressors in later life [6]. Thus, insights into the molecular mechanisms by Cold stress induces many cellular alterations that in turn lead to various neuroendocrine, physiological, and immunological adaptations [7]. Animals living in extreme climates show a marked seasonal variation in both energy homeostasis (energy intake and expenditure) and intermediary metabolism as a response to the changing metabolic requirements imposed by differences in environmental temperature [8]. These variables are mediated by complex molecular networks that are still not completely defined.

Depending on the type, degree and duration of the stress, cells can develop highly efficient stress response and protein quality control systems to ensure their survival or activate stress signaling cascades that proceed into cell-death pathway. Stress rapidly initiates the increased synthesis of a group of stress proteins belonging to the heat shock protein (HSP)

families. These ubiquitously expressed molecular chaperones are classified into about six families (HSP-10 to HSP-100) on the basis of their monomeric molecular weight [9]. They carry out crucial housekeeping functions and orchestrate folding/unfolding and assembly/disassembly of protein complexes to maintain normal cell function. HSPs are regulated at transcriptional levels through HSP transcription factors (HSF1-4) that bind to heat shock response element (HSE) in the upstream promoter regions of HSPs [10]. Additionally, HSPs are subjected to various post-translational modifications such as acetylation, S-nitrosylation and glycosylation [11–13].

Emerging evidence indicates that the regulation of energy homeostasis and the stress response are coupled physiological processes [14]. We and other groups have previously shown that HSP-70 gene expression is regulated by various feeding-related hormones [15–18]. We also showed that acute cold stress alters the expression of key genes (leptin and uncoupling protein, UCP) involved in the regulation of energy intake and expenditure in 5-week old broiler chickens. Several recent studies investigating the effect of acute or chronic severe cold exposure (-15 to 12°C) on neuropeptides, antioxidant, immune, immunoglobulin and cytokine systems in immune organs, heart, hypothalamus and gastrointestinal tract in old (15 days to 4 weeks) chickens have been reported [5,19–24]. However, data related to the effect of chronic mild cold conditioning (CMCC) on metabolic-related genes in young chicks are scarce.

Therefore, the present study was designed to determine the effects of CMCC on the expression of hypothalamic feeding-related neuropeptides, hepatic lipogenesis- and muscle energy expenditure-related genes, and the expression of HSPs and their related transcription factors HSFs in three metabolically important tissues: brain (the main site of feed intake control),

liver (the main site of lipogenesis), and muscle (the main site for thermogenesis and energy expenditure) in young chicks.

3.3. Materials and Methods

3.3.1. Ethic Statement

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Animal Care and Use Committee under protocol 13026.

3.3.2. Animals

Male broiler chicks (Cobb males from the Cobb 500 female line) were hatched from a single flock of 29-week-old hens in their third week of lay. At day one post-hatch, chicks (n = 80) were weighed, divided in four body weight-matched groups and placed into four randomly assigned floor pens (20 birds/pen) containing fresh pine shavings in two separate environmentally controlled rooms. Control groups were maintained at 32°C for the first week. Cold-stressed groups were maintained at 26.7°C for the first day and the ambient temperature was reduced gradually by 1°C every day to reach 19.7°C at the seventh day. Chicks were given *ad libitum* access to clean water and a complete starter diet (12.7 MJ metabolizable energy Kg-1 and 220 g crude protein Kg-1). A relative humidity of * 55% and a 23 h light/1h dark cycles were maintained until the end of the experiment (7 days). Body weight and feed intake were recorded weekly. Body temperature was measured daily using a Braun Thermoscan IRT4520 thermometer (KazInc, Southborough, MA). EDTA-treated whole blood and serum were collected and assayed for standard hematology and metabolites. Birds were cervically dislocated

and whole brain, liver and leg muscle tissues were collected, snap frozen in liquid nitrogen and stored at -80°C until use.

3.3.3. Measurement of circulating metabolites

Commercial colorimetric diagnostic kits were used to measure plasma glucose (Ciba Corning Diagnostics Corp., OH), triglycerides, cholesterol and creatine kinase (Chiron Diagnostics, CergyPontoise, France), lactate dehydrogenase (LDH, Bayer Healthcare, Dublin, Ireland), and uric acid levels (Pointe Scientific Inc, Canton, MI) with an automated spectrophotometer according to manufacturer's recommendations. Briefly, glucose concentration was estimated by the formation of NADH at 340 nm absorbance. When phosphorylated with ATP and hexokinase, glucose yields glucose-6- phosphate and ADP which, in turn, is catalyzed by G-6-PD to form 6-phosphogluconate and NADH. NADH formation is directly proportional to the amount of glucose in the sample. Triglyceride levels were enzymatically determined based on the action of lipase, glycerol kinase and glycerol-phosphate oxidase at 540 nm. Cholesterol lev-els were enzymatically estimated in the presence of cholesterol esterase, cholesterol oxidase and peroxidase at 500 nm. Creatine kinase levels were determined based on the rate of NADPH formation measured at 340 nm in the presence of HK and G-6-PD. LDH activity was estimated following the oxidation of lactate to pyruvate at 340 nm. Uric acid levels were measured using the coupling of 4-aminoantipyrine with 2-hydroxy-2,4,6-tribromobenzoic acid and hydrogen peroxide in the presence of peroxidase at 520 nm.

3.3.4. RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was extracted from chick tissues by Trizol reagent (Life Technologies) according to manufacturer's recommendations, DNAse treated and reverse transcribed (Quanta Biosciences). RNA integrity and quality was assessed using 1% agarose gel electrophoresis and

RNA concentrations and purity were determined for each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (Life Technologies). Oligonucleotide primers used for chicken hypothalamic neuropeptides, HSPs, HSFs, lipogenesis- and energy expenditure-related genes are summarized in Table 3.1. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the 2– Ct method [25].

3.3.5. Western blot analysis

Total proteins were extracted from chick tissues, quantified, and subjected to Western blot as we previously described [26,27]. The rabbit polyclonal anti-phospho mechanistic target of rapamycin (mTOR) Ser2448 (#2971), anti-mTOR (#2972), anti-phospho AMP-activated protein kinase alpha (AMPKα)Thr172 (#2531), anti-AMPKα1 (#2795), anti-AMPKα2 (#2757) anti-phospho acetyl-CoA carboxylase alpha (ACCα)Ser79 (#3661), anti-ACCα (#3662), anti-HSP90 (#PA5-17610), and mouse monoclonal anti-HSP70 (#MAI-91159) were used. Protein loading was assessed by immunoblotting with the use of rabbit anti-β actin (#4967) or rabbit anti-vinculin (#V4139). Pre-stained molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (BioRad). All primary antibodies were purchased from Cell Signaling

Technology, except for the anti-HSP70 and anti-HSP90 which were purchased from Pierce Thermo Scientific, and anti-vinculin which was purchased from Sigma-Aldrich. The secondary anti-bodies were used (1:5000) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences) and captured by FluorChem M MultiFluor System (Protein simple). Image Acquisition and Analysis were performed by Alpha-View software (Version 3.4.0, 1993–2011, Protein simple).

3.3.6. Statistical analysis

Growth performance (feed intake FI, body weight gain BWG, and feed conversion ratio FCR), plasma metabolite parameters (cholesterol, glucose, triglyceride, uric acid, LDH and creatine kinase), and gene and protein expression data were analyzed by the Student's unpaired t-test. Body temperature data were analyzed using two-way repeated-measures ANOVA with time as the repeated measure and treatment (TN vs cold exposure) as factors. Data are expressed as the mean ± SEM and analyzed using Graph Pad Prism software (version 6 for windows). Significance was set at $P<0.05$.

3.4. Results

3.4.1. CMCC improves growth performances in chicks

As shown in Fig 3.1a, both weekly and total (whole 3-wk period) cumulative feed intake did not differ between the control and CMCC chicks, however the total body weight gain (3-wk period) was significantly higher in CMCC group compared to the control resulting in significant lower FCR ($P<0.05$, Fig 3.1b and 1c). The body temperature (BT) of the control chicks significantly increased from day 3 to day 7 of the first week compared to day 1, however the BT of CMCC chicks remained the same during the first 7 days (Fig 3.1d). When BT of both groups

were plot-ted together, CMCC chicks exhibited higher BT at the first day and lower BT from day 3 to day 7; however, the difference was not statistically discernable (Fig 3.1d).

3.4.2. CMCC alters circulating metabolite levels

CMCC significantly increased the circulating levels of cholesterol (Chol) and creatine kinase (CK) without affecting circulating glucose (Glc), triglyceride (TG), uric acid (UA) and L-lactate dehydrogenase (LDH) levels (Fig 3.2a and 2b).

3.4.3. CMCC affects the expression of hypothalamic feeding-related genes in chicks

Since the effect of some neuropeptides in chicken is not fully understood and established, we classified them in the present section based on their effects in mammals. CMCC significantly down-regulated the expression of the orexigenic hypothalamic neuropeptide NPY, adiponectin (Adpn) and its related receptors AdipoR1 and AdipoR2 compared to the control group ($P<0.05$, Fig 3.3a). The expression of hypothalamic agouti-related protein (AgRP), orexin (Ox) and its related receptors (Ox1R and Ox2R) remained unchanged between the control and CMCC groups (Fig 3.3a). Among the anorexigenic neuropeptides, only cocaine and amphetamine regulated transcript (Cart) was significantly down-regulated in CMCC compared to control chicks ($P<0.05$, Fig 3.3a). CMCC significantly down regulated the expression of hypo-thalamic fatty acid synthase (FAS) and mechanistic target of rapamycin (mTOR), and up regulated the expression of AMP-activated protein kinase alpha 1 and 2 (AMPKα1 and α2) ($P<0.05$, Fig 3.3b) and AMPKγ1/2 ($P<0.05$, S1 Fig). The expression of acetyl-CoA carboxylase alpha (ACCα), stearoyl-CoA desaturase1 (SCD-1), ribosomal p70 S6 kinase (S6K1), and AMPKβ1/β2 and γ3 remained unchanged between the cold and the control groups (Fig 3.3b and S1 Fig). Neither HSP (HSP70 and HSP27), nor their related transcription factors (HSF1-4) mRNA abundances, were affected by CMCC compared to control group (Fig 3.3c).

3.4.4. CMCC down-regulates hepatic lipogenic gene expression in chicks

CMCC significantly down-regulated the expression of hepatic FAS, ACCα, malic enzyme (ME), sterol regulatory element binding protein 1 and 2 (SREBP-1 and 2) and mTOR com-pared to the control group ($P<0.05$, Fig 3.4a). The expression of ATP citrate lyase (ATPcl), stear-oyl-CoA desaturase 1 (SCD-1), AMPKα1/2, AMPKβ1/2, AMPKγ1/2/3, and insulin induced gene 1 and 2 (INSIG-1 and 2) did not differ between the control and CMCC groups (Fig 3.4a and S1 Fig). Concomitant with these changes, CMCC decreased the phosphorylated levels of mTORSer2448 and increased the phosphorylated levels of ACCαSer79 compared to the control group (Fig 3.4b and 4c). CMCC induced the hepatic HSP27 and HSP70 mRNA levels and HSP70 protein expression compared to the control group ($P<0.05$, Fig 3.5b and 5c), however hepatic expression of HSF1-4 remained unchanged between the two groups (Fig 3.5a).

3.4.5. CMCC alters the expression of metabolic-related genes in young chick muscles

CMCC did not affect the expression of uncoupling protein (UCP), adenine nucleotide translocator (ANT), nuclear respiratory factor 1 (NRF-1) and the avian sarcoma viral oncogene homolog Ski, key genes involved in the regulation of mitochondrial function and energy expenditure (Fig 3.6a). However, it significantly induced the expression of carnitine palmitoyl transferase 1 (CPT-1), AMPKγ3 and mTOR and down-regulated the expression of peroxisome proliferator-activated receptor alpha (PPARα) compared to the control group ($P<0.05$, Fig4.6a and S1 Fig). The expression of PPARγ, PPARγ coactivator alpha and beta (PGC-1α and PGC-1β), SREBP-1/2, AMPKα1/2, AMPKβ1/2, and AMPKγ1/2 did not differ between the two groups (Fig 3.6a and S1 Fig). In line with the variation of gene expression, CMCC increased the phosphorylated levels of mTORSer2448 compared to the control group ($P<0.05$, Fig 3.6b and 3.6c). Total AMPKα2 protein levels were significantly decreased in the CMCC compared to the

control group (Fig 3.6b). Only HSF-1 gene expression was higher in CMCC chicks compared to the control group ($P<0.05$, Fig 3.7a); however, the expression of HSP70, HSP60, HSP27, HSF2-4 did not differ between the two groups.

3.5. Discussion

Low and high environmental temperatures are the most challenging stressors impacting both poultry and livestock industries. Cold stress, for instance, has been reported to cause an estimated total annual economic loss to the Chinese poultry industry of 100 million in currency [5]. A tremendous amount of seminal work has been done to identify the animal physiological and behavioral responses to cold stress and improve management strategies, however, the underlying molecular mechanisms are still not completely defined. In the present study, we aimed to determine the effect of CMCC on growth performance, plasma metabolite levels, and the expression of key metabolic-related genes.

The standard rearing practice in poultry industry is to maintain the average environmental temperature at 32°C during the first week of chick age and gradually decrease it (2 to 3°C/ week) to about 22°C at 4 weeks. Although chickens are homeothermic and able to engage adaptive and/or protective mechanisms to cope with cold stress and re-establish their body temperature homeostasis, sudden severe cold stress could be detrimental. The strong negative effect of severe cold stress on avian growth performance, health and welfare is well documented [4,7,28,29]. In the present study, growth performances (BWG and FCR) were improved by CMCC during the first week and at the end of the experimental period (3 weeks) compared to the control group, corroborating previous studies in chickens exposed to short-term cold conditioning [6,30]. Shinder et al. [31] found that embryonic cold conditioning also improved growth performance and reduced ascites incidence in chickens. In contrast, Baarendse et al. [32] reported that

exposure of chickens to a moderate reduction in house temperature during early post-hatching period have long-term negative effects on growth performance. The discrepancies between these findings might be related to several factors including chicken strain (Hubbard HY vs. Cobb chickens in Baarendse's [32] and our studies, respectively), duration of cold exposure (5 vs. 7 days in Baarendse's and our studies, respectively), and/or experimental conditions (diet composition, stress, density and feeding system).

In an attempt to better understand the mechanisms underlying the adaptation and acclimatization to CMCC, we measured circulating metabolic substrates in both chick groups. In agreement with previous studies in mammals and chickens [33–35], plasma CK and cholesterol levels were both higher in CMCC chicks compared to control group. CK, which catalyzes the reaction of ATP and creatine to phosphocreatine, is released into the circulation following changes in the permeability of the sarcolemma in response to various stressors [36] and is generally accepted as an indicator of muscle damage. As LDH levels were not affected during CMCC exposure in the present study, the increased levels of circulating CK indicates its potential role in maintaining high ATP turnover at low temperature as previously reported [37]. It is possible that the increased levels of plasma CK, observed in our study, is a result of an enhanced skeletal muscle mass [38,39] that is mirrored in higher BWG in CMCC compared to control chicks. Although the mechanisms underlying the CMCC-induced cholesterol elevation are not known at this time, we hypothesize that chicks heavily rely on mobilizing energy from body fat to satisfy their higher energy requirement.

As changes in growth performances (BWG and FCR) are mainly caused by feed intake, energy expenditure and intermediary metabolism, we next performed in depth analysis of key regulators gene expression in three metabolically important tissues; the brain (main site for feed

intake regulation) [40], the liver (main site for lipogenesis) [41], and the muscle (main site for thermogenesis and energy expenditure) [42]. The brain contains the satiety and hunger centers and two separate populations of neuronal cell types. One synthesizes orexigenic neuro-peptide Y (NPY) and agouti-related peptide (AgRP) [43,44], while the other produces anorexigenic pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) [45,46]. These neuropeptides interact in a complex way with each other and with the central melanocortin system, melanin-concentrating hormone, orexin, ghrelin and adiponectin, to mention a few, to regulate energy homeostasis in mammals [for review see [47]]. A recent study, using microarray analysis, reported 24h-cold exposure-induced changes in chicken hypothalamic gene expression [5]. Our work complements and adds to this study by showing that CMCC down regulated the expression of the potent orexigenic NPY and anorexigenic CART genes which may explain the similar feed intake observed between the control and CMCC chicks. Central adiponectin (Adpn) and its related receptors (AdipoR1 and AdipoR2) were down regulated by chronic CMCC in our experimental conditions. Although the role of adiponectin in the regulation of feed intake in mammals is controversial [48,49], its role has not been previously addressed in avian species. As we did not see an effect of CMCC on feed intake despite the significant decrease in central adiponectin system, we speculate that adiponectin might be involved in the regulation of thermogenesis, energy expenditure and/or lipid metabolism as previously reported in mammals [50]. We also identified central FAS-AMPK-mTOR as a new pathway involved in chronic mild cold response. This pathway has been reported to be involved not only in the regulation of energy homeostasis in both mammalian and avian species [51–54], but also in the regulation of energy expenditure [55] and lipid metabolism [56]. It is likely that during cold stress and in order to maintain their normal body temperature, the chicks increased their metabolic heat production

by facultative/adaptive thermogenesis which in turn activates the key cellular energy sensor AMPK and thereby inhibits mTOR (another energy/nutrient-sensitive kinase) activation and the switch from anabolic to catabolic state.

Consistent with our hypothesis and in agreement with previous studies [57], CMCC down regulated the hepatic expression of the key lipogenic genes FAS (which catalyzes the synthesis of long-chain fatty acid synthesis through the condensation of acetyl-CoA and malonyl-CoA in a complex seven-step reaction), ACCα (multifunctional enzyme which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis) and ME (which catalyzes the oxidative decarboxylation of malate to pyruvate and generates NADPH for fatty acid biosynthesis). The concomitant decrease in hepatic SREBP1/2 mRNA abundance indicates that CMCC down regulated the lipogenic gene expression at transcriptional levels through these key transcriptional factors [58]. ACCα is also regulated at translational levels and by phosphorylation/dephosphorylation of targeted serine residues. The increase of ACCα phosphorylation at Ser79 indicates its inactivation by CMCC and further support the notion of fatty acid synthesis inhibition [59]. Furthermore, the decrease of hepatic mTOR phosphorylation consolidates the concept of activated catabolic pathways in the liver of chicks exposed to CMCC. mTOR has been found to play a role in lipogenesis with the finding that rapamycin (mTOR inhibitor) blocks the expression of lipogenic genes and impairs the nuclear accumulation of the SREBPs [60]. Although the exact molecular mechanisms by which SREBPs are regulated by mTOR are not well defined, it is believed to be mediated by ribosomal S6 kinase 1 (S6K1) [61]. As recently reported, mTOR may also regulate the SREBP-lipogenic gene transcriptional networks through the negative regulation of lipin 1 [62]. Several other molecular

mediators such as PPARα/δ, Akt and PKA are not ruled out [63,64] and further studies are warranted.

Nonshivering thermogenesis (NST) is a common adaptive response to cold found in a number of mammalian and avian species [65], however the site and mechanisms appear to be different. While brown adipose tissue is well known to account for a large proportion of mammalian NST [66] via activation of UCP-1, skeletal muscle, however is the main site of avian NST [42]. Mechanisms involved in avian NST are still unclear but may involve reduced energetic coupling in skeletal muscle mitochondria through activation of an avian homolog of mammalian UCP1. We have previously identified avian muscle UCP [67] and we and others found that it was up regulated by short severe cold exposure [68–70]. Here, neither UCP nor other mitochondrial-related genes (ANT, NRF-1 and Ski) are altered by CMCC indicating that our experimental conditions are not harsh. The difference observed between this and previous studies may be related to severity and duration of environmental temperature at which birds were acclimated. Indeed, Duchamp's group recently reported that muscle NST, UCP expression and the intensity of mitochondrial oxidative phosphorylation increased in proportion with the harshness of cold [69]. CMCC induced CPT-1, the rate-limiting enzyme in the β-oxidation of long chain fatty acids [71]. Taken together our data indicate that the decreased activity of ACCα causes a decrease in malonyl-CoA concentration which in turn induces CPT-1activity and enhances fatty acid β-oxidation. The increased mRNA levels and phosphorylation of mTOR in the muscle of CMCC chicks indicate that mitochondrial fatty acid utilization and oxidation are very likely regulated by mTOR [72].

Finally, to assess whether our experimental conditions were stressful or not, we determined the mRNA abundance and protein expression of HSPs, known as key stress markers,

and their related transcription factors HSFs in all three tissues. During stress, HSPs are rapidly synthesized and are involved in folding/unfolding and assembly/disassembly of protein complexes to protect stressed cells. In our study, CMCC induced the expression of avian HSP-70 and HSP-27 only in the liver but not in the muscle or the brain, indicating a tissue-specific regulation of HSPs by cold exposure. Additionally, our data suggested that liver tissue may be more sensitive to cold stress compared to other tissues and supports a protective function of HSPs as previously reported in myocardium [20,73].

In summary, we provided new evidence that CMCC can improve growth performances (BWG and FCR) in young chicks through a modulation of key metabolic-related genes. AMPK and mTOR seem to be a key molecular signatures orchestrating cellular fatty acid utilization (inhibition of hepatic lipogenesis and increase of muscle fatty acid β-oxidation) to satisfy energy requirement during cold exposure. These findings open a new vista on the role of the AMPK-mTOR pathway in cold acclimation and further studies are needed to determine the up- and down-stream mediators that can be used in genetic selection to improve avian thermo (cold)-tolerance.

3.6. Supporting Information

S1 Fig. Effect of CMCC on AMPKβ and AMPKγ subunits in young broiler chickens. Relative expression of AMPKβ1/β2 and AMPKγ1/2/3 in the brain (a), liver (b), and muscle (c) was measured by qPCR using 2- Ct method. Data are mean ± SEM (n = 6). Indicate a significant difference between cold and the control group ($P<0.05$).

(TIF)

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3.8. Author Contributions

Conceived and designed the experiments: SD GH. Performed the experiments: PN EG PI. Ana-lyzed the data: PN EG PI. Contributed reagents/materials/analysis tools: SD AD WB. Wrote the paper: SD.

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Table 3.1. Oligonucleotide qPCR primers

Gene	Accession number	Primer sequence (5' →3')	Orientation	Product size (bp)
NPY	NM_205473	CATGCAGGGCACCATGAG	Forward	55
		CAGCGACAAGGCGAAAGTC	Reverse	
AgRP	AB029443	GCGGGAGCTTTCACAGAACA	Forward	58
		CGACAGGATTGACCCCAAAA	Reverse	
Ox	AB056748	CCAGGAGCACGCTGAGAAG	Forward	67
		CCCATCTCAGTAAAAGCTCTTTGC	Reverse	
Ox1R	AB110634	TGCGCTACCTCTGGAAGGA	Forward	58
		GCGATCAGCGCCCATTC	Reverse	
Ox2R	XM_004945362	AAGTGCTGAAGCAACCATTGC	Forward	61
		AAGGCCACACTCTCCCTTCTG	Reverse	
Adpn	AY786316	ATGGACAAAAGGGAGACAAAGG	Forward	64
		TCCAGCACCCATATACCCAAA	Reverse	
AdipoR1	NM_001031027	CCGGGCAAATTCGACATC	Forward	58
		CCACCACGAGCACATGGA	Reverse	
AdipoR2	NM_001007854	TTGCCACTCGGAAGGTGTTT	Forward	60
		AGTGCAATGCCAGAATAATCCA	Reverse	
Pomc	AB019555	GCCAGACCCCGCTGATG	Forward	56
		CTTGTAGGCGCTTTTGACGAT	Reverse	
Cart	KC249966	GCTGGAGAAGCTGAAGAGCAA	Forward	60
		GGCACCTGCCCGAACTT	Reverse	
Ob-R	NM_204323	GCAAGACCCTCTCCCTTATCTCT	Forward	70
		TCTGTGAAAGCATCATCCTGATCT	Reverse	
Ghrl	AY303688	CACTCCTGCTCACATACAAGTTCA	Forward	75
		TCATATGTACACCTGTGGCAGAAA	Reverse	
GHS-R1a	NM_204394	GCACAAATCGGCAAGGAAA	Forward	61
		GTGACATCTCCCAGCAAATCC	Reverse	
CRH	NM_001123031	TCAGCACCAGAGCCATCACA	Forward	74

Table 3.1. Oligonucleotide qPCR primers (cont.)

Gene	Accession number	Primer sequence (5' →3')	Orientation	Product size (bp)
		GCTCTATAAAAATAAAGAGGTGACA TCAGA	Reverse	
FAS	JO3860	ACTGTGGGCTCCAAATCTTCA	Forward	70
		CAAGGAGCCATCGTGTAAAGC	Reverse	
ACCα	NM_205505	CAGGTATCGCATCACTATAGGTAAC AA	Forward	74
		GTGAGCGCAGAATAGAAGGATCA	Reverse	
SCD-1	NM_204890	CAATGCCACCTGGCTAGTGA	Forward	52
		CGGCCGATTGCCAAAC	Reverse	
AMPKα1	NM_001039603	CCACCCCTGTACCGGAAATA	Forward	68
		GGAAGCGAGTGCCAGAGTTC	Reverse	
AMPKα2	NM_001039605	GCGGAGAGAATCTGCTGGAA	Forward	62
		TGTAAGCATGGACGTGTTGAAGA	Reverse	
AMPKβ1	NM_001039912	TTGGCAGCAGGATCTGGAA	Forward	60
		AAGACTGTTGGTCGAGCTTGAGT	Reverse	
AMPKβ2	NM_001044662	TGTGACCCGGCCCTACTG	Forward	56
		GCGTAGAGGTGATTGAGCATGA	Reverse	
AMPKγ1	NM_001034827	CAAGCCGTTGGTCTGCATCT	Forward	56
		GGGAGGAGACGGCATCAA	Reverse	
AMPKγ2	NM_001278142	TGCCATGCCATTCTTGGA	Forward	62
		CCACCTTGCGAGAAGCATTT	Reverse	
AMPKγ3	NM_001031258	CCCAAGCCACGCTTCCTA	Forward	57
		ACGGAAGGTGCCGACACA	Reverse	
mTOR	XM_417614	CATGTCAGGCACTGTGTCTATTCTC	Forward	77
		CTTTCGCCCTTGTTTCTTCACT	Reverse	
ME	AF408407	AGATGAAGCTGTCAAAAGGATATGG	Forward	62
		CACGCCCCTTCACTATCGA	Reverse	

Table 3.1. Oligonucleotide qPCR primers (cont.)

Gene	Accession number	Primer sequence (5' →3')	Orientation	Product size (bp)
ATPcl	NM_001030540	CTTTTAAGGGCATTGTTAGAGCAAT	Forward	65
		CCTCACCTCGTGCTCTTTCAG	Reverse	
SREBP-1	AY029224	CATCCATCAACGACAAGATCGT	Forward	82
		CTCAGGATCGCCGACTTGTT	Reverse	
SREBP-2	AJ414379	GCCTCTGATTCGGGATCACA	Forward	63
		GCTTCCTGGCTCTGAATCAATG	Reverse	
INSIG-1	NM_001030966	TGGCGCTGGTGCTGAAC	Forward	63
		TGACCTCGTCGGGAAACAG	Reverse	
INSIG-2	NM_001031261	CAGCGCTAAAGTGGATTTTGC	Forward	65
		CAATTGACAGGGCTGCTAACG	Reverse	
UCP	NM_204107	TGGCAGCGAAGCGTCAT	Forward	59
		TGGGATGCTGCGTCCTATG	Reverse	
ANT	AB088686	GCAGCTGATGTCGGCAAA	Forward	56
		CAGTCCCCGAGACCAGAGAA	Reverse	
NRF-1	NM_001030646	GGCCAACGTCCGAAGTGAT	Forward	55
		CCATGACACCCGCTGCTT	Reverse	
Ski	M28517	GGCCCTGCTGCTTTCTCA	Forward	75
		AGGTTCCGCTGGGTCTTTG	Reverse	
CPT-1	AY675193	GCCCTGATGCCTTCATTCAA	Forward	60
		ATTTTCCCATGTCTCGGTAGTGA	Reverse	
S6K1	NM_001109771	GTCAGACATCACTTGGGTAGAGAAAG	Forward	60
		ACGCCCTCGCCCTTGT	Reverse	
PGC-1α	NM_001006457	GAGGATGGATTGCCTTCATTTG	Forward	62
		GCGTCATGTTCATTGGTCACA	Reverse	
PGC-1β	XM_414479	TTGCCGGCATTGGTTTCT	Forward	66
		CACGGGAAGCCACAGGAA	Reverse	
PPARα	AF163809	CAAACCAACCATCCTGACGAT	Forward	64

Table 3.1. Oligonucleotide qPCR primers (cont.)

Gene	Accession number	Primer sequence (5' →3')	Orientation	Product size (bp)
		GGAGGTCAGCCATTTTTTGGA	Reverse	
PPARδ	NM_001001460	CACTGCAGGAACAGAACAAAGAA	Forward	67
		TCCACAGAGCGAAACTGACATC	Reverse	
HSP70	JO2579	GGGAGAGGGTTGGGCTAGAG	Forward	55
		TTGCCTCCTGCCCAATCA	Reverse	
HSP60	NM_001012916	CGCAGACATGCTCCGTTTG	Forward	55
		TCTGGACACCGGCCTGAT	Reverse	
HSP27	XM_001231557	TTGAAGGCTGGCTCCTGATC	Forward	58
		AAGCCATGCTCATCCATCCT	Reverse	
HSF1	L06098	GAGACGGACCCGCTGATCT	Forward	58
		GGTCGAACACATGGAAGCTGTT	Reverse	
HSF2	NM_001167764	GCCCAGCAACCAGCTTATCA	Forward	63
		TGTTCATCCAACACCAAGAAACTC	Reverse	
HSF3	L06126	CAGAGCGACGACGTCATCTG	Forward	66
		CCGCTGCTCATCCAGGAT	Reverse	
HSF4	NM_001172374	CAAAGAGGTGCTGCCCAAGT	Forward	60
		AGCTGCCGGACGAAACTG	Reverse	
18S	AF173612	TCCCCTCCCGTTACTTGGAT	Forward	60
		GCGCTCGTCGGCATGTA	Reverse	

[a] Accession number refer to Genbank (NCBI). ACC, acetyl-CoA carboxylase; AdipoR, adiponectin receptor; Adpn, adiponectin; AgRP, agouti related peptide; AMPK, AMP-activated protein kinase; ANT, adenine nucleotide translocator; ATPcl, ATP citrate lyase; Cart, cocaine and amphetamine regulated transcript; CPT, carnitine palmitoyltransferase; CRH, corticotropin releasing hormone; FAS, fatty acid synthase; Ghrl, ghrelin; GHS-R1a, ghrelin receptor; HSF, heat shock factor; HSP, heat shock protein; INSIG, insulin induced gene; ME, malic enzyme; mTOR, mechanistic target of rapamycin; NPY, neuropeptide Y; NRF, nuclear respiratory factor; Ob-R, leptin receptor; Ox, orexin; OxR, orexin receptor; PGC, PPARδ coactivator; Pomc, pro-opiomelanocortin; PPAR, peroxisome proliferator activator of transcription; SCD, stearoyl-CoA desaturase; Ski, avian sarcoma viral oncogene homolog; S6K1, ribosomal S6 kinase; SREBP, sterol regulatory element binding protein; UCP, uncoupling protein.

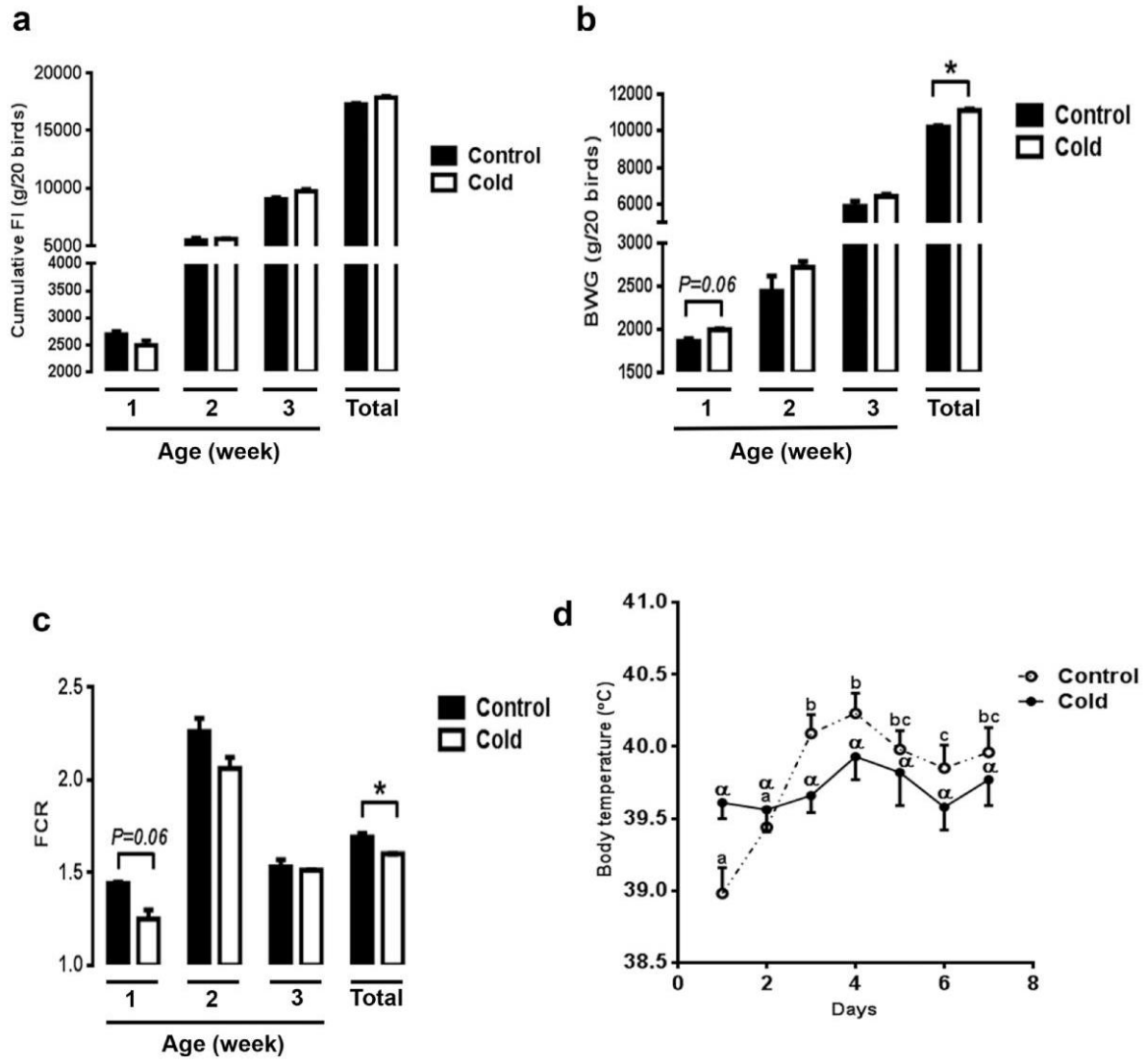

Fig 3.1. Effect of CMCC on growth performances in young broiler chicks. Cumulative feed intake FI (a), body weight gain BWG (b), feed conversion ratio FCR (c), and body temperature (d). Data are presented as mean ± SEM (n = 40) for each week and for the total experimental period (3 weeks). *P<0.05. Different letters indicate daily difference in body temperature within each group (a-c and α, difference within control and cold group, respectively).

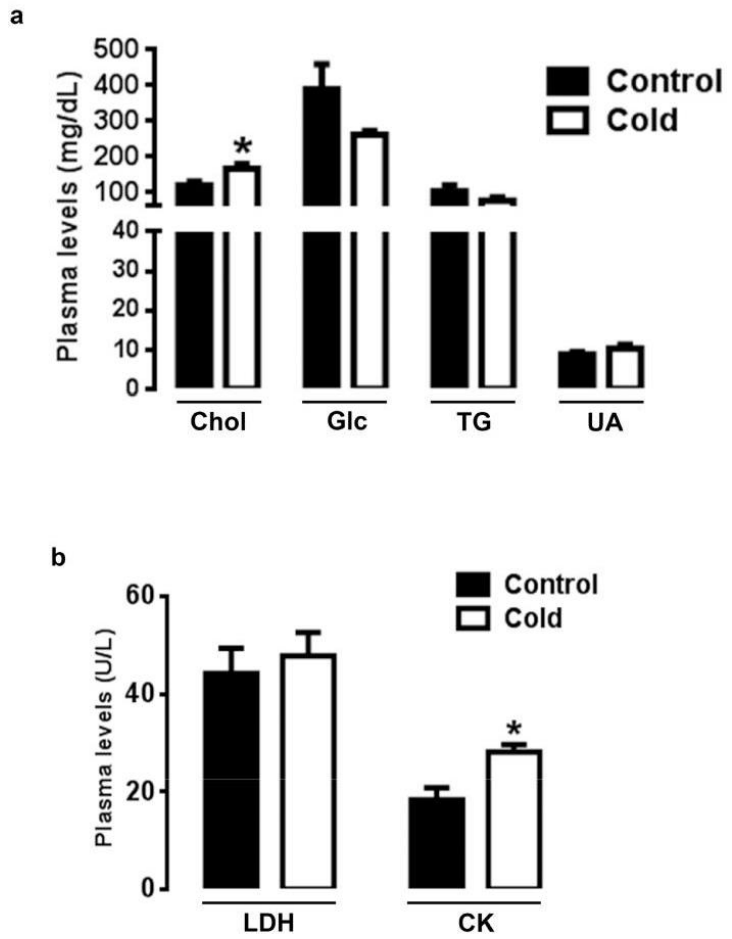

Fig 3.2. Effect of CMCC on plasma metabolite levels. Plasma levels of Chol, Glc, TG, UA (a), LDH and CK (b) were determined at the first week using commercial kits as described in materials and methods. Data are presented as mean ± SEM (n = 6). *Indicate a significant difference between cold and control group ($P<0.05$).

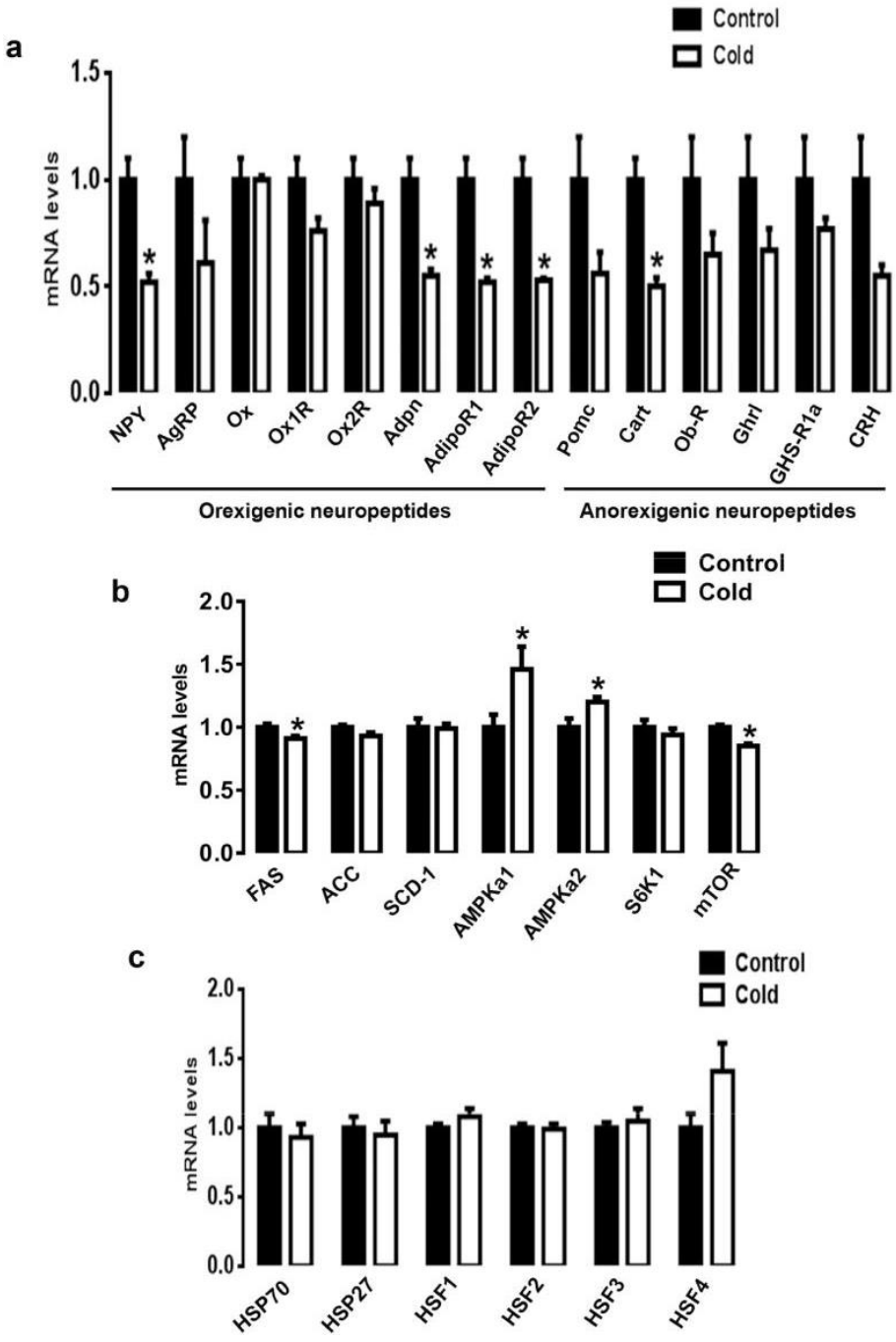

Fig 3.3. Effect of CMCC on feeding-related genes and HSPs in the brain of young broiler chicks. Relative expression of hypothalamic feeding-related neuropeptides (a), lipogenic genes (b), HSPs and HSF (c) were determined by qPCR using 2- Ct method [25]. Data are presented as mean ± SEM (n = 6). * Indicate a significant difference between cold and control group ($P<0.05$).

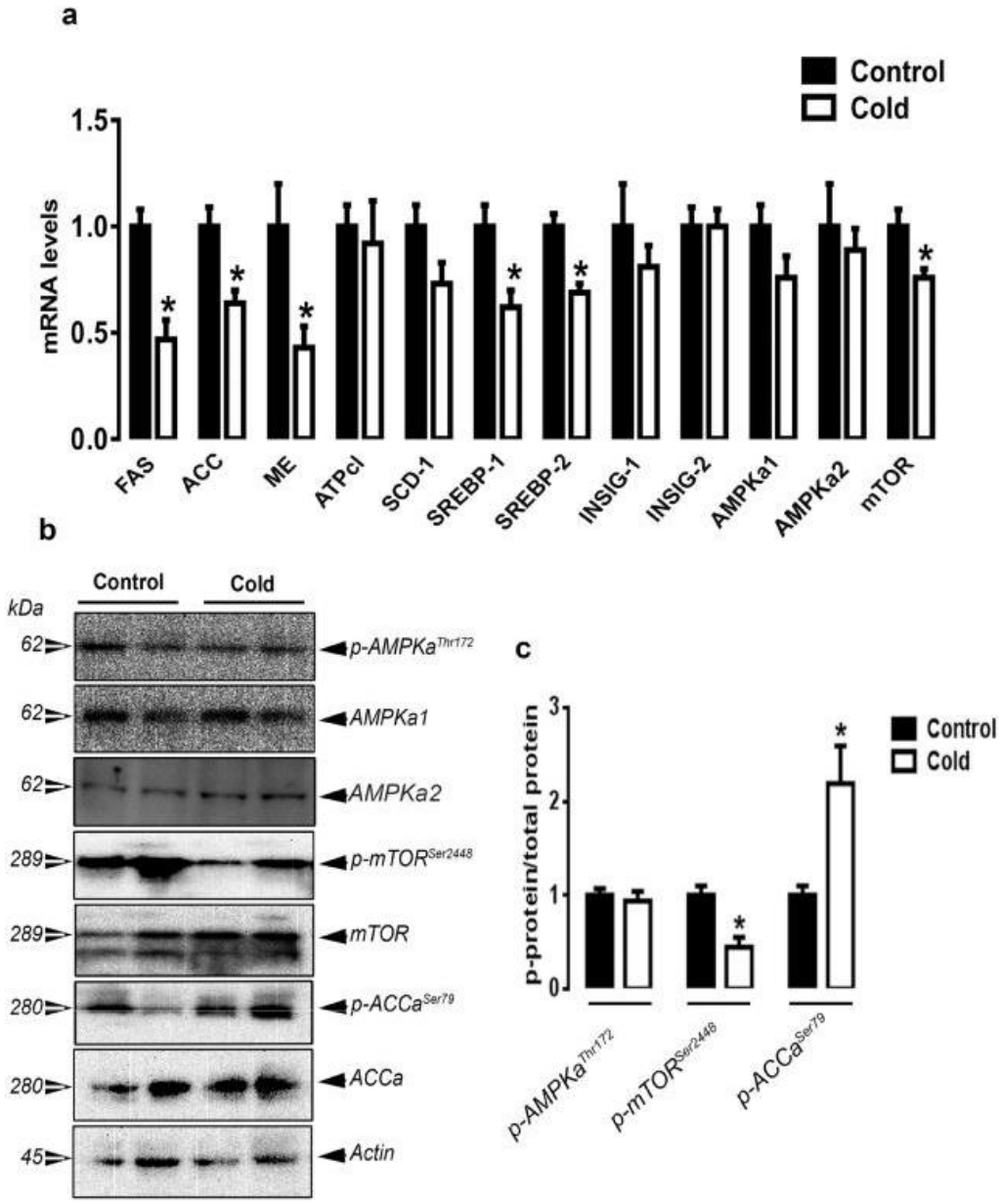

Fig 3.4. Effect of CMCC on lipogenesis-related genes in liver of young broiler chicks. Relative expression of lipogenic genes and their related transcription factors (a) was measured by qPCR as described in material and methods. Phosphorylated and total protein levels of AMPK, mTOR and ACC were determined by Western blot (b) and presented as p-protein/total protein ratio (c). β-actin was used as loading and housekeeping control. Data are presented as mean ± SEM (n = 6). *Indicate a significant difference between cold and control group ($P<0.05$).

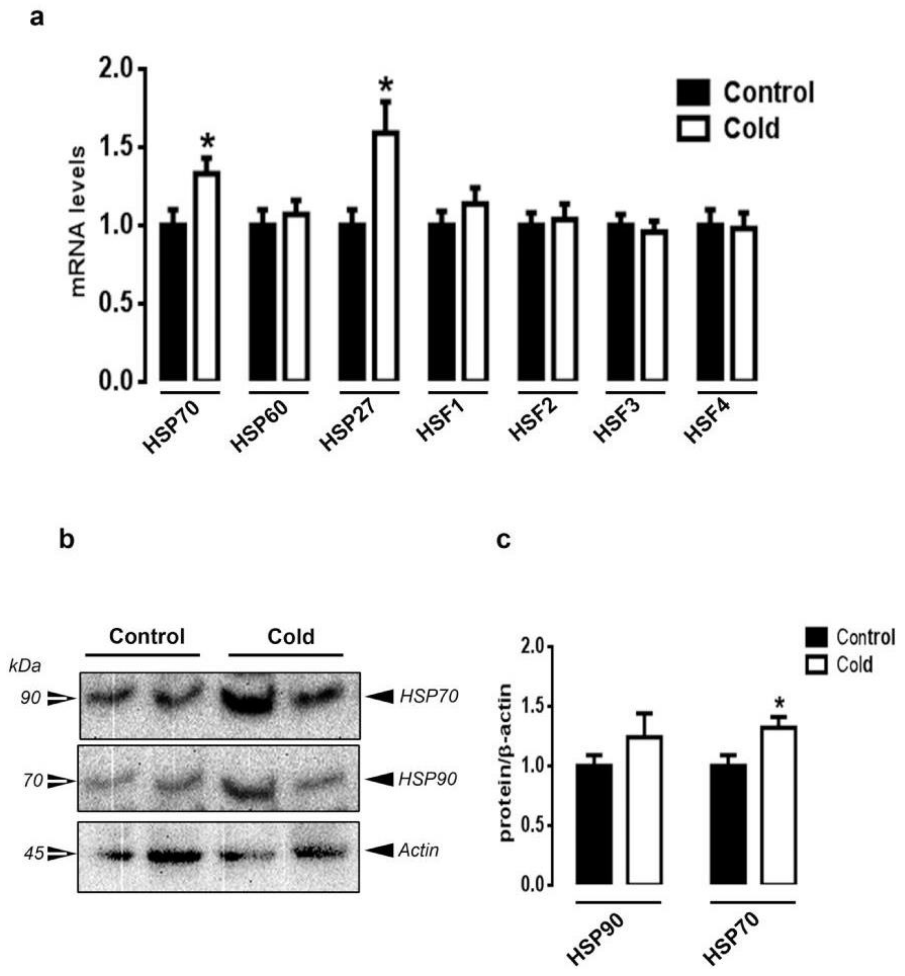

Fig 3.5. Effect of CMCC on HSP and HSF expression in liver of young broiler chicks. HSP and HSF mRNA levels were measured by qPCR (a). HSP70 and HSP90 protein levels were determined by Western blot (b) and presented as normalized ratio to β-actin (c). The values represent the mean ± SEM (n = 6). *Indicates a significant difference between cold and control group ($P<0.05$)

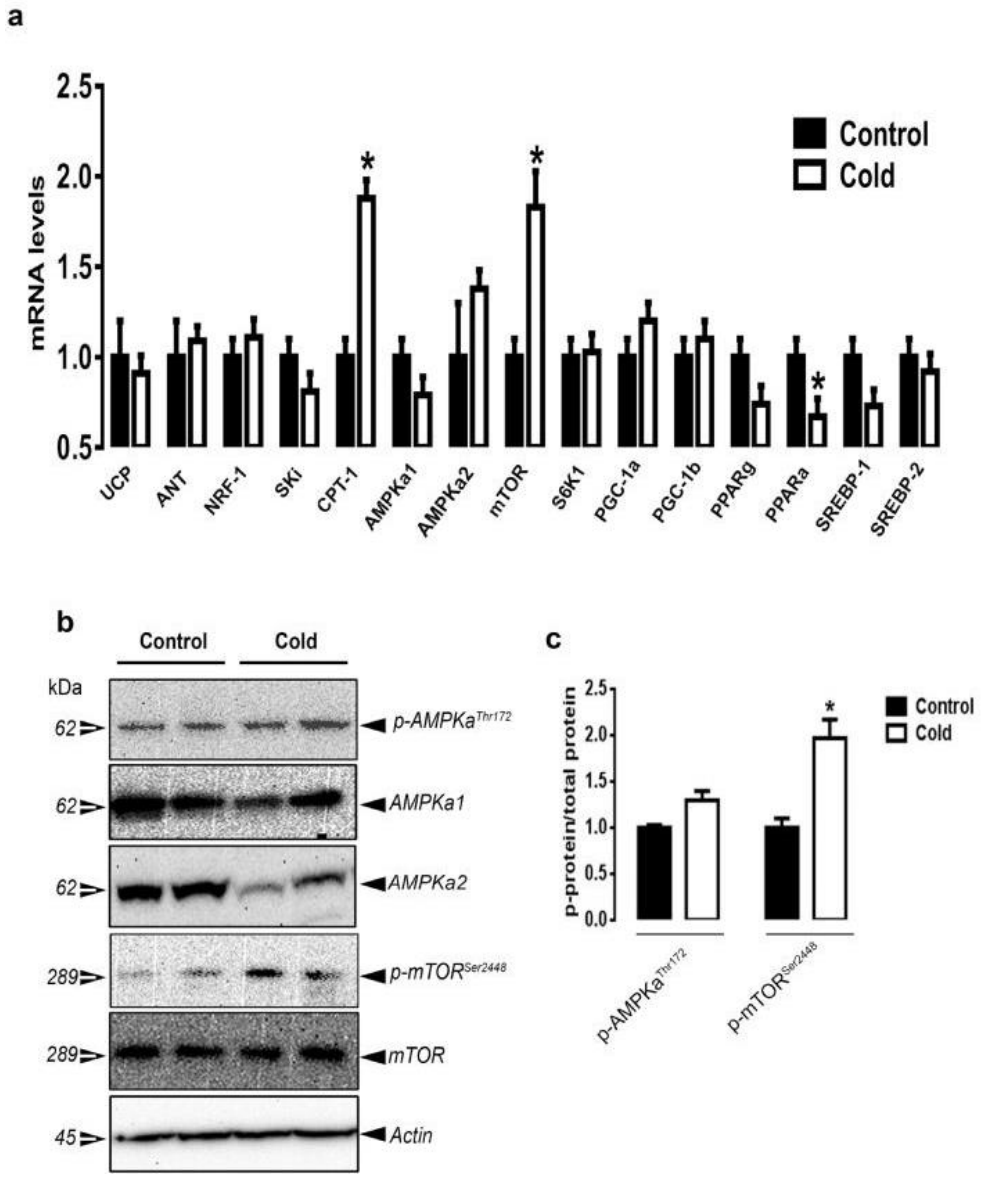

Fig 3.6. Effect of CMCC on metabolic-related genes in muscle of young broiler chicks. Relative expression of mitochondrial- and metabolic-related genes was determined by qPCR (a). Phosphorylated and total protein levels of AMPKα1/α2 and mTOR were determined by Western blot (b) and presented as p-protein/total protein ratio (c). The values represent the mean ±SEM (n = 6). * Indicates a significant difference between cold and control group ($P<0.05$).

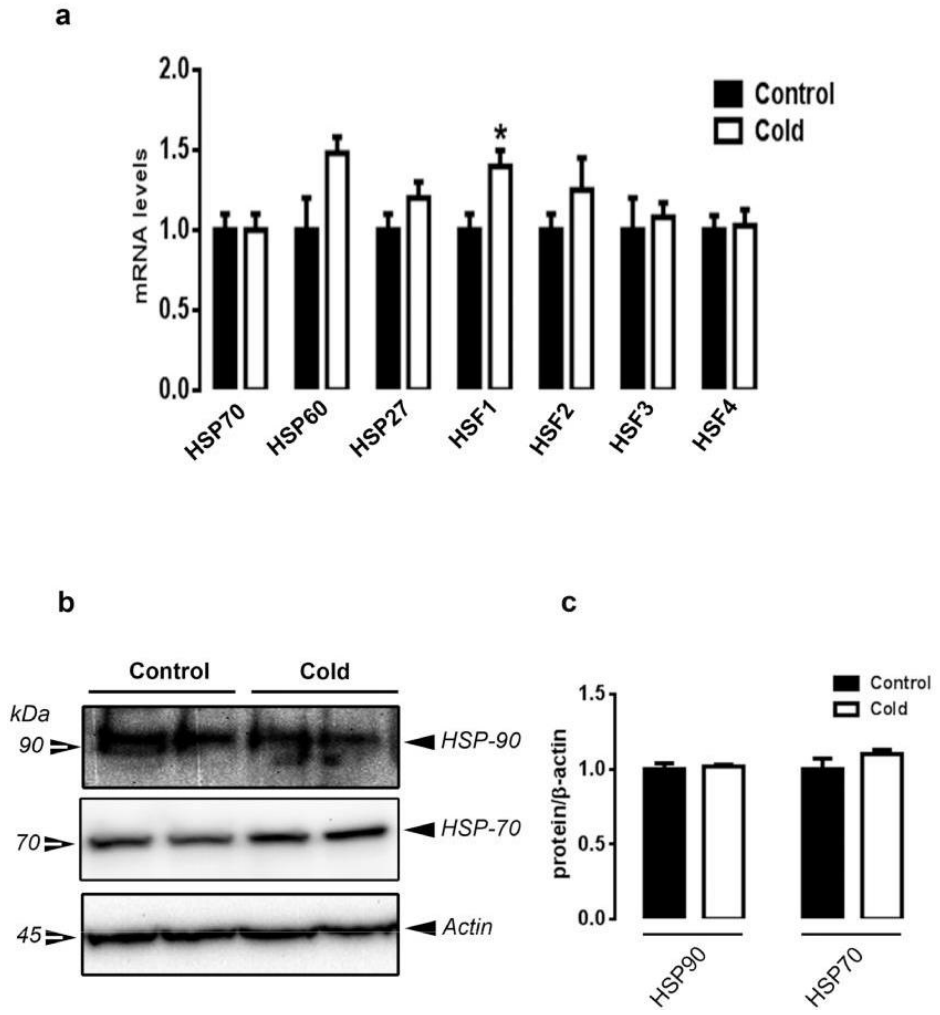

Fig 3.7. Effect of CMCC on HSP and HSF expression in the muscle of young broiler chicks. HSP and HSF mRNA levels were measured by qPCR (a). HSP70 and HSP90 protein levels were determined by Western blot (b) and presented as normalized ratio to β-actin (c). The values represent the mean ± SEM (n = 6). *Indicates a significant difference between cold and control group ($P<0.05$).

CHAPTER 4
A New Insight into Cold Stress in Poultry Production

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Since about 1950, numerous examples of extreme climate events have been recorded. These include not only the increase in temperature which has caused frequent and intense heat waves, melting ice caps, and high sea level, but also an extreme low winter temperature along with higher precipitation in different regions in the world. Further changes in the climate system are predicted to increase over the course of the 21st century.[1] Since growing animals are vulnerable to extreme temperature, climate changes become an important critical constraint to several species in the world.[1,2] In poultry production, while heat stress has been a rising concern for producers and scientists, cold stress has also caused economic loss worldwide. In China, winter conditions and low temperature caused almost 20 million poultry deaths and an economic loss of 100 million (Chinese currency) in 2008.[3] Early research in poultry exposed to acute cold stress has shown a clear suppression in development, survival and egg production.[4-6]

At the molecular level, an acute hypothermal condition significantly up-regulated gene expression of hepatic leptin and muscle UCP in 5-wk-old broilers.[7] Exposure at 4 ˚C for 24 hours resulted in changes in some genes involved in lipid metabolism in broilers pituitary. This suggests that cold stress can affect lipid metabolism.[3] At younger age (7-14 days old), although a chronic cold stress (20 ˚C) did not affect body weights and feed intake, it significantly increased chick body heat production and *avUCP* gene expression in the leg muscle.[8] Birds exposed to cold stress had severely injured liver and increased gene expression of AMPKα– PPARα pathway.[9] Moreover, there is clear evidence that cold stress affects thyroid hormones (T3 and T4) which play a key role in energy expenditure and body temperature homeostasis.[8,10] Yet, the effect of cold exposure on growth performance is still controversial. Indeed, Baarendse and colleagues[11] observed that moderate cold exposure (28 ˚C, reducing 1 ˚C every day in five-day period) during the early post-hatching period caused long-term negative effects onchicken

growth performance. However, Shinder and colleagues reported that acute cold exposure at late embyogenesis improved growth performance.[10,12] These discrepancies might be due to several factors including experimental conditions (environmental temperature, age, chicken strain, and/or exposure duration). We recently investigated the effect of Chronic Mild Cold Conditioning (CMCC) and its underlying molecular mechanisms on growth performances in broilers. Our data show that CMCC improved the growth performance of chicks during the first week post-hatch and their later lives in terms of body weight gain and Feed Conversion Ratio (FCR).[13] Plasma cholesterol and creatine kinase (CK) levels increased indicating a potential role of CK in maintaining high ATP turnover in a hypothermal condition.[14] Hypothalamic orexigenic neuropeptide Y (NPY) and anorexigenic cocaine and amphetamine regulated transcript (CART) gene expression were significantly down-regulated in the brain of cold group which may explain the reduction of feed intake in CMCC compared to the control group. CMCC also modulated the hepatic expression of lipogenic genes, which implies the inhibition of fatty acid synthesis in cold stress chicks.[13] Moreover, CMCC enhanced muscle fatty acid β-oxidation through affecting the gene and protein expression of carnitine palmitoyl transferase-1 (CPT-1) and phosphorylated mTOR.[16,17]

In summary, the CMCC used in our study could improve later growth performance of young chicks (body weight gain and FCR). This is new evidence that gives us a broader view of how young birds can adapt to and prepare for changes in their environment. In addition, gene expression analyses provide insight into the roles of the AMPK-mTOR pathway in cold acclimation; thus further studies are needed to understand the regulation of these genes for better management *via* genetic selection and/or nutritional strategies to improve cold tolerance and feed efficiency.

CONFLICTS OF INTEREST: None.

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CHAPTER 5
Expression of Orexin and Its Related Receptors in Avian Muscle Tissues and Cells under Heat and Oxidative Stress

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5.1. Abstract

Orexins (ORX A and ORX B) or hypocretins (hypocretin 1 and hypocretin 2) and their receptors (ORXR1 and ORXR2) have been found mainly in the brain of many species including avian. Recently, we identified orexin system in avian muscle cell and tissue. Since orexins and their related receptors have roles in stress response, and heat and oxidative stress adversely impact poultry production, their regulation and functions in avian muscle under these stressors needs to be elucidated. In this study, two Japanese quail (*Coturnix coturnix Japonica*) lines divergently selected for susceptibility (S) or resistance (R) to restraint stress at 4 weeks of age, were exposed to acute heat stress (37^{o}C for 1.5h) or maintained at thermoneutral conditions (22^{o}C). In addition, a quail muscle (QM7) cell line was exposed to heat stress (45^{o}C) for 0.5, 1, 2, or 4 h; or exposed to over-expressed HSP70 and exposed to heat stress for 4 h and recovered 1h. The control cells were maintained at 37^{o}C. The cells were also treated with hydrogen peroxide (H_2O_2) at 10, 50, 100 or 200 µM for 3 hours and 4-Hydroxynonenal (4-HNE) at 10, 20 or 30 µM levels for 24 hours. The *in vivo* results revealed that acute heat stress significantly down regulated ORX, ORXR1, and HSP70 protein expression but up regulated mRNA levels of ORX receptors in R quail muscle. Heat stress down regulated the orexin system (protein and gene expression) in the muscle cell line (QM7) in a time dependent manner. Over-expression of HSP70 in QM7 cells suppressed ORX system expression under thermoneutral conditions ($P<0.05$), but not under heat stress. Oxidative stress induced by H_2O_2 reduced protein levels of the ORX system, but 4-HNE up-regulated ORX and its related receptor protein expression. These results suggest that orexin and its related receptors expression was affected by heat stress and oxidative stress, therefore they may have potential as bio-markers for these stressors, and

further understanding their regulation can help alleviate heat stress and oxidative stress effects in birds.

Key words: Heat stress, oxidative stress, orexin, muscle

5.2. Introduction

The fast increase in average weather temperature due to climate change within the past 30 years (Hansen et al. 2012) has affected agriculture worldwide (Skuce et al. 2013). Poultry production is also markedly influenced by a hot climate or heat stress which suppresses the growth, egg production, and welfare of poultry (Knox et al. 2012; Lara & Rostagno 2013). Heat stress reduces sodium, potassium, partial CO_2 pressure, and lymphocytes in blood (Borges et al. 2004). It also alters heat shock proteins (particularly HSF3 and HSP70) in the liver, leg muscle and brain of chickens (Zhang et al. 2013). Heat stress has also been shown to up regulate HSP70 gene expression in chicken embryos (Gabriel et al. 2003). Noticeably, the effects of heat stress in poultry are different among breeds and genetic selection has been leading to more heat stress susceptible birds (Soleimani et al. 2011; Zhang et al. 2013).

In addition, there is evidence showing the relationship between heat stress and oxidative stress. Mujahid and colleagues found that acute heat stress induced the production of superoxide in skeletal muscle mitochondria of broilers which caused a significant weight loss in the birds (Mujahid et al. 2005). It has also been shown that heat exposure leads to oxidative stress in these birds (Mujahid et al. 2005; Lin et al. 2006; Mujahid et al. 2007a; Mujahid et al. 2007b). Oxidative stress plays an important role in biological damage and influences on poultry growth (reviewed by (Fellenberg & Speisky 2006).

Orexins (orexin A and orexin B) or hypocretins (hypocretin 1 and hypocretin 2) were first identified in and around the lateral and posterior hypothalamus in the rat brain (de Lecea et al. 1998; Sakurai et al. 1998). These neuropeptides bind to two G-protein couple receptors named orexin receptor 1 (ORXR1) and orexin receptor 2 (ORXR2) (Sakurai et al. 1998; Trivedi et al. 1998). Their expression has since been found in many species including birds (reviewed by

(Wong et al. 2011). In avian species, orexins/hypocretins and their receptors are distributed in brain regions such as: the pituitary gland, the adrenal gland and the testis/ovary (Ohkubo et al. 2003; Miranda et al. 2013). Interestingly, in 2015, our group characterized orexin and its receptors in chicken muscle tissue and quail muscle (QM7) cell line and proposed autocrine, paracrine and/or endocrine roles for orexins (Lassiter et al. 2015). It is widely recognized that hypocretins/orexins and their receptors are involved in feed intake regulation (Sakurai et al. 1998; Haynes et al. 2000), drinking behavior (Kunii et al. 1999), sleeping and wakefulness (Ohno & Sakurai 2008), cardiovascular and respiratory functions in rodents (Li & Nattie 2014). Recently, orexin was shown to participate in arousal and stress regulation (Berridge et al. 2010); but it is not involved in all forms of stress (Furlong et al. 2009). In poultry, the roles of orexin have not been well understood; but do not appear to be the same as in mammals. For instance, Miranda and colleagues found that hypothalamic orexin did not control the sleep-wake cycle in chicken; however, it was up-regulated when feed intake was restricted in broilers (Miranda et al. 2013). In peripheral tissues, ORX system gene and protein expression were observed to be significantly down-regulated in heat stressed quail livers and LMH (leghorn male hepatoma) cells. In the same study, oxidative stress caused by H_2O_2 treatment reduced ORX protein; but significantly increased its mRNA level in a dose-dependent manner (Greene et al. 2016). Taken together, this shows the regulation of the ORX system and suggests its key role in heat and oxidative stress response in avian livers (Greene et al. 2016). The role of orexins/ hypocretins in avian muscles has been not examined.

The significant impacts of heat and oxidative stress in poultry have been studied recently; however, there is still a paucity of information about the molecular mechanism of these stressors in the avian species. Orexins have been shown to be regulators in stress and are expressed in

avian muscle. Since muscle is the main site of energy expenditure, the aim of this study is to investigate and understand more about the regulation of orexins/hypocretins in avian muscle tissues and cells under heat and oxidative stress.

5.3. Material and methods

5.3.1 In vivo experiment

This study was conducted according to the recommendations in the guide for the care and use of laboratory animals by the National Institutes of Health and the protocol was approved by the University of Arkansas Animal Care and Use Committee under protocols 13039 and 10025. It was described previously by Greene and colleagues (Greene et al. 2016).

Briefly, two lines of Japanese quail (*Coturnix coturnix Japonica*) males were used. These lines were selected for circulating corticosterone level to restraint stress. The resistant stress line (R) had 66% lower blood corticosterone levels compared to the sensitive stress line (S) (Satterlee & Johnson 1988). The two quail lines in this study were from generation 46. They were hatched and reared separately in floor pens at the University of Arkansas poultry farm. Birds were warm-brooded for 10 days at 32^{o}C and the temperature was gradually reduced each week to 22^{o}C (thermal neutral, TN) at 4 weeks of age. At 4 weeks of age, 6-10 birds of each line were exposed to acute heat stress (37^{o}C for 1.5h) and 6-10 birds were kept at thermoneutral (22^{o}) conditions as controls. The relative humidity of the experiment was 50 ± 5%. By the end of the experiment; the quails were humanly euthanized by cervical dislocation and leg muscle tissues were collected, immediately frozen in liquid nitrogen, and stored at -80^{o}C for later analysis.

5.3.2 In vitro experiment

Quail muscle (QM7) cell lines were grown in M199 medium (Life Technologies, Grand Island, NY) complemented with 10% FBS (Life Technologies), 10% tryptose phosphate (Sigma-

Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO2 and 95% air. The medium was changed every 48h and cells were treated, during their exponential phase of growth, with the following treatments: time course heat stress exposure at 45C for 0.5, 1, 2 or 4 h and then recovered for 1h at 37°C, over-expression of HSP70 in QM7 cells at 37°C, or at 45°C for 4h, and then recovered for 1h at 37°C, hydrogen peroxide (H2O2, 10, 50, 100, or 200 µM) for 3h, and 4-Hydroxynonenal (4-HNE, 10, 20, or 30 µM) for 24h treatment. The control cells were transfected with a null vector (control for HSP-70 overexpression study), or maintained at 37°C (control for the heat stress study), or untreated (control for H_2O_2 and 4HNE studies).

5.3.3. RNA isolation, reverse transcription and real-time quantitative PCR

Total RNAs were extracted from quail muscle tissues and QM7 cells by Trizol reagent according to the manufacturer's recommendations (Life Technologies, Grand Island, NY). As in Piekarski and colleagues description (Piekarski et al. 2014), total RNAs were treated with DNAse, then reverse transcribed and amplified by qPCR using oligonucleotide primers specific for chicken genes. They are orexin (ORX): forward, 5'-CCAGGAGCACGCTGAGAAG-3' and reverse, 5'-CCCATCTCAGTAAAAGCTCTTTGC; orexin receptor 1 (ORXR1): forward, 5' – TGCGCTACCTCTGGAAGGA-3' and reverse, 5'-GCGATCAGCGCCCATTC-3'; orexin receptor 2 (ORXR2): forward, 5'-AAGTGCTGAAGCAACCATTGC-3' and reverse, 5'-AAGGCCACACTCTCCCTTCTG-3'; HSP70: forward, GGGAGAGGGTTGGGCTAGAG and reverse, TTGCCTCCTGCCCAATCA; and ribosomal 18S forward, 5'-TCCCCTCCCGTTACTTGGAT-3' and reverse, 5'-GCGCTCGTCGGCATGTA-3', as a house keeping gene. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method

(Schmittgen & Livak 2008). For the *in vivo* and *in vitro* studies, thermoneutral R quails and control cells were used as calibrators.

5.3.4. Protein extraction and Western blot analysis

Harvested muscle tissues and QM7 cells were homogenized, the protein extracted and measured for concentrations as described by Lassiter and colleagues (Lassiter et al. 2015). 30 µg and 100 µg of total proteins per sample for cells and tissues, respectively, were assessed by immunoblotting (Lassiter et al. 2015) using the following antibodies: rabbit anti-mouse ORX, rabbit anti-rat ORXR1 and 2 (Interchim, Montlucon, France), mouse monoclonal anti-HSP70, GAPDH, or β-actin as house-keeping proteins (Cell signaling Technology, Danvers, MA). Pre-stained molecular weight marker (precision plus protein Dual color) was used as a standard (Biorad, Hercules, CA). The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by FlourChem M MultiFlour System (Protein simple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993-2011, Protein simple, Santa Clara, CA).

5.3.5. Immunoflourescene

Immunoflourescene staining was performed as previously described (Lassiter et al. 2015) using rabbit anti-ORX, anti-ORXR1, or anti-ORXR2 antibody (1:200, Interchim, Montlucon France) and mouse monoclonal anti-HSP70. After incubation with Alexa Flour 488- or 594-conjugated secondary antibody (Molecular probes, Life Technologies, Grand Island, NY) and DAPI (Vector Laboratories, Burlingame, CA); images were obtained and analyzed using the Zeiss Imager M2 and Axio Vision software (Carl Zeiss Microscopy, GmbH 2006-2013).

5.3.6. Statistics

Data from the *in vivo* experiment were analyzed by two-factor ANOVA with temperature conditions and genotype (R and S quail lines) as classification variables. The data from oxidative stressed cells were analyzed by one-way ANOVA, and Tukey's test. Over-expression of HSP70 and heat stressed cell data analysis used Student's t-test. All data were analyzed using Graph Pad Prism software (version 6, La Jolla, CA). $P<0.05$ was set as significantly different.

5.4. Results

5.4.1. Acute heat stress altered ORX and ORXR1/2 expression in quail muscles

As shown in figure 5.1 (a-b), exposure to acute heat stress reduced the protein expression of ORX and ORXR1 in leg muscle of R quail, while there was no change of ORX and its related receptor protein expression in S quail. Under thermoneutral conditions, the protein expression of ORXR1 in S quail muscle was significantly lower than that of R line ($P<0.05$); and ORX protein expression in S quail was markedly higher than that of R quail under acute heat exposure ($P<0.05$). In the same pattern, heat shock protein 70 (HSP70) protein expression was also down regulated by heat stress in the R line muscle ($P<0.05$) without any change in the S line. In contrast, ORX mRNA levels in leg muscles of both two quail lines were the same after acute heat stress exposure. ORXR1 gene expression significantly increased in R and S quail muscles, but muscle ORXR2 gene expression only significantly increased in the R line and no change was detected in the S line under heat stress (Figure 5.1c-e).

5.4.2. Heat stress down regulated orexin system (protein and gene expression) in muscle cell line (QM7) in a time dependent manner

Figure 5.2a and b show that ORX and its receptor (protein and mRNA levels) were reduced gradually when duration of exposure time increased. Noticeably, the mRNA level of

ORX was significantly reduced at 4h of heat stress (45^{o}C) and 1h of recovery (37^{o}C); and ORXR 1/2 mRNA levels were significantly reduced from 2 to 4h under heat compared to control groups.

5.4.3. Over-expression of HSP70 in QM7 cells shows the regulation of ORX system expression via HSP70 under thermoneutral and heat stress condition

From the data in over-expressed HSP70-QM7 cells under thermoneutral conditions, heat stress experiment was conducted on these cells. Over-expression of HSP70 in quail muscle (QM7) cells significantly down-regulated the gene and protein expression of ORX and its related receptors ($P<0.05$) (Figure 5.3 a-b). The mRNA levels of HSP27, HSF1 and HSF2 were also significantly reduced ($P<0.05$) (Figure 5.3b).

Conversely, figure 5.4 (a-b) shows the significant increase of HSP70 protein expression after 4h of heat exposure (45^{o}C) and 1 h recovery in over-expressed QM7 cells. Noticeably, protein levels of ORX and its related receptors did not reduce under heat stress ($P>0.05$); but mRNA level of ORX was markedly up-regulated ($P<0.05$). These results were opposite to the observed data in normal QM7 cells which showed the reduction of ORX system expression under heat stress (figure 5.2 a-b).

5.4.4. H_2O_2 down regulated the expression of ORX and its related receptors in QM7 cells

H_2O_2 reduced ORX and ORXR1/2 protein levels in a dose dependent manner, but increased HSP70 protein expression at the highest dose of H_2O_2 (200μM) (Figure 5.5a). Therefore, the highest concentration of H_2O_2 (200μM) was chosen for the immunofluorescence staining. It decreased protein expressions of ORX and its related receptors, but increased HSP70 as shown in figure 5.5f.

5.4.5. 4-HNE up-regulated the expression of ORX and its related receptors in QM7 cell lines

4-HNE altered ORX and ORXR1/2 protein levels, and caused the highest expression of ORX system at 20μM 4-HNE (Figure 5.6a). However, 30μM of 4-HNE significantly increased mRNA level of ORXR2 in QM7 cells (Figure 5.6d). 20μM of 4-HNE was chosen for the treatment in immunofluorescence staining, and it increased protein expressions of ORX and its related receptors, and increased of HSP70 (Figure 5.6f).

5.5. Discussion

Heat stress has been negatively affecting poultry health and production, due to the increasing global climate change (Hansen et al. 2012; Knox et al. 2012; Lara & Rostagno 2013). Studies have found that orexin and its related receptors are expressed in avian muscle tissues (Lassiter et al. 2015), and involved in heat and oxidative stress response in chicken liver and LMH (leghorn male hepatoma) cells (Greene et al. 2016). Therefore, this study was conducted to understand the regulation of the ORX system in avian muscle tissue under heat and oxidative stress.

Heat stress down regulated orexin and its related receptor 1 protein expressions in R line quail muscle and QM7 cells; this suggests a regulation of orexin system in muscle under acute heat stress. These results are in agreement with the results from a previous study in R quail liver and LMH cells (Greene et al. 2016); but not in the S quail line. Zhang and colleagues observed that the loss of orexin in orexin neuron-ablated mice (ORX-AB) decreased stress-induced hyperthermia (Zhang et al. 2010). Moreover, orexin neurons were proved to be inhibited under the increase of temperature (from 2 to 4^{o}C between 26 and 40°C) in acute rat brain slices (Parsons et al. 2012). Therefore, the reduction in orexin system expression may relate to body temperature change in the animal body or cells under heat exposure. In addition, there was a lack

of correlation between the protein expression and gene expression of the ORX system in muscle tissues. This could be due to post-transcriptional mechanisms (Michinaga et al. 2011). In cell culture, ORX is secreted into the medium of QM7 cells (Lassiter et al. 2015) and may inhibit the gene expression of its own system which was noticed as a positive correlation between protein and gene expression of ORX and its related receptors in QM7 cells treated under heat stress from 2 to 4h.

Interestingly, this study shows almost no alteration under heat stress in the protein and gene expression of ORX and its receptors in the muscle of S line quails which have a 66% higher plasma corticosterone levels than the R line quails. This may be the result of the relationship between ORX and plasma corticosterone levels. Intra-cerebroventricular injection of ORX-A significantly increased plasma corticosterone level in male rats (Samson et al. 2002). Furthermore, corticosterone was observed to reverse the reduction of ORX A – induced feeding caused by adrenalectomy (ADX) in the rat brain (Ford et al. 2005). Another study showed that the deficiency of corticosterone significantly elevated ORX expression in the lateral hypothalamus of Pomc−/− mice (López et al. 2007). The unchanged ORX protein or gene expression in S quail muscle tissues, may indicate the link between ORX system and corticosterone in bird muscle under heat stress.

As noticed, the reduction in protein and gene expression of the ORX system in QM7 cells under heat stress and over-expressed HSP70-QM7 cells accompanied by the increase in HSP70 expression. HSP70 has the role in thermotolerance, as it protects and repairs cell from damage (Sanchez et al. 1993; Zhao et al. 2013).It was found that heat shock (43-45^{o}C) elevated HSP70 expression in chicken reticulocytes (Banerji et al. 1984). The increase of HSP70 after heat exposure shows its protective role under heat stress in QM7 cells. The results from QM7 cells

and over-expressed HSP70- QM7 cells may suggest regulation of ORX and its related receptors via HSP70 under heat stress. Therefore, this relationship needs to be elucidated in further study. In addition, heat stress was found to induce oxidative stress by producing superoxide in skeletal muscle mitochondria of broilers (Mujahid et al. 2005). Heat stress enhanced intracellular lipid peroxidation (LPO), particularly 4-hydroxy-t-2,3-nonenal (4-HNE) and reactive oxygen species (hydrogen peroxide, H_2O_2) (Cheng et al. 2001). Therefore, in this study we used H_2O_2 and 4-HNE to study the effects of oxidative stress in QM7 cell.

Hydrogen peroxide (H_2O_2) has been used to induce oxidative stress, and it also can induce LPO (Cheng et al. 2001; Chen et al. 2013). H_2O_2 was found to down-regulated the protein levels of ORX system in a dose dependent manner in leghorn male hepatoma (LMH) cell (Greene et al. 2016). This result is in agreement with our study in QM7 cell. The effect of hydrogen peroxide on QM7 cells in this study may increase the accumulation of HSP70 as found in cucumber plants (Li et al. 2014). This study indicates that ORX system may involve in oxidative stress.

4-HNE is a product of lipid peroxidation, and has been generally proved to be an oxidative stress inducer (Uchida 2003; Chen & Niki 2006). It is found that treating 4-HNE at 50 μM for 30 minutes in rat L6 muscle cells reduced the insulin-induced glucose up take via the decrease of insulin signaling (Pillon et al. 2012). In addition, incubation ORXA in human hepatocellular carcinoma (Hep3B) cell line (10^{-7}M) increased glucose uptake, and glucose utilization. The study also shows that ORXA may enhance mitochondrial glucose oxidation (Liu et al. 2016). Taken together, 4-HNE and ORX seem to have effect on glucose uptake and metabolism. In this study, 4-HNE induced oxidative stress increased ORX and ORXR1/2 protein

levels in a dose dependent manner. This may be due to the decreased glucose uptake effect of 4-HNE on QM7 cells that triggered the expression of ORX system.

5.6. Conclusion

This study shows that orexin and its related receptors expression was affected by heat stress and oxidative stress (H_2O_2 and 4-HNE) in avian muscle tissues and cells; therefore, they may have potential as bio-markers for these stressors. Further understanding their regulation can help alleviate heat stress and oxidative stress effects in birds.

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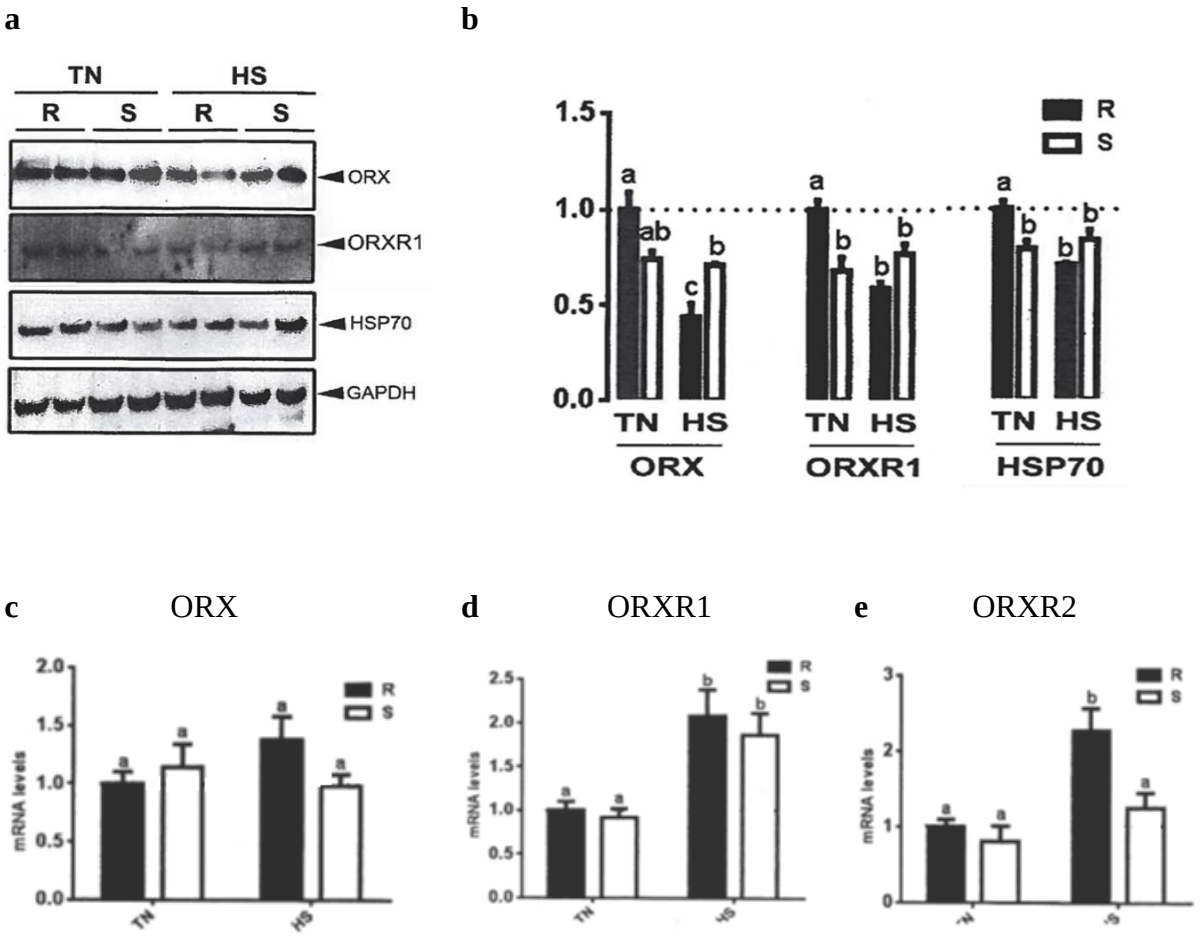

Figure 5.1. Acute heat stress (90 minutes) altered ORX system expression in quail muscles. It down-regulated ORX, ORXR1, and HSP70 protein expression in R quail muscle was shown in Western Blot image **(a)** and protein quantitation **(b)**; mRNA levels of ORX **(c)** ORXR1 **(d)** and ORXR2 **(e)** in muscle of R and S quail lines under thermoneutral (TN) and heat stress (HS) conditions. Data are mean ± SEM (n=4). Means without a common letter differ, $P<0.05$.

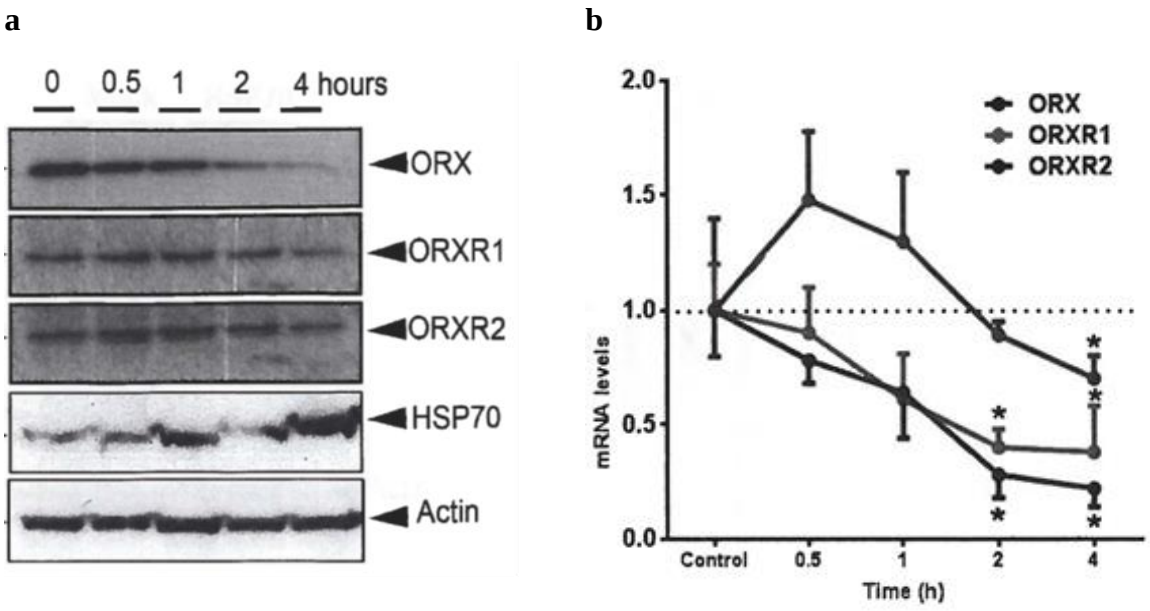

Figure 5.2. Heat stress down-regulated ORX system expression in QM7 cells in a time dependent manner. QM7 cells were exposed to heat stress (45^{o}C for 0.5, 1, 2, and 4h) and then 1h recovery at 37^{o}C or maintained at 37^{o}C as a control. ORX and ORXR1/2 protein levels were determined by Western blot **(a)**. ORX and ORXR1/2 mRNA levels were measured by qPCR using $2^{-\Delta\Delta Ct}$ method **(b)**. * Shows a significant difference ($P<0.05$).

a b

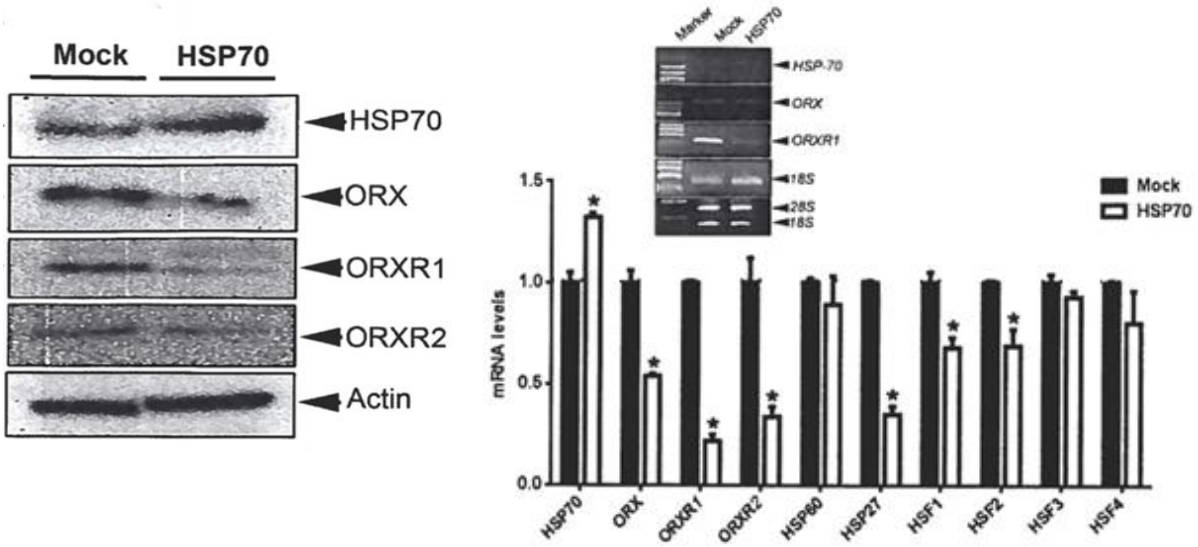

Figure 5.3. Overexpression of HSP70 decreased ORX and ORXR1/2 expression in QM7 cells under thermoneutral temperature (37^{o}C). QM7 cells were over-expressed with HSP70, the blank treated cells were used as control (Mock). ORX, ORXR1/2, and HSP70 protein levels were determined by Western blot **(a)**. ORX, ORXR1/2, HSP70, HSP27, HSF1, HSF2, HSF3 and HSF4 mRNA levels were measured by qPCR using $2^{-\Delta\Delta Ct}$ method **(b)**. * Shows a significant difference ($P<0.05$).

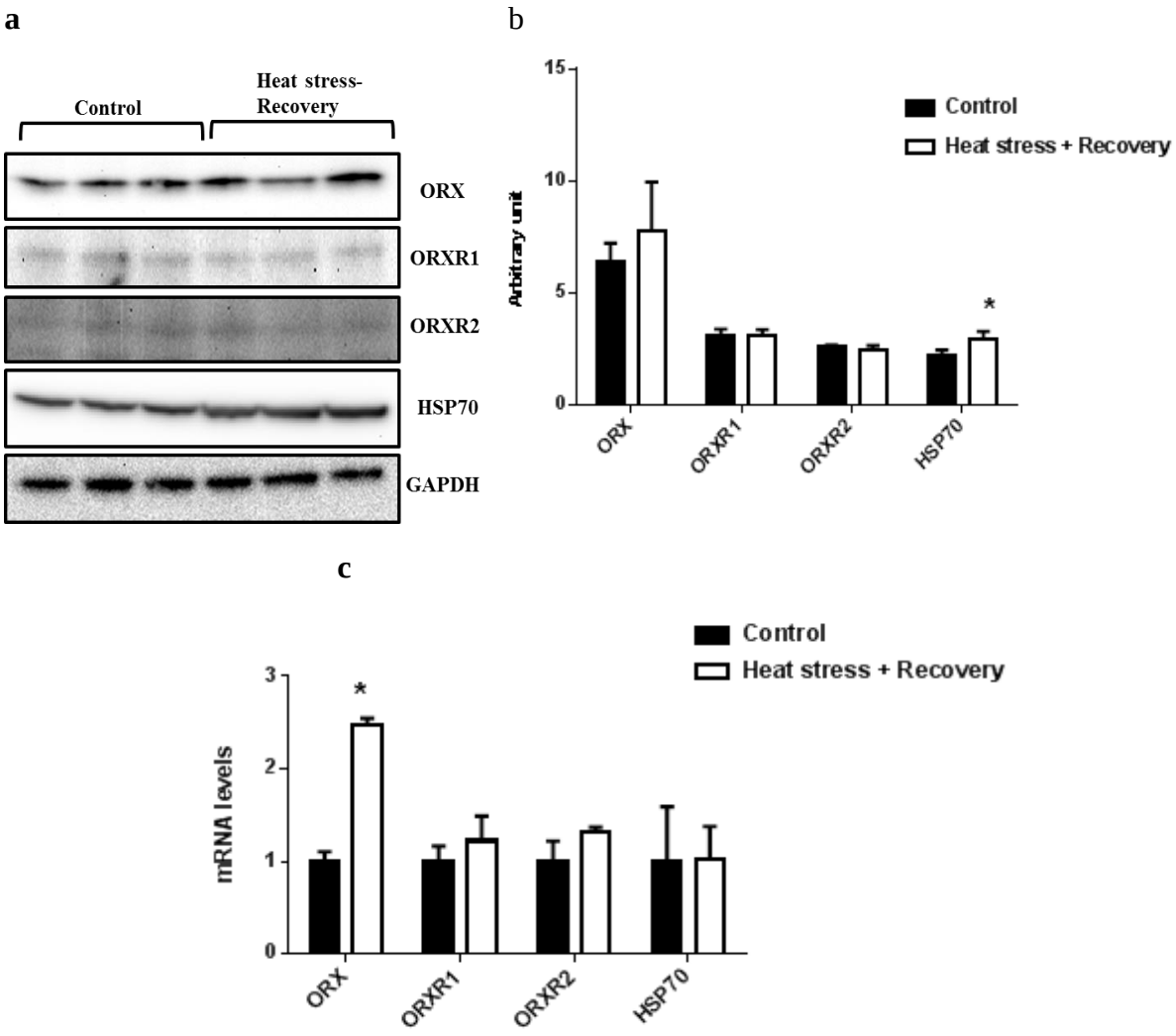

Figure 5.4. Heat stress (45ºC for 4h and 1h of recovery) increased ORX gene expression of over-expressed HSP70 - QM7 cells. Over-expressed HSP70-QM7 cells were incubated at 45ºC for 4h and 1h of recovery or kept at 37ºC as control. ORX, ORXR1/2 and HSP70 protein levels were determined by Western blot image **(a)** and protein quantitation **(b)**. ORX, ORXR1/2 and HSP70 mRNA levels were measured by qPCR using $2^{-\Delta\Delta Ct}$ method **(c)**. * Shows a significant difference ($P<0.05$).

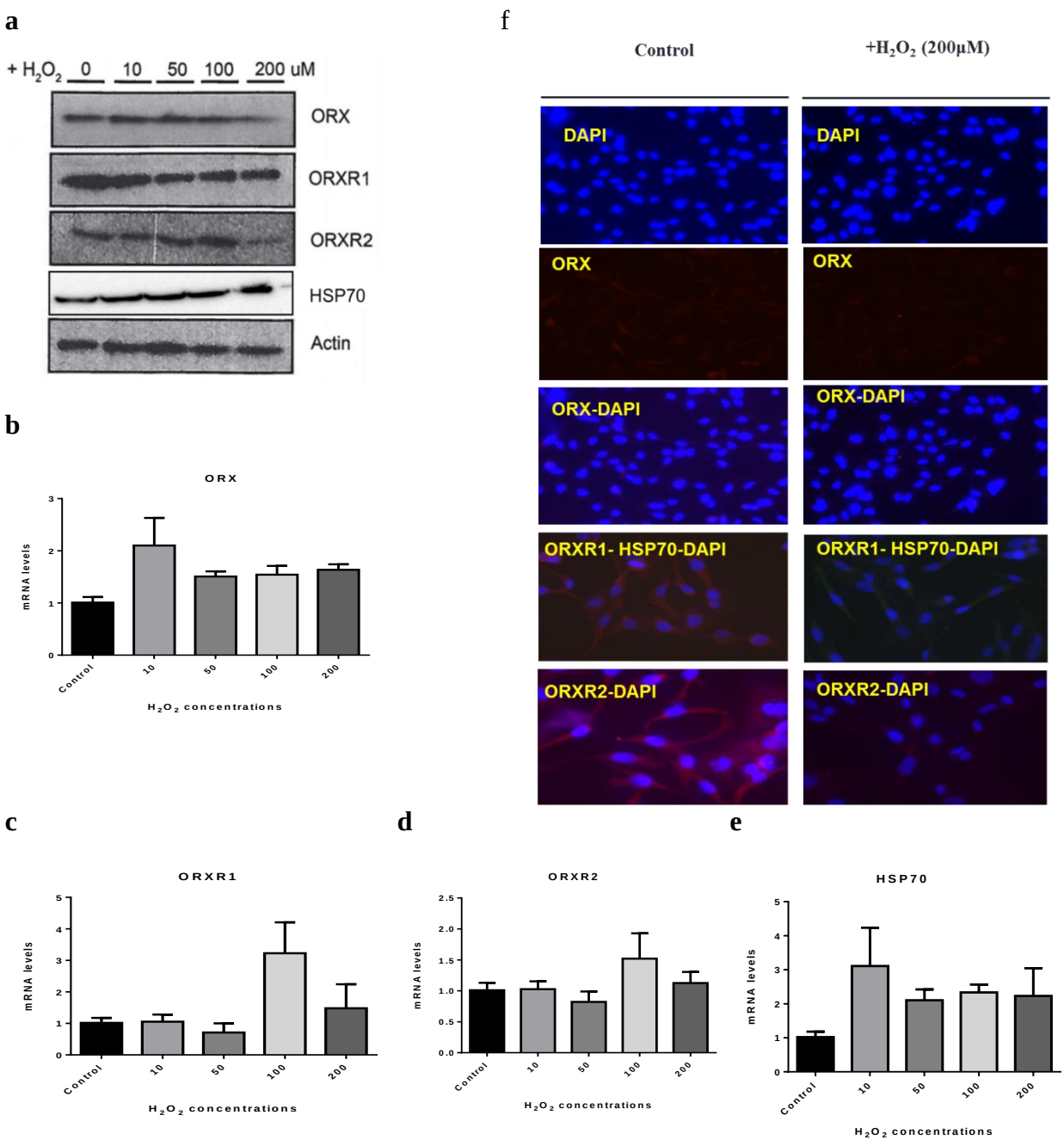

Figure 5.5. Dose-dependent alteration of ORX and ORXR1/2 expression in QM7 cells by H_2O_2. QM7 cells were treated with 10, 50, 100, and 200 µM of H_2O_2. Untreated cells were used as control. ORX, ORXR1/2, and HSP70 protein levels were determined by Western blot **(a)**. ORX, ORXR1/2 and HSP70 mRNA levels were measured by qPCR using $2^{-\Delta\Delta Ct}$ method **(b-e)**. Immunofluorescene shows the expression of ORX, ORXR1/2 in red and HSP70 in green **(f)**.

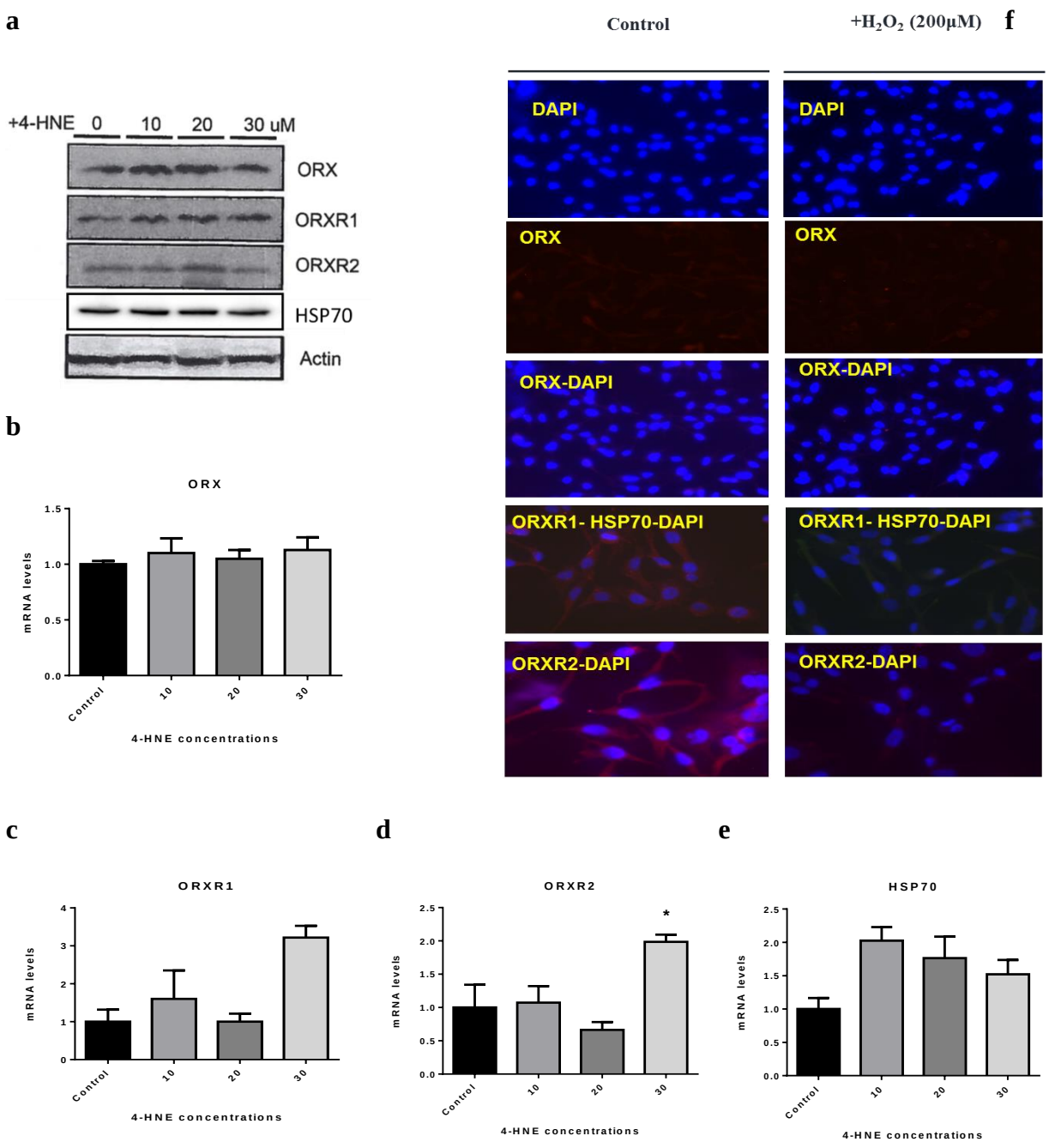

Figure 5.6. Dose-dependent alteration of ORX and ORXR1/2 expression in QM7 cells by 4-HNE. QM7 cells were treated with 10, 20 and 30 µM of 4-HNE. Untreated cells were used as control. ORX, ORXR1/2, and HSP70 protein levels were determined by Western blot **(a).** ORX, ORXR1/2, and HSP70 mRNA levels were measured by qPCR using $2^{-\Delta\Delta Ct}$ method **(b-e)**. Immunofluorescene shows the expression of ORX, ORXR1/2 in red and HSP70 in green **(f)**. * Shows a significant difference ($P<0.05$).

CHAPTER 6
HSPA9/Mortalin in Avian Species: Effect of Sex, Genotype and Environmental Stress

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6.1. Abstract

Heat shock protein 9 (HSPA9)/mortalin is a multipotent chaperone regulating cellular processes ranging from stress response to energy homeostasis. It has been extensively studied in mammals; however, there is a paucity of information in avian species. The present study aimed to characterize HSPA9 gene and to determine its regulation by gender, genotype and heat stress in avian tissues and cell lines. Using chicken (*Gallus gallus*) and two Japanese quail lines (*Coturnix japonica*) which are susceptible (S) or resistant (R) to restraint stress, it was found that HSPA9 is ubiquitously expressed in both chicken and quail. Its expression seemed to be tissue-, gender-, and genotype-dependent. Importantly, under thermoneutral conditions, S line quail liver only had half of the amount of HSPA9 mRNA levels compared to R line, but the heat stress significantly up regulated its mRNA level in the S line ($P<0.05$). In muscle, protein levels of HSPA9 are significantly lower in the S line compared with the R line under control or acute heat stress conditions ($P<0.05$). In vitro studies used SH-SY5Y (human neuroblastoma cell), QM7 (Quail muscle clone 7), CEL-im (immortalized chicken embryo liver) treated with three treatments: exposure 37^{o}C for 1h as control, 45^{o}C for 1h and then recovered for 1h at 37^{o}C, and 45^{o}C for 1h. Heat stress significantly down-regulated gene expression of HSPA9 in SH-SY5Y cells and Cel-im cells, but not QM7 cells ($P<0.05$). Interestingly, HSPA9 protein levels were significantly up-regulated in QM7 cells under the heat stress treatment ($P<0.05$). In conclusion, this study found that HSPA9 is ubiquitously expressed in avian species and it is responsive to heat stress.

Key words: chicken, gene expression, HSPA9, heat stress, quail

6.2. Introduction

Within just a few decades, climate change has increased its effects on weather patterns and temperature globally. Comparing the 1950s to the1980s, the temperatures in most regions have been rising, whilst some areas have lower temperatures than before (Hansen et al. 2012). This creates two opposite trends of extreme weather: hot and cold and further changes in the climate system are predicted to increase in the 21st century (Pachauri et al. 2014). In addition, the modern broilers, selected for higher growth rate due to the ever-increasing demand for high quality, affordable, and sustainable sources of food, particularly protein, have become more sensitive to disease and stress under extreme climate conditions (Cahaner & Leenstra 1992; Havenstein et al. 1994; Soleimani et al. 2011). Thermal stress in avian species has been causing heavy economic losses to the poultry industry. In the U.S., the poultry industry loses $128 to $165 million per year due to heat stress (St-Pierre et al. 2003). Heat stress studies in birds observed a series of changes from behavior thermoregulation to reduced growth performance (Lu et al. 2007; Kadim et al. 2008). In 2008, snow and low temperatures resulted in 19.55 million poultry deaths and an economic loss of about 14.5 million US dollars (Chen et al. 2014). Early research in poultry has clearly shown suppression effect of acute cold stress in development, egg production, and survival (Sagher 1975; Renwick et al. 1985; Sahin et al. 2003). There are studies that have shown one cellular response in birds to thermos-stress is an elevation of heat stress proteins (HSPs) (Hoekstra et al. 1998; Givisiez et al. 2001; Zhao et al. 2014).

GRP75/mortalin/mtHSP70/HSPA9 (hereafter referred to as HSPA9) was first identified in the cytosolic fraction of mouse embryonic fibroblasts with a molecular weight of 75 kDa and was categorized to the heat shock protein 70 (HSP70) family. It was initially considered to be involved in cell death and cell growth (Wadhwa et al. 1993; Starenki et al. 2015). By different

techniques, its presence was reported first in mitochondria, and later in endoplasmic reticulum, cytoplasmic vesicles, and the cytosol (as reviewed by Ran et al. 2000; Wadhwa et al. 2002). HSPA9 was found to have several roles in mitochondrial protein import, intracellular trafficking, the endocytic pathway in mammalian cells, stress and immune responses, and prevention of apoptosis (Voos & Röttgers 2002; Kaul et al. 2007; Wittrup et al. 2010; Yang et al. 2011). In glucose deprived PC12 cells, HSPA9 overexpression reduced the accumulation of ROS (Reactive oxygen species) (Liu et al. 2005). Further findings in glucose deprivation stress in cells indicate that HSPA9 overexpression inhibited apoptosis may be via activating protein kinase B (PKB or AKT), which has anti-apoptotic effect in cells (Yang et al. 2011). For example, in a study of amino acid deprivation, HSPA9 protein content was increased two-fold after 5-10 hours of amino acid free culture in Chinese Hamster Ovary cells (CHO) (Heal & McGivan 1997). Over-expression of HSPA9 was reported to prevent rat liver injury from oxidative stress and the same has been observed in a normal human liver cell line (HL-7702). Treatment with H_2O_2 caused oxidative stress, high cytochrome c protein expression, and a rapid increase of ROS in control cells, but these effects were lowered in over-expressed HSPA9 cells. Tail vein injection of HSPA9-overexpressing vector significantly helps to alleviate serum aspartate aminotransferase and alanine transaminase, cytochrome c protein expression in rats. This data suggests the protective ability of HSPA9 in oxidative stressed livers (E et al. 2013).

Numerous studies of HSPA9 were conducted in humans and rodents. These approaches discovered multiple roles of HSPA9 in diseases and stress, both of which are major concerns in poultry. Therefore, the aim of this study is to characterize HSPA9, and to determine its regulation and role in avian species, particularly under environmental stress.

6.3. Materials and methods

6.3.1. Identification of HSPA9 gene in avian tissues and cells

Tissue samples from male and female Jungle fowls; and stress resistant and susceptible quails were collected for conventional PCR includes heart, breast muscle, brain, liver, gizzard, kidney, testis, leg muscle, lung, and intestine. HSPA9 gene expression was also examined in avian liver cell lines: Leghorn male hepatoma (LMH), and immortalized chicken embryo liver cell (siCEH), and quail muscle clone 7 cell (QM7).

Total RNA was extracted from all samples and were reverse transcribed as described in the 6.3.4.1 section. Long fragments of GRP75 (802 bp) were amplified by PCR using oligonucleotide primers specific for chicken GRP75: forward 5'-TGTGTCAGCCAAGGACAAAG-3', and reverse 5'-GAGGATCCACCAGGATTTCA-3' and 18S (515 bp) forward 5'-CTGCCCTATCAACTTTCG-3', and reverse 5'-CATTATTCCTAGCTGGAG-3'. PCR was performed in 50 µL reaction containing 5 µL of the reverse transcription reaction, 1 µL of forward and reverse primers, and 43 µL of platinium PCR SuperMix (Life Technologies, grand Island, NY). Thermal cycling parameters were as follows: 1 cycle of 95°C for 4 min, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 min with a final extension at 72°C for 10 min. The amplified fragments were separated on a 1% agarose gel and the appropriate bands were removed, purified by using spin column DNA gel extraction kit (Biobasic, Amherst, NY) and stored at −20°C for later sequencing.

6.3.2. In vivo study

6.3.2.1. Heat stress

This experiment was previously described in Chapter 5. Briefly, two lines of Japanese quail (*Coturnix coturnix Japonica*) males that were stress resistant (R) and the high stress birds

called sensitive (S) were used. They were selected for circulating corticosterone level in response to restraint stress (Satterlee & Johnson 1988). All the birds were warm-brooded for 10 days at 32^{o}C and the temperature was gradually reduced each week to 22^{o}C (thermoneutral, TN) at 4 weeks of age. Six to ten birds of each line were exposed to 37^{o}C for 1.5h (acute heat stress – HS) and six to ten birds were kept at 22^{o} (TN) conditions as controls. The relative humidity of the experiment was 50 ± 5%. Control and heat-stressed groups were housed in separate environment controlled rooms. The birds were humanly euthanized by cervical dislocation, and heart, breast muscle, brain, liver, gizzard, kidney, testis, leg muscle, lung and intestine were collected, immediately frozen in liquid nitrogen, and stored at -80^{o}C for later analysis.

6.3.2.2. Cold Stress

This experiment was described in Chapter 3 (Nguyen et al. 2015). Briefly, 80 Cobb male broiler chicks from the Cobb 500 female line were assigned to two separate environmentally controlled rooms: thermoneutral/control and cold. Birds of each treatment were placed into four randomly assigned pens (20 birds/pen) at day one post-hatch. Control room has temperature started at 32°C for the first week. The cold room temperature was maintained at 26.7°C for the first day, and then gradually decreased by 1°C every day to reach 19.7°C on day 7. The seventh day, birds were euthanized humanly and whole brain, liver and leg muscle tissues were collected, immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

These experiment were conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Animal Care and Use Committee under protocols 13039, 10025, and 12050.

6.3.3. In vitro study

SH-SY5Y (human neuroblastoma cell) not mentioned earlier, QM7 (Quail muscle clone 7), CEL-im (immortalized chicken embryo liver) cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Grand Island, NY), M199 medium (Life Technologies, Grand Island, NY), and Waymouth's standard growth medium (Life Technologies, Grand Island, NY), respectively. These media were complemented with 10% FBS (Life Technologies) and 1% penicillin-streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO2 and 95% air. Each cell type was exposed to three temperature treatments: 37ºC for 1h (control), 45ºC for 1h followed by 1h recovery at 37ºC, and 45ºC for 1h.

6.3.4. RNA isolation, reverse transcription and real-time quantitative PCR

Total RNAs were extracted from brain, liver, and leg muscle quail, and SH-SY5Y, QM7 and CEL-im cells by Trizol reagent (Life Technologies, Grand Island, NY) according to manufacturer's recommendations. Total RNAs were treated with DNAse, then reverse transcribed and amplified by qPCR using oligonucleotide primers specific for chicken GRP75: forward 5'-TGCAGCAGGCTTCCTTGAA-3' and reverse, 5'-CTCGTTCGGAGGCCATCTT-3', and ribosomal 18S forward, 5'-TCCCCTCCCGTTACTTGGAT-3' and reverse, 5'-GCGCTCGTCGGCATGTA-3', as a house keeping gene as previously described (Piekarski et al. 2014). Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008).

6.3.5. Protein extraction and Western blot analysis

Harvested tissues and cells were homogenized, protein extracted and measured for concentrations as described in the previous description (Lassiter et al. 2015). Protein expression was assessed by immunoblotting (Lassiter et al. 2015) using the following antibodies: anti-

HSPA9, and GAPDH or β-actin as house-keeping proteins (Cell signaling Technology, Danvers, MA). Pre-stained molecular weight marker (precision plus protein Dual color) was used as a standard (Biorad, Hercules, CA). The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by FlourChem M MultiFlour System (Proteinsimple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993-2011, Proteinsimple, Santa Clara, CA).

6.3.6. Immunoflourescene

Immunoflourescene staining was performed as previously described (Lassiter et al. 2015) using anti-HSPA9. After incubation with Alexa Flour 594-conjugated secondary antibody (Molecular probes, Life Technologies, Grand Island, NY) and DAPI (Vector Laboratories, Burlingame, CA), images were obtained and analyzed using Zeiss Imager M2 and Axio Vision software (Carl Zeiss Microscopy, GmbH 2006-2013).

6.3.7. Statistics

Gene and protein expression data from the cold stress experiment were analyzed by Student's "t"-test and quail experiment data were analyzed by two-factor ANOVA with temperature conditions and genotype (R and S quail lines) as classification variables. The data from three cell types were analyzed by one-way ANOVA, and Tukey's test. All data were analyzed using Graph Pad Prism software (version 6, La Jolla, CA), and $P<0.05$ was set as significantly different.

6.4. Results

6.4.1. Characterization of heat shock protein A9 (HSPA9) gene

HSPA9 was characterized and identified by PCR in chicken liver, Leghorn male hepatoma cells (LMH), immortalized chicken embryo liver cell (siCEH), and quail muscle clone 7 (QM7). Figure 6.1 shows that HSPA9 was expressed in chicken liver and avian cell types.

6.4.2. Differential expression of HSPA9 in avian tissues

Figure 6.2 (a, b, c, d) shows the gene and protein expression of HSPA9 by conventional PCR and Western Blotting techniques in different tissues of male quails, and female and male jungle fowls. Western Blot images show that the protein levels of HSPA9 were highly expressed in heart, breast muscle, brain, liver, gizzard, kidney, testis, and leg muscle of male quail, while its expression in lung and intestine was very low (Figure 6.2a, b). Similarly, HSPA9 protein levels were high in heart, liver, kidney, and intestine of male and female jungle fowl, while it is quite low in breast muscle, brain, gizzard, and leg muscle, and very low in lung, ovary or testis (Figure 6.2 d, f).

The gene expression of HSPA9 in avian species is tissue specific, gender, and genotype dependent. HSPA9 mRNA levels were significantly higher in heart, intestine, and ovary of female jungle fowl compared with other tissues, and lung had the lowest gene expression. HSPA9 mRNA levels were significantly higher in heart, kidney, and intestine, followed by breast muscle, testis, and leg muscle, while brain, lung, liver, and gizzard had the lowest gene expression. Furthermore, HSPA9 expression in the ovary of female birds was significantly higher than that in the testis of males ($P=0.002$) (Figure 6.3a). In quail, the S line had a significantly higher gene expression of HSPA9 in heart, and testis, followed by kidney, gizzard, and intestine, then breast muscle, brain, lung, liver, and leg muscle ($P<0.0001$). However, R line

had a higher gene expression of HSPA9 in all tissues compared with that in S line. HSPA9 mRNA levels in heart and testis are significantly lower than in those of S line ($P<0.0001$).

6.4.3. HSPA9 expression under environmental stress in avian tissues and cells

Gene and protein expression of HSPA9 were not significantly different between chronic mild conditional cold stress and thermal neutral groups in brain, liver, and leg muscle of young chicks (Figure 6.4).

Figure 6.5d shows the higher HSPA9 mRNA level in R quail livers compared with that in S quail livers under thermoneutral conditions. Acute heat stress significantly up-regulated mRNA levels of HSPA9 in the livers of S quails ($P=0.009$), but did not significantly alter HSPA9 levels in the R line (Figure 6.5d). Furthermore, figure 6.5i shows that protein levels of HSPA9 are lower in the S line leg muscles compared with that of the R line muscles under control or acute heat stress conditions ($P<0.05$).

Figure 6.6 (a, b, c) shows that HSPA9 is distributed abundantly in intracellular of QM7, Cel-im and SH-SY5Y cells. Heat stress (45^{o}C for 1h) significantly down-regulated HSPA9 gene expression in SH-SY5Y cells and Cel-im cells, but not in QM7 cells ($P<0.05$) (Figure 6.7 a, d, g). Interestingly, HSPA9 protein levels were significantly up-regulated in QM7 cells under the heat stress treatment ($P<0.05$) (Figure 6.7i).

6.5. Discussion

HSPA9 was initially identified as a 75 kDa protein and was categorized to the heat shock protein 70 family (Wadhwa et al. 2002), a group of highly conserved molecular chaperones recognized for their roles as stress-related proteins (Morimoto et al. 1994). HSPA9 was found to be expressed in multiple tissues of rats (heart, brain, skeletal muscle, lung, liver, kidney, testis, and spleen). HSPA9 expression in rats is tissue specific and is rather similar to that in avian

species. For example, in both avian species and rats, heart is where HSPA9 expression is the highest, and lung has the lowest expression (Kaul et al. 1997). Moreover, HSPA9 gene expression was observed to be gender specific in jungle fowls, with testis/ovary showing the greatest differences between males and females. A study conducted to investigate the gender specific nature of HSPs (HSP60, HSP70, HSP90) expression in the gastric mucosa of male and female rats revealed that estrogen may play a role in the different expressions (Takahashi et al. 2010). Thus, the significant difference in HSPA9 expression in various tissues between male and female jungle fowls in our study may be due to estrogen levels in tissues of male and female birds.

In this study, two lines of quails selected for high resistant ability (R) and susceptible (S) were used. The R line has 66% lower plasma corticosterone levels compared with their high stress counterpart (Satterlee & Johnson 1988). Therefore, the marked difference of HSPA9 mRNA levels between two lines indicates the genotype effect on this gene. Over-expression of HSPA9 protects rat liver and normal human liver cell line (HL-7702) from injury due to oxidative stress, suggesting the protective ability of HSPA9 in stress conditions (E et al. 2013). From the significantly higher HSPA9 mRNA levels in heart and testis of S line quails compared to those of R line quails, we believe that HSPA9 serves a protective role in these tissues in the susceptible quail.

Interestingly, HSPA9 gene expression in R quail livers is significantly higher than that of S quail livers under control condition ($P<0.05$). A previous study using taurine zinc to alleviate stress caused by gastric ulceration in mouse has reduced the plasma corticosterone levels and up-regulated HSP70 expression in mouse gastric mucosa (Yu et al. 2015). Because HSPA9 belongs to the HSP70 family and the R line quails has 66% lower plasma corticosterone levels than that

of the S line quails, we hypothesize that the plasma corticosterone levels in these birds affects the expression of HSPA9. Conversely, the higher expression of HSPA9 in heart and testis of S line quails that we discussed above may follow the tissue specific characteristic of this gene in S line birds.

It is indicated that HSPA9 is a heat non-inducible protein (Deocaris et al. 2006); and this current study shows the non-significant change in HSPA9 expression of R quail livers after heat stress. However, the significant increase of HSPA9 expression in S quail livers under acute heat stress condition indicates that this gene is affected by heat and the stress resistant ability of birds. The increase may help to prevent liver damage under heat stress. Furthermore, genotype effect may result in the lower protein levels of HSPA9 in S quail leg muscles compared with those in R quail leg muscles under both control and acute heat stress.

HSPA9 was initially found in mitochondria where it plays an important role in assisting mitochondrial protein import (Voos & Röttgers 2002). As heat stress induces oxidative stress and causes mitochondrial damage (Slimen et al. 2014), this is expected to change the expression of HSPA9. It is observed that extreme heat stress induced apoptosis in human umbilical vein endothelial cells (HUVEC) via transcription-independent p53-mediated mitochondrial pathways (Gu et al. 2014). HSPA9 has been known for its role in binding and inactivating p53 which prevents apoptosis, and therefore low levels of HSPA9 activates apoptosis via p53 (Londono et al. 2012). In this study, heat stress at 45^{o}C for 1 hour is considered intense heat stress to SH-SY5Y cells and Cel-im cells; thus, it can cause apoptosis to these cell types. HSPA9 mRNA levels in heat stressed SH-SY5Y cells and Cel-im cells were significantly down-regulated ($P<0.05$), but not in QM7 cells. However, HSPA9 protein levels was remarkably up-regulated in QM7 cells. Similar to these findings, the induction of HSPA9 protein expression was not

correlated with its mRNA level in amino acid deprived CHO cells (Heal & McGivan 1997). These suggest that HSPA9 expression under heat stress is different, due to the characteristics of different cell types.

6.6. Conclusions

HSPA9 gene is distributed differently in various tissues of avian species and it is gender and genotype specific. It is expressed abundantly in intracellular of different cell types including SH-SY5Y, CEl-im and QM7 cells. HSPA9 expression is not affected by conditioning chronic cold stress in young chicks. However, acute heat stress alters its gene expression in S quail livers, SH-SY5Y cells and Cel-im cells. Moreover, acute heat stress also increases HSPA9 protein levels in QM7 cells. There is a need for further investigations to determine the mechanisms by which HSPA9 is regulated in avian species.

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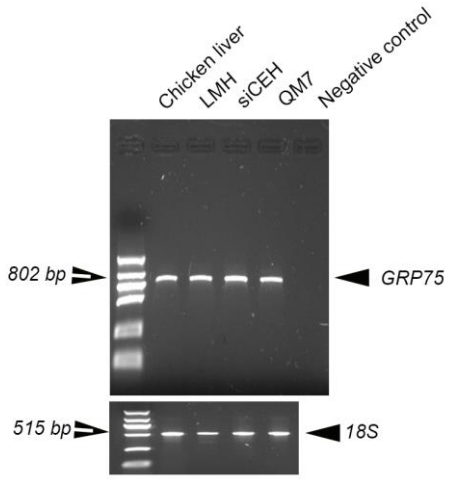
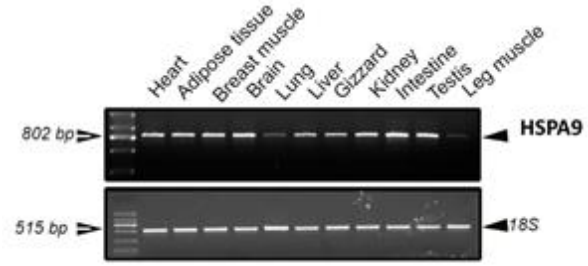
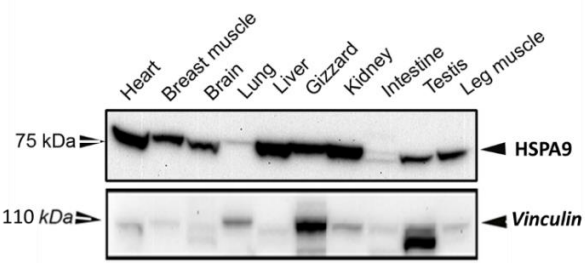


Figure 6.1. Gene expression of HSPA9 in chicken liver tissues and different avian cell lines (LMH, siCEH and QM7)

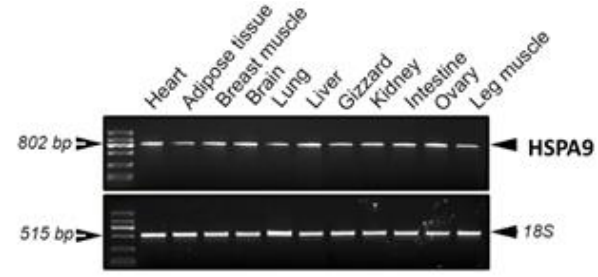
a - Quail



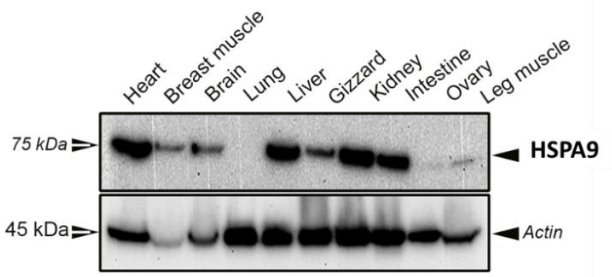
b - Quail



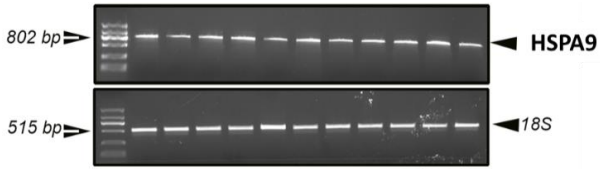
c – Female Jungle fowl



d – Female Jungle fowl



e - Male Jungle fowl



f - Male Jungle fowl

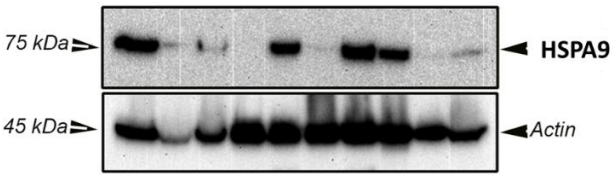
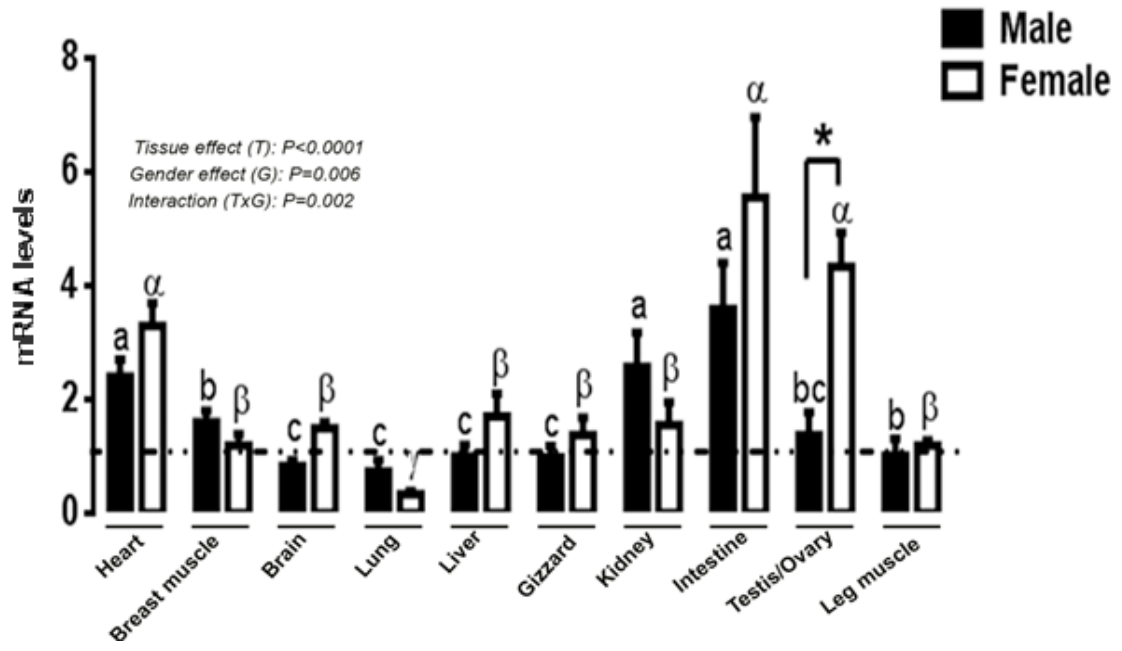


Figure 6.2. HSPA9 is expressed in several tissues of avian species (Jungle fowl and quail) including heart, adipose tissue, breast muscle, brain, lung, liver, gizzard, kidney, intestine, ovary/testis, leg muscle. HSPA9 gene expression by conventional PCR and protein expression by Western blotting in multiple tissues of male quail (**a, b**). HSPA9 gene expression by conventional PCR and protein expression by Western blotting in multiple tissues of female jungle fowl (**c, d**) and male jungle fowl (**e, f**).

a



b

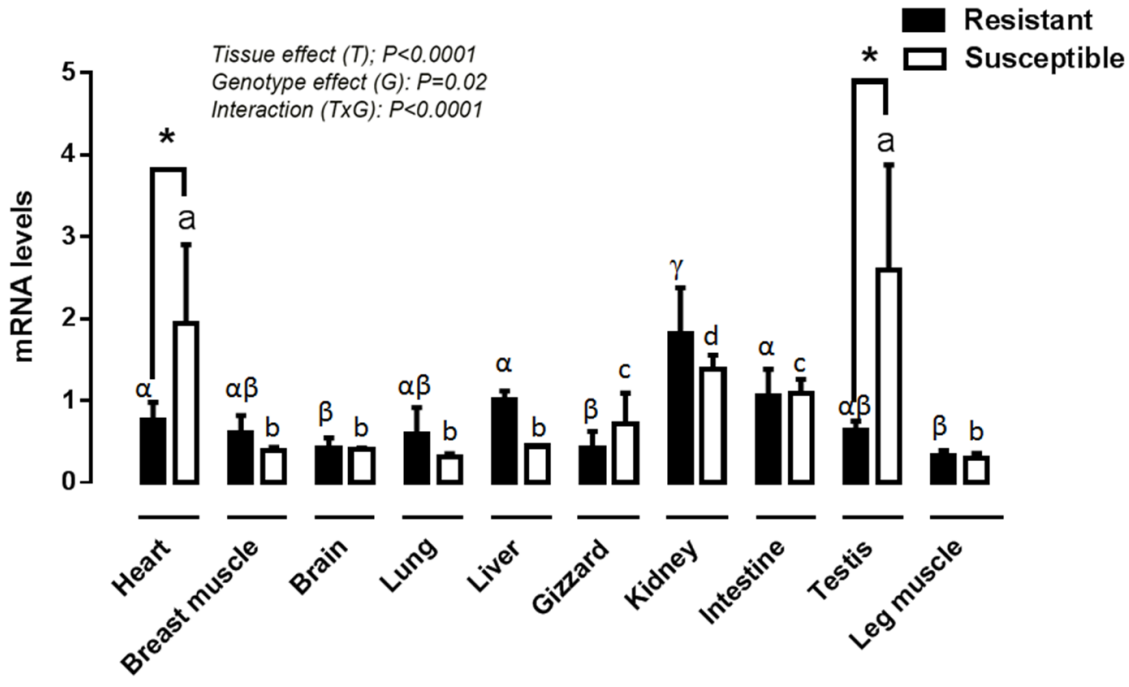
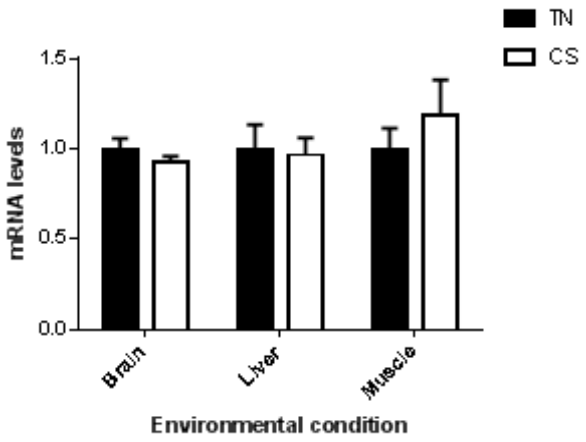
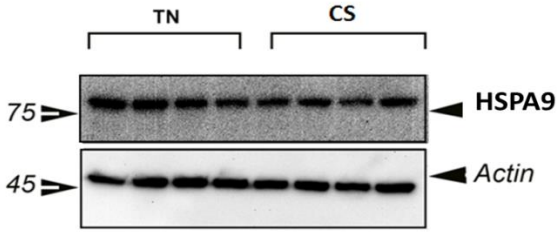


Figure 6.3. The expression of HSPA9 in avian species is tissue, gender and genotype dependent. HSPA9 gene expression in different tissues of male and female jungle fowl **(a).** HSPA9 gene expression in different tissues of R and S quail lines **(b).** Different letters show significant difference. * P<0.05

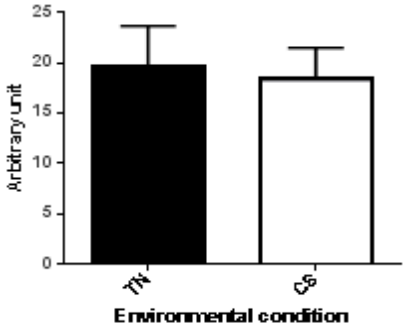
a



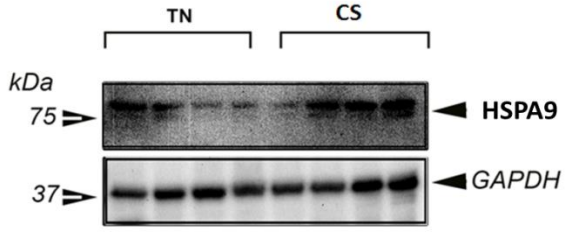
b - Liver



c - Liver



d – Leg muscle



e – Leg muscle

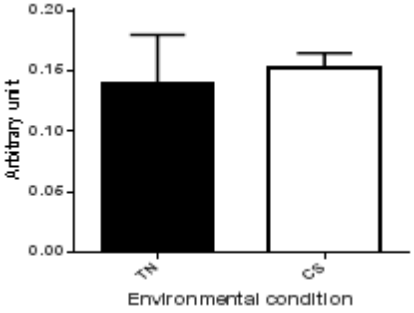


Figure 6.4. HSPA9 expression in brain, liver and leg muscle tissues of cold chronic cold exposed chicks. HSPA9 mRNA levels in brain, liver and muscle of chronic cold exposed chicks was examined by qPCR (n=6) **(a).** Protein expression images and protein quantitation analysis of HSPA9 in livers **(b-c)** and muscle **(d-e)** of chronic cold exposed chicks (n=4). Thermoneutral condition (TN). Cold stress (CS)

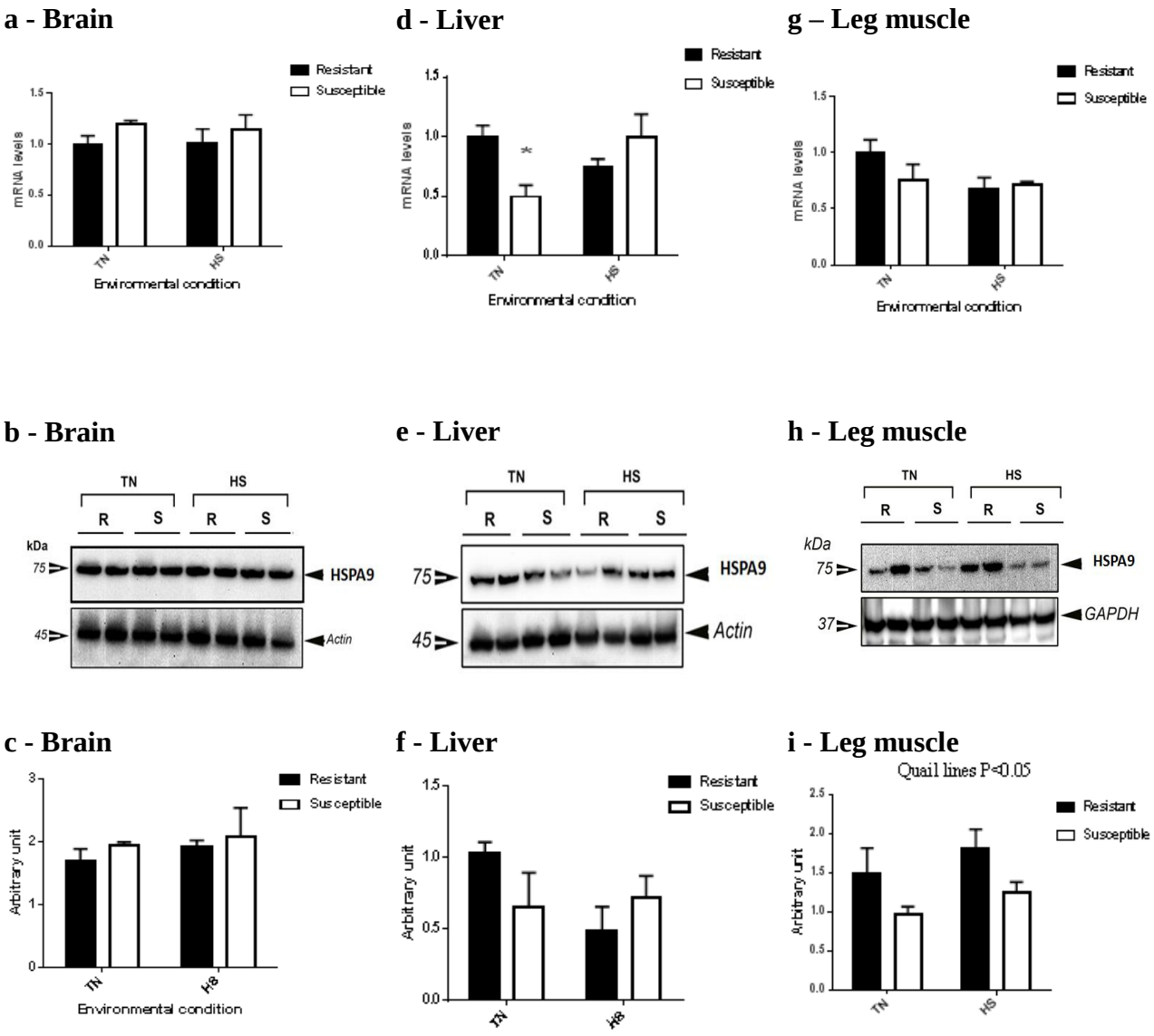


Figure 6.5. Expression of HSPA9 in brain, liver, and leg muscle of R and S quail lines under heat stress. HSPA9 mRNA levels in brain, liver and muscle of R and S lines under thermoneutral (TN) and acute heat stress (HS) conditions were accessed by qPCR (n=4-5) (**a, d, g**). HSPA9 protein expression images in brain, liver and muscle of R and S lines under thermoneutral and acute heat stress conditions were accessed by Western Blot (**b, e, h**) and the protein levels were quantitated **(c, f, i)** respectively (n=4). * P<0.05.

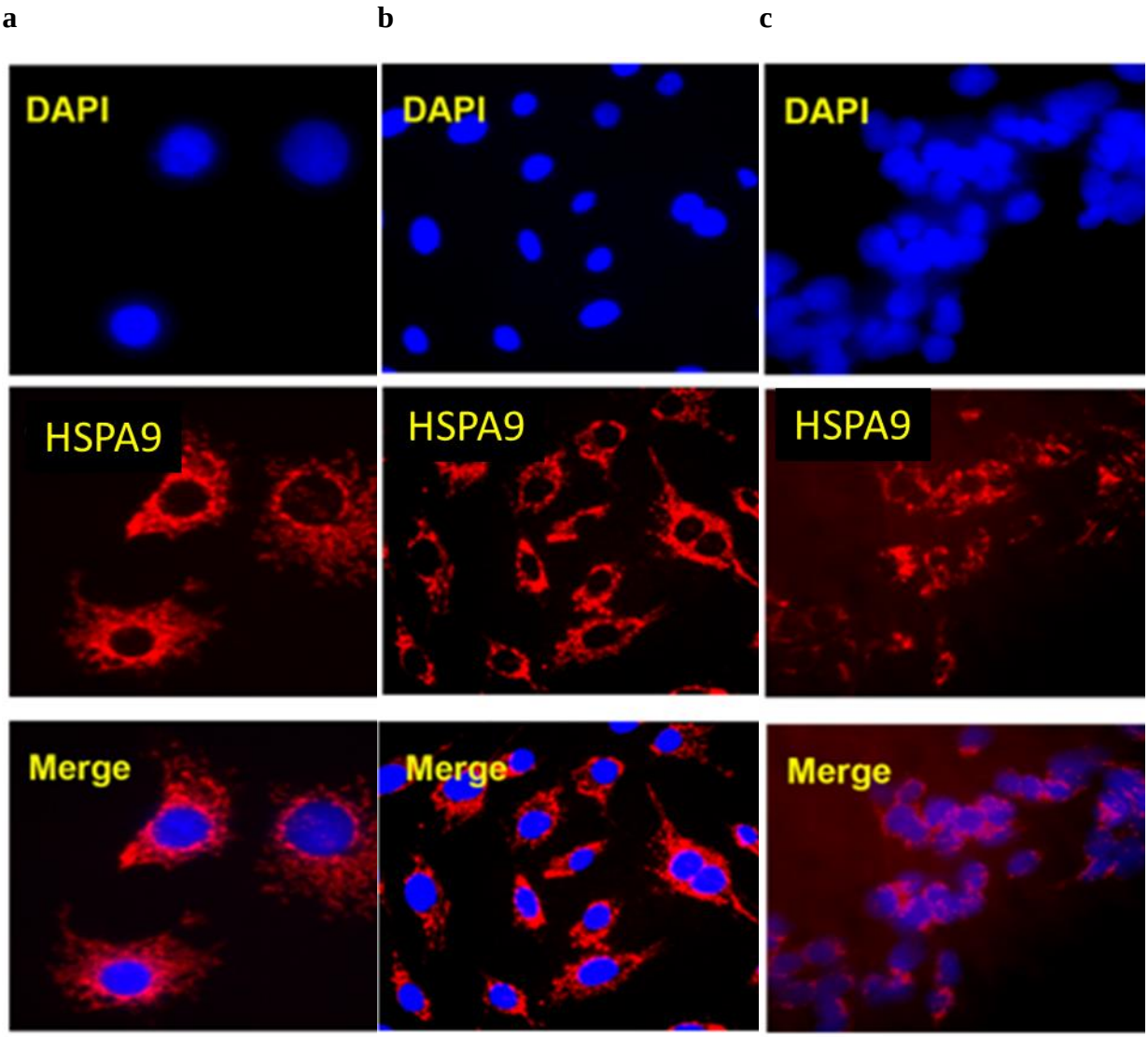


Figure 6.6. Immunofluorescence staining. Intracellular HSPA9 distribution visualized by fluorescent microscope in presence of a secondary antibody conjugated with Alexa Flour 594- (red) and DAPI (Blue). Images of HSPA9 distribution in QM7 cells **(a),** CEL-im cells **(b)**,and SH-SY5Y cells **(c)**.

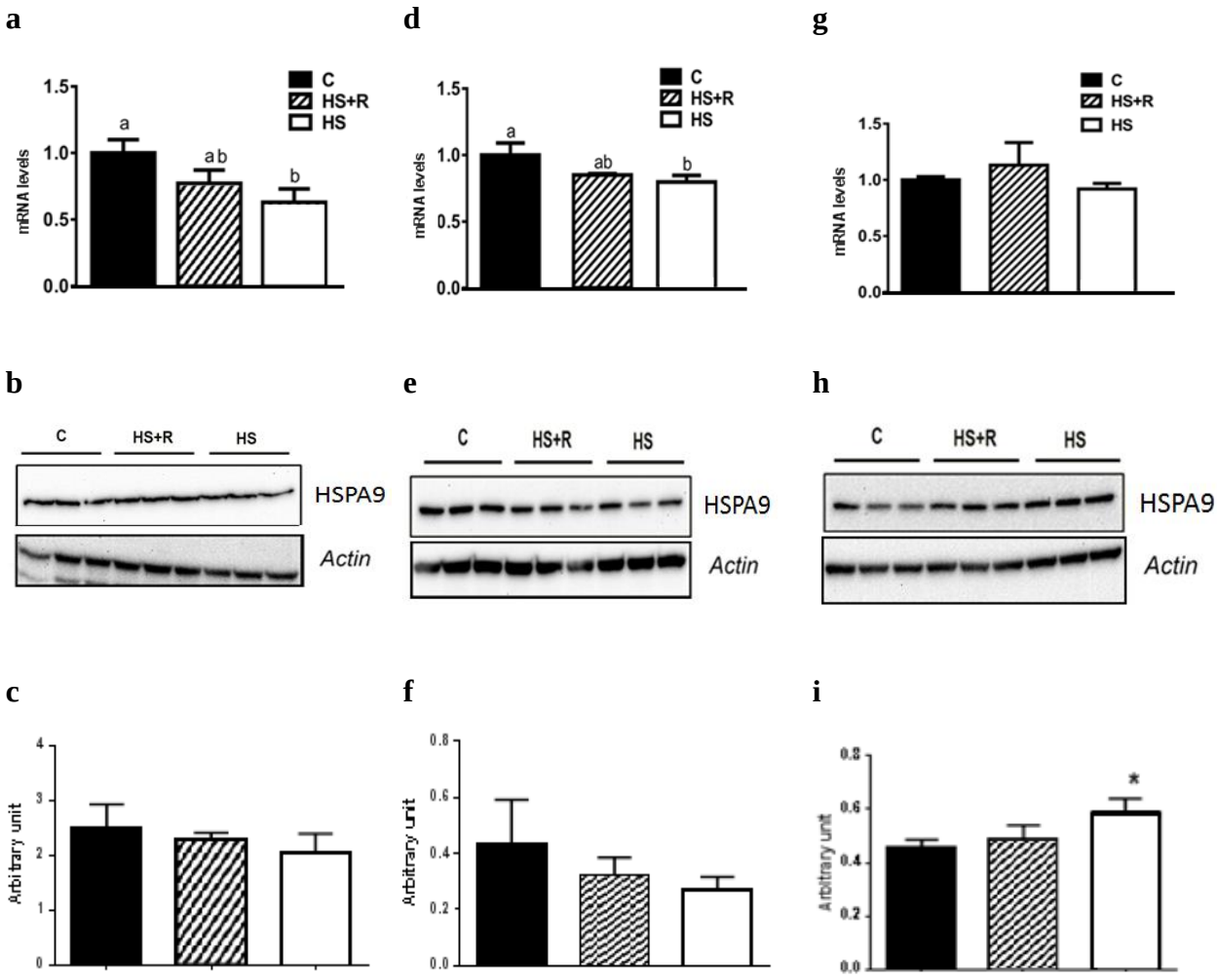


Figure 6.7. HSPA9 mRNA and protein levels in different cell types. SH-SY5Y, QM7, CEL-im cell lines were treated with three treatments: exposure 37ºC for 1h as control (C), 45ºC for 1h and then recovered for 1h at 37ºC (HS+R), and 45ºC for 1h (HS). mRNA levels of HSPA9 were measured by qPCR (n=3), and HSPA9 protein levels were accessed by Western Blot (n=3). mRNA levels of HSPA9 in SH-SY5Y cells **(a)**, Cel-im cells **(d),** and QM7 cells **(g)** under different treatments. Protein expression of HSPA9 presented by Western Blot images and quantitation analysis in SH-SY5Y cells **(b, c),** Cel-im cells **(e, f**), and QM7 cells (**h, i**) under different treatments. Different letters show significant difference. * P<0.05.

CHAPTER 7

Expression and Regulation of Dicer1 in Nutritional Stressed Avian Tissues and Cells

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7.1. Abstract

Dicer is a key enzyme in microRNA biogenesis which plays key roles in many cellular processes including metabolism. In avian species, the role of Dicer 1 and its regulation are still unknown. Therefore, this study aims to examine the regulation of Dicer 1 by nutritional state and genetic factors in avian tissues and cells. 16 week-old Japanese quails were caged individually and subjected to three nutritional states: fasting for 24 or 48h with refeeding for 3h, and an *ad libitum* fed group as a control. In a separate set of experiment, four commercial broiler crosses followed a typical commercial breeder feed restriction program from 14 to 56 d of age, and were then assigned to one of two feeding regimes equally and randomly by line: restricted or *ad libitum* for two weeks. To investigate the mechanism by which Dicer 1 expression is regulated during fasting, an *in vitro* study was conducted: serum-deprived for 24 h with immortalized chicken embryo liver (CEL-im) cells and 12, 24, and 48 h with neuroblastoma (SH-SY5Y) cells. Additionally, for further understanding of Dicer regulation, the SH-SY5Y cell line was treated with 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), Bisindolylmaleimide I (BIM I) or Rapamycin to stimulate AMPK activity, to inhibit PKC, and to inhibit mTOR, respectively. Dicer 1 gene expression was significantly up-regulated in the liver of 48h fasted quails and was then down-regulated after 3h-refeeding ($P<0.05$). In chicken, feed restriction upregulated Dicer 1 gene expression in the liver ($P<0.05$). Serum fasting alters the expression of Dicer 1, genes and proteins involved in energy homeostasis in cells. Our data indicates that DICER1 is responsive to the nutritional state (fasting) and it may be involved in the regulation of energy homeostasis.

Keywords: DICER, quail, chicken, feed restriction

7.2. Introduction

Dicer is a ribonuclease III enzyme that cleaves single-stranded pre-miRNAs and dsRNA to mature miRNAs and siRNA (Bernstein et al. 2001; Kurzynska-Kokorniak et al. 2015). miRNAs play several important roles in growth, death, stress, glucose and lipid metabolism, and metabolic diseases (Ambros 2003; Lynn 2009; Fernández-Hernando et al. 2013). In animals, two Dicer proteins have been identified termed Dicer 1 and Dicer 2 (Kurzynska-Kokorniak et al. 2015). While Dicer 1 is involved in the processing of miRNA, Dicer 2 is necessary for siRNA biogenesis in *Drosophila melanogaster* (Lee et al. 2004). A study by Mukherjee and colleagues tracing the evolution of the Dicer gene showed that only Dicer 1 homologs spanned in vertebrates, not Dicer 2 (Mukherjee et al. 2013). Dicer 1 has been found to be involved in energy homeostasis and stress response of animals. Its disruption in mouse hepatocytes resulted in steatosis and glycogen depletion in the cells which leads to the impairment of lipid and glucose metabolism in disrupted Dicer hepatocytes of mice (Sekine et al. 2009). Knockdown of Dicer 1 inhibited lipid accumulation and adipocyte differentiation in 3T3-L1 cells by suppressing gene expression of PPARγ (Peroxisome proliferator-activated receptor gamma), C/EBPα (CCAAT-enhancer-binding protein α), and FAS (Fatty acid synthase) (Fujimoto et al. 2012). High glucose exposure for 24 h in human pancreatic islet cells resulted in the significant up-regulation of abundant proteins including Dicer 1 indicating the role of Dicer 1 in glucose regulation (Schrimpe-Rutledge et al. 2012). Furthermore, Dicer is an energy sensor as its mRNA expression is regulated by nutrient availability. Fasting for 16 h up-regulated Dicer gene expression in mouse hypothalamus. Dicer deletion in POMC-expressing cells alters energy homeostasis in mutant mice such as increase of body weight, hyperphagia, plasma leptin, blood triglycerides concentration, adiposity and defecting in glucose homeostasis (Schneeberger et al. 2013). Other

studies have shown that Dicer 1 is a cellular stress response factor. Different stressors (reactive oxygen species, Ras oncogene and phorbol esters) inhibited the protein expression of Dicer 1 in JAR trophoblast cells and IMR-90 cells (Wiesen & Tomasi 2009). In addition, Dicer 1 knockdown in mice resulted in a hypersensitive response to oxidative stress and reduction of stress tolerance in *C. elegans*, but its overexpression assisted intestine against stress (Mori et al. 2012).

Dicer has been studied extensively in human and mammals; there is, however, a paucity of Dicer 1 research in avian species. Tian and co-authors reported that the chicken Dicer gene is located on chromosome 5 at 1040 cRs, which is consistent with human. They also found that Dicer genes of chicken and human have a high percentage of homology (87%) (Tian et al. 2007). Additionally, Carraco and colleagues observed broad gene expression of Dicer 1 in multiple tissues of the stage HH6 embryo (Carraco et al. 2014).

In addition, the poultry industry has applied several feed restriction regimens due to chickens' tendency for hyperphagy, risk of fat deposition, and reproductive decline (Siegel 1980). Studies on feed-deprivation have shown changes in growth rates, reproduction, chemical blood index, physiological behavior, immune system, and cellular alterations in birds. Feed restriction causes both behavioral and nutritional stresses to birds (De Jong et al. 2002). Fasting for 2 days affected the LKB1/AMPK pathway in chicken hypothalamus by increasing phosphorylated-AMPKα:total AMPKα and phosphorylated-LKB1:total LKB1 ratios, and re-feeding restored this ratio (Song et al. 2012). In the liver of 4-week old chicken subjected to 16 or 48 hours of fasting, genes involved in gluconeogenesis, ketogenesis, and peroxisomal fatty acid beta-oxidation were significantly increased, whereas genes involved in the synthesis of fatty acids and cholesterols were markedly down-regulated (Yang et al. 2010).

Since Dicer 1 is involved in energy homeostasis, metabolism, and stress, we conducted a study of nutritional stress via short term fasting and long-term feed restriction in avian species, and serum fasting in different cell lines to gain understanding of the regulation of Dicer 1.

7.3. Materials and method

7.3.1. Animals

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Animal Care and Use Committee under protocols 13039, 16084 and 15039.

7.3.1.1. Short term fasting in quails

Thirty 16 week-old Japanese quails from the Arkansas Heavy RBC were gifted from Dr. Nick Anthony (Center of Excellence, the University of Arkansas). The birds evaluated in this study were derived from a reciprocal cross between the heavy weight line (HW) quail developed at The Ohio State University (Bacon et al., 1982) and P-line as described by Lepore and Marks (1968). Both quail populations were initiated from a different genetic base and had been independently selected for high 4 week body weight for more than 50 and 100 generations respectively. Reciprocal crosses between the HW and P-line were conducted to form the AR Heavy currently maintained at the University of Arkansas. The AR Heavy line was maintained and randomly mated for 8 generations prior to this experiment. Birds were caged individually and subjected to one of two nutritional states: fasting for 24 or 48h and refeeding for 3h after each fasting period. *Ad libitum* fed group was used as a control. There were 5-6 replicates in each treatment. The birds had free access to water. At the end of each study period, animals were

humanely euthanized by cervical dislocation, and whole brain, liver and leg muscle tissues were removed, immediately frozen in liquid nitrogen, and stored at -80^{o}C until analysis.

7.3.1.2. Feed deprivation in chickens

Thirty-two birds from four commercial broiler cross breeds (as indicated in table 7.1) followed a feed restriction program from the Cobb breeder management guide from 14 to 56 days of age on an 8 hour light/16 hour dark cycle. Then they were assigned to two feeding regimes equally and randomly by line: restricted or *ad libitum* for next 2 weeks. The animals had free access to water. At the end of the experiment, birds were humanly euthanized by carbon dioxide asphyxiation, and hypothalamus, liver, and leg muscle tissues were collected, immediately frozen in liquid nitrogen, and stored at -80^{o}C until analysis.

7.3.2. Cell culture

Immortalized chicken embryo liver (CEL-im) (Lee et al. 2013) cell line and human neuroblastoma (SH-SY5Y) cell line were grown in Waymouth's medium (Life Technologies, Grand Island, NY) and DMEM/F-12 (Life Technologies, Grand Island, NY), respectively, complemented with 10% FBS (Life Technologies) and 1% penicillin-streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO2 and 95% air. At 80% confluence, cells were washed with PBS, and growth media was replaced with serum-free media. For the CEL-im cells, treatment was for 24 h. Treatment on SHSY-5Y cells was for 12, 24, and 48 h. Standard growth media was used as a control.

In order to understand the mechanism of Dicer 1 regulation in energy homeostasis, the SH-SY5Y cell line was treated for 24 hours with: the AMPK activator 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Cell Signaling, USA) at 500 μM, the PKC inhibitor

Bisindolylmaleimide I (BIM I) (Cayman chemical, USA) at 2 μM, or the mTOR inhibitor Rapamycin (Sigma, USA) at 100 nM.

7.3.3. Sample collection and analysis

7.3.3.1. RNA isolation, reverse transcription and real-time quantitative PCR

Total RNA was extracted from brain, hypothalamus, liver, and leg muscle tissues, CEL-im cells and SH-SY5Y cells by Trizol reagent (Life Technologies, Grand Island, NY) following manufacturer's recommendations and as described in (Piekarski et al. 2014). Total RNA was DNAse treated, reverse transcribed and amplified by qPCR using oligonucleotide primers specific for chicken as in table 7.2. Relative expression of target genes was determined by the 2-ΔΔCt method (Schmittgen & Livak 2008). For the *in vitro* studies, control cells were used as calibrators.

7.3.3.2. Protein extraction and Western blot analysis

Protein extraction and concentration measurement were conducted as previously described (Lassiter et al. 2015). Total proteins for tissues (100 μg) and for cells (30 μg) were separated by SDS-PAGE, and individual proteins were assessed by Western Blotting (Lassiter et al. 2015) using the following antibodies: rabbit anti-Dicer (Santa Cruz Biotechnologies), anti-phospho AMP-activated protein kinase alpha (AMPKα) Thr172 (#2531), anti-phospho acetyl-CoA carboxylase alpha (ACCα) Ser79 (#3661), and anti-ACCα (#3662), anti-ATP Cl (Lifespan BioSciences, USA), anti-AMPKα (23A3), anti-phospho-ERK1/2, anti-phospho-JNK, anti-phospho-p38,anti- ERK1/2, anti- JNK, anti- p38, anti-phospho-mTOR (Ser2481), anti-mTOR, anti-phospho-PKCα/βII, anti-phospho-PKCβII (Ser660), anti-phospho-PKD-PKCμ, anti-phospho-PKCδ-θ (Ser643/676), anti-phospho-PKCζ/λ (Thr410/403), anti-total-PKCα, anti-total-PKD/PKCμ, anti-PKCδ, anti-total-PKCζ (all from Cell Signaling), anti-FAS (Novus Biologicals,

USA), anti-ME3 (AVIA systems biology, USA) were used. Protein loading was assessed by immunoblotting with the use of rabbit anti-β actin (#4967, Cell Signaling) or GAPDH (Santa Cruz Biotechnology, Inc.). Pre-stained molecular weight marker (Precision Plus Protein Dual Color, Bio-rad, Hercules, CA) was used as standard. The signal was visualized by enhanced chemiluminescence (ECL plus, GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by FlourChem M MultiFlour System (Proteinsimple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993-2011, Proteinsimple, Santa Clara, CA).

7.3.4. Data analysis

Effect of fasting on Dicer 1 expression in quail tissues was analyzed by one way ANOVA, with fasting regimen as the main factor. Effect of feed restriction on Dicer 1 expression was analyzed by two-way ANOVA with feeding regimes and breeds as factors. The data from the *in vitro* studies were analyzed by the Student's unpaired t-test with comparison between control and fasted treatment in CEL-im cells or in SH-SY5Y cells. All data were analyzed using Graph Pad Prism software (version 6, La Jolla, CA) and were presented as means ± SEM. Significance was set at $P<0.05$.

7.4. Results

7.4.1. Short term fasting up-regulates Dicer gene expression in quail liver

Fasting for 48 h and refeeding 3 h upregulate Dicer protein expression ($P=0.07$, Figure 7.1b). Fasting 48 h significantly increased Dicer gene expression in quail livers compared with 48 hour fasting and refeeding ($P<0.05$, Figure 7.2a). However, there was no significant difference in gene and protein expression of Dicer in quail brain (Figure 7.1a), and no difference in protein expression of Dicer in quail livers among all treated groups (Figure 7.2b). Also,

different short term fasting treatments did not statistically affect the gene expression of Dicer in quail leg muscles (Figure 7.3).

7.4.2. Long term feed restriction alters Dicer 1 expression in different breeds of chicken

In this study, chicken hypothalamus was only used for only RNA extraction to analyze gene expression. The results show no significant difference in Dicer gene expression in chicken hypothalamus between different feeding regimes and breeds (Figure 7.4). Nevertheless, feed restriction markedly upregulated Dicer gene expression in chicken livers from different breeds ($P<0.05$) (Figure 7.5a). No difference was observed in Dicer protein expression between treated chicken groups (Figure 7.5b). Conversely, the gene expression of Dicer in chicken leg muscles was not affected by feeding regimes and breeds (Figure 7.6a).

7.4.3. Dicer 1 regulation in 24 hour-serum fasted CEL-im cell line

Twenty-four hours of serum fasting did not affect gene expression of Dicer 1 in CEL-im cells. However, serum fasting significantly up-regulated gene expression of MITF, a transcription factor of Dicer 1 ($P<0.05$) (Figure 7.7). Fasting down-regulated FAS in Cel-im cells ($P<0.05$), and there is a decrease of ATPCl gene level in the fasted group with $P=0.063$ (Figure 7.7).

Figure 7.8 shows that fasting Cel-im cells markedly upregulated pAMPKα protein level, down regulated pACC and total ACC protein expression ($P<0.05$).

7.4.4. Serum fasting down-regulates Dicer 1 expression in a time dependent manner in SH-SY5Y cell line

As shown in Figure 7.9a, serum fasting affects the gene expression of Dicer in a time dependent manner. Fasting for 12 h reduced Dicer gene level with $P=0.06$, while this gene level did not change after 24 h and was significantly down-regulated after 48 h (Figure 7.9a).

Furthermore, Dicer protein expression was gradually reduced as the serum fasting time increased from 12 h to 24 h and 48 h (Figure 7.9b). For further investigation regarding role of Dicer 1 in fasting, we chose to work with 24 h serum fasting. Twenty – four hour fasting reduced the protein levels of Dicer, phospho- mTOR and total mTOR, but it increased pAMPKα:total AMPK ratio (Figure 7.10a). Moreover, in the MAPK pathway, fasting significantly down-regulated the pERK:total ERK ratio, but did not affect the expression of pP38:P38 and pJNK:JNK ratios (Figure 7.10b). Twenty – four hour serum fasting also reduced protein expression of pPCKα-βII:total PKCα ratio, and total PKCζ (Figure 7.10c).

Treating SH-SY5Y with 500 μM AICAR for 24 h increased pAMPKα: Total AMPK protein expression ratio and down-regulated Dicer protein level (Figure 7.11a). This treatment also reduced the protein expression of pPKCα-βII, total PKCα, pmTOR, and pERK : total ERK ratio (Figure 7.11a). Rapamycin treated SH-SY5Y (100 nM of Rapamycin for 24 h) had lowered Dicer, pmTOR, and total mTOR compared with the control. However, there was an increase in pERK : total ERK ratio in the treated group; while there is no change in pAMPKα : total AMPK ratio (Figure 7.11b). Moreover, pPKCα and total PKCα protein expression was down-regulated in Rapamycin treated group (Figure 7.11b). Inhibition of PKC by 2μM BIM I for 24 h reduced protein expression of Dicer, pPKCα-βII:total PKCα ratio, but this increased pAMPKα, and pERK; and did not have an effect on protein expression of p-mTOR, total mTOR.

7.5. Discussion

Dicer is an essential enzyme in miRNA biogenesis which plays key roles in many cellular processes including metabolism. Dicer dysregulation leading to alterations of miRNA biogenesis has been causally linked to several human diseases. In non-mammalian species, the regulation of Dicer under normal and stress conditions is unknown. This study aimed to examine the

regulation of Dicer in chicken tissues under feed deprivation as well as delineate a possible mechanistic pathway in Cel-im and SH-SY5Y cells during serum fasting. The *in vivo* studies showed significant up-regulation of Dicer gene expression in 48 hour-fasted quail livers (Figure 7.2a) and during long term feed restriction in different chicken strains (Figure 7.5a). These results are similar to those documented by Schneeberger and colleagues where Dicer mRNA levels increased in mouse hypothalamus and Dicer was also found to play a role in energy homeostasis as an energy sensor (Schneeberger et al. 2013). Wiesen and Tomasi found that different stressors (reactive oxygen species, Ras oncogene and phorbol esters) inhibited the protein expression of Dicer in JAR trophoblast and IMR-90 cells (Wiesen & Tomasi 2009). In this study, the decrease of Dicer protein expression due to metabolic stress in 24 hour-fasted SH-SY5Y cells is similar to the report of Wiesen and Tomasi. This study further supports previous evidence showing that Dicer gene and protein expression are not positively correlated (Wiesen & Tomasi 2009).

Based on the results from the fasting experiments, the effects of certain pharmacological activator and inhibitors of AMPK, PKC, and mTOR on Dicer expression was examined. Interestingly, in SH-SY5Y cells, the regulation of related proteins (AMPK, Dicer, PKCα and mTOR) after AMPK activation treatment was the same as those in the fasting treatment. AMP-activated protein kinase (AMPK), a protein complex that plays vital role in energy homeostasis by sensing the ratio of adenosine monophosphate (AMP) to adenosine triphosphate (ATP), has been shown to regulate the stress response (Reid & Kong 2013). In SH-SY5Y, endoplasmic reticulum (ER) stress caused by homocysteine-induced neurotoxicity was significantly reduced by AMPK activation (Park et al. 2013). As a response to cell energy stress, AMPK inhibits the rapamycin-sensitive mTOR pathway in order to increase cellular energy

(Gwinn et al. 2008). A similar mechanism happens when AMPK activation by AICAR causes repression of mTOR signaling in rat liver (Reiter et al. 2005). This may explain the increased protein level of pAMPK, and reduction of pmTOR in fasted CEl-im cells, fasted SH-SY5Y cells, and AICAR treated SH-SY5Y cells observed in the present study. Furthermore, fasting condition or AMPK activation by AICAR treatment in SH-SY5Y cells for 24 hours inhibited ERK phosphorylation. Another study conducted in rat cardiac fibroblasts showed inhibitory crosstalk between AMPK and ERK when treated with AICAR (Du et al. 2008). This is similar to what we observed in our study when using AICAR in the SH-SY5Y cell line. A study conducted using vascular smooth muscle showed increased phosphorylation of AMPKα at Thr172 was negatively correlated with the diminished expression of miR-144/451 (Turczyńska et al. 2013). It was found that miR-33 is involved in posttranscriptional repression of key genes regulating fatty acid oxidation such as AMPK (Fernández-Hernando & Moore 2011). As Dicer, a ribonuclease III enzyme, plays an important role in miRNA biogenesis (Bernstein et al. 2001; Kurzynska-Kokorniak et al. 2015), we sought to explore the relationship between Dicer and AMPK in energy homeostasis. In this study, fasting increased phosphorylation of AMPKα and down-regulated Dicer protein levels in SH-SY5Y cells. This is opposite of the observations of Loomba, Blandino and colleagues (Blandino et al. 2012; Loomba 2014). These studies showed the upregulation of Dicer following AMPK activation by AICAR for 12 hours in an estrogen independent breast cancer cell line (SUM159PT) (Blandino et al. 2012). It may be possible that the relationship between AMPK and Dicer in Cel-im and SH-SY5Y cell lines under 24-hour fasting (or AMPK activation by AICAR is more related to energy sensing and stress, as well as different from the results of previous studies in different cell types.

In this study, pharmacological inhibition of PKC by BIM I resulted in the down-regulation of Dicer protein levels and activation of AMPK and ERK1/2. The reduction of Dicer protein expression is in agreement with the reports of Matskevich and Moelling , as well as Jiang and colleagues when they inhibited PKC (Matskevich & Moelling 2008; Jiang et al. 2015). These similar results suggest that suppression of Dicer expression follows the inhibition of PKC via apoptosis in Hela, T24 and 5637 cells (Matskevich & Moelling 2008; Jiang et al. 2015). It is hypothesized that the down-regulation of PKCα initiated Dicer reduction which took place synchronously through the caspase-3-PARP pathway. The authors also suggested a possible link involved in the PKCα- Dicer pathway that could be the P53 which was shown to regulate Dicer expression and has an interwoven linkage with PKCα (Delphi et al. 1997; Smith et al. 2012; Muller et al. 2014; Jiang et al. 2015). Furthermore, AMPK is known for its role in cell survival during glucose deprivation by inducing p53 phosphorylation. This will allow cells to survive and reenter the cell cycle when a carbon source is available (Jones et al. 2005). Further studies examining the role of p53 in the regulation of dicer under fasting conditions are warranted. Previous studies have shown that knocking down PKCα by siRNA-PKCα transfection or by a PKC inhibitor (Go¨6976) did not alter ERK protein expression in human hepatoma SK Hep-1 cells and adreno chromaffin cells, respectively (Sugano et al. 2006; Hsieh et al. 2007). In this study, we therefore proposed that PKCα does not have an effect on ERK expression. The reduction of mTOR phosphorylation by rapamycin did not alter pAMPK expression and is the same as previous study in which 1nM rapamycin treatment failed to affect AMPK phosphorylation in decidualized mouse stromal cells (cells in preparation for, and during, pregnancy) suggesting that AMPK is upstream of mTOR (Deng et al. 2016).

Interestingly, treatments with PKC or mTOR inhibitors (BIM I or rapamycin) increased ERK phosphorylation). Moreover, rapamycin was found to be ineffective in the activation of ERK (Du et al. 2008), and mTOR was shown to be down-stream of ERK1/2 (Muscella et al. 2014). Therefore, it is believed that ERK was independently activated by mTOR after mTOR inhibitor (rapamycin) treatment. Moreover, a study showed that rapamycin (10μM) treatment for 24 hours inhibited PKCα phosphorylation in human glioblastoma cell line LN-18 (Chandrika et al. 2016). This observation is similar to these results treating SH-SY5Y cells with rapamycin (200nM) for 24 hours which caused a decrease in pPKCα protein level. In addition, Dridi and colleagues showed that reduced Dicer 1 protein level activated ERK1/2 in retinal pigmented epithelium (RPE) of human eyes in geographic atrophy (GA) (Dridi et al. 2012). This is similar with our results where down-regulation of Dicer resulted in an increase of ERK1/2 phosphorylation after treatment with BIM I or rapamycin for 24 hours. In the current study, pharmacological inhibition of PKC by BIM I resulted in the down-regulation of Dicer protein levels, and activation of AMPK and ERK

The results of this study presenting serum fasting in cells and inhibition of related proteins, reveal the link between AMPK, mTOR, PKC, ERK and Dicer in the regulation of energy homeostasis and stress.

7.6. Conclusion

The data indicates that DICER1 is responsive to the nutritional state (fasting) and it may be involved in the regulation of energy homeostasis in avian species. Results from cells indicates that the regulation of Dicer 1 in fasted cells may involve in AMPK/ mTOR pathway.

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Table 7.1. Name tags of birds and their relative breeds

Name tags	Strain
5000	Cobb MX × Cobb 500
11000	Hubbard × HIFF
7000	ROSS 544 × ROSS 708
14000	ROSS Y+ × ROSS 308

Table 7.2. Oligonucleotide qPCR primers

Gene	Accession number[a]	Primer sequence (5' → 3')	Orientation
Dicer 1		TTTAAACACTGGCTCAGGGAAGA	Forward
		AAATCCCCCCTGATCTGATAGG	Reverse
DROSHA		TGGCCATGCTGGCTAAGAAG	Forward
		GTCCGGACCATGAGCATAAAG	Reverse
DGCR8		TGGCTGTAGCGCACGTACTG	Forward
		AGAGAAGACGTACTGCAATCACTCA	Reverse
AGO2		ACCAGTCTATGCGGAGGTGAA	Forward
		AACACACTGCGTAGCCATTCC	Reverse
MITF		AAACCAGCCCGGTGATCA	Forward
		GGGCGCACTGCTTCCA	Reverse
SCAP		TGGCCCAGAGACTCATCATG	Forward
		GCAGGATCCGTATAAACCAGGAT	Reverse
FAS	JO3860	ACTGTGGGCTCCAAATCTTCA	Forward
		CAAGGAGCCATCGTGTAAAGC	Reverse
ACCα	NM_205505	CAGGTATCGCATCACTATAGGTAACAA	Forward
		GTGAGCGCAGAATAGAAGGATCA	Reverse
SCD-1	NM_204890	CAATGCCACCTGGCTAGTGA	Forward
		CGGCCGATTGCCAAAC	Reverse
AMPKα1	NM_001039603	CCACCCCTGTACCGGAAATA	Forward
		GGAAGCGAGTGCCAGAGTTC	Reverse
ME	AF408407	AGATGAAGCTGTCAAAAGGATATGG	Forward
		CACGCCCCTTCACTATCGA	Reverse
ATPcl	NM_001030540	CTTTTAAGGGCATTGTTAGAGCAAT	Forward
		CCTCACCTCGTGCTCTTTCAG	Reverse
SREBP-1	AY029224	CATCCATCAACGACAAGATCGT	Forward

Table 7.2. Oligonucleotide qPCR primers (cont.)

Gene	Accession number[a]	Primer sequence (5' → 3')	Orientation
		CTCAGGATCGCCGACTTGTT	Reverse
SREBP-2	AJ414379	GCCTCTGATTCGGGATCACA	Forward
		GCTTCCTGGCTCTGAATCAATG	Reverse
18S	AF173612	TCCCCTCCCGTTACTTGGAT	Forward
		GCGCTCGTCGGCATGTA	Reverse

a Accession number refer to Genbank (NCBI).

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ATPcl, ATP citrate lyase; FAS, fatty acid synthase; ME, malic enzyme; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element binding protein;

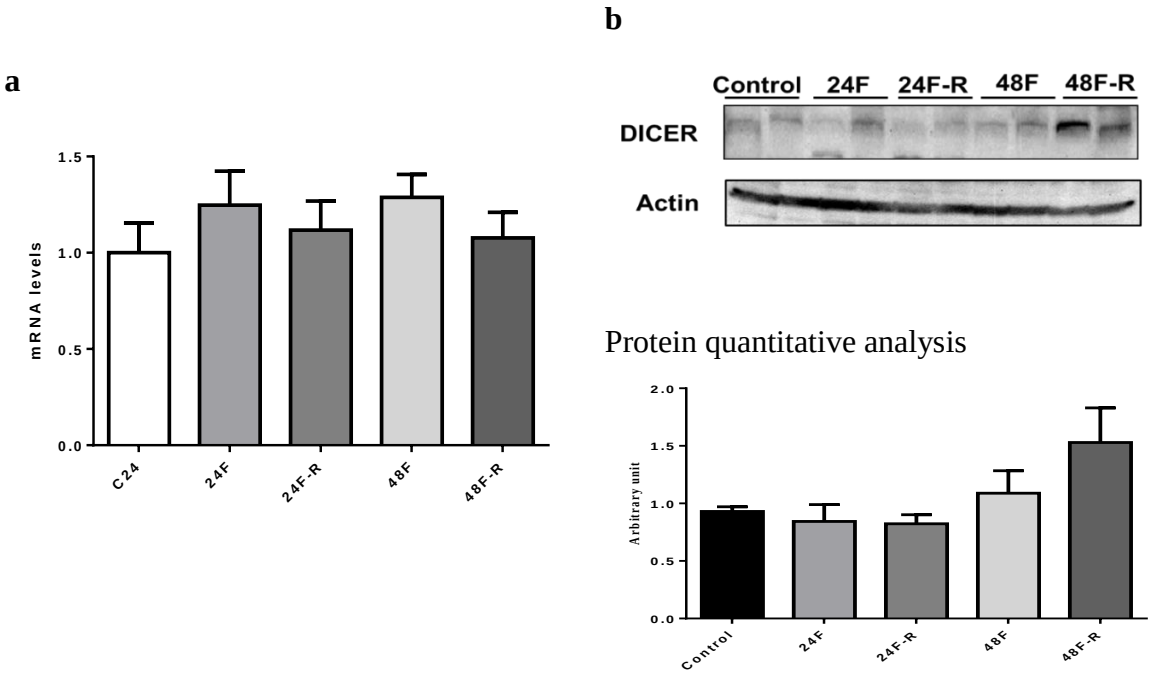

Figure 7.1. Dicer 1 expression in fasted quail brains. Dicer 1 gene expression **(a)**. Western blot image and quantitative analysis of Dicer protein expression **(b)**. Data are presented as mean ± SEM (n = 5-6/treatment for gene expression data, n=4/treatment for protein expression data). The birds were caged individually and subjected to two nutritional states: fasting for 24 or 48h and refeeding for 3h after each fasting period. Ad libitum fed group was used as a control.

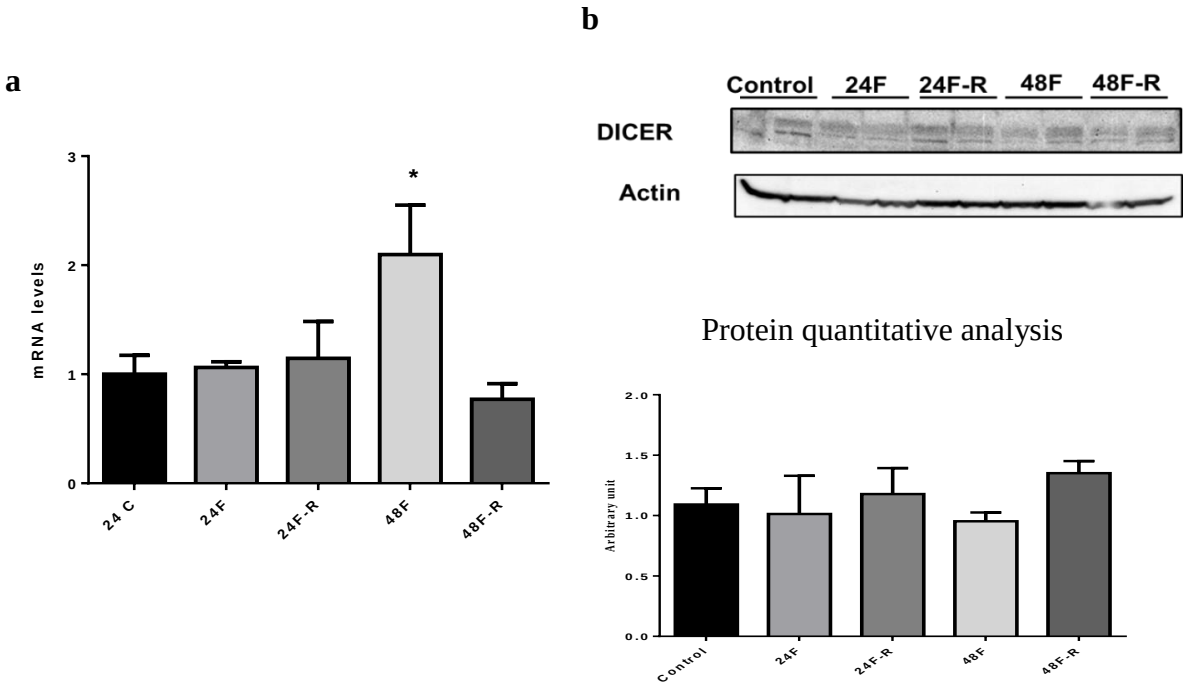

Figure 7.2. Dicer 1 expression in fasted quail livers. Dicer 1 gene expression (a). Western blot image and quantitative analysis of Dicer protein expression **(b)**. Data are presented as mean ± SEM (n = 5-6/treatment for gene expression data, n=4/treatment for protein expression data). The birds were caged individually and subjected to two nutritional states: fasting for 24 or 48h and refeeding for 3h after each fasting period. *Ad libitum* fed group was used as a control. *P<0.05.

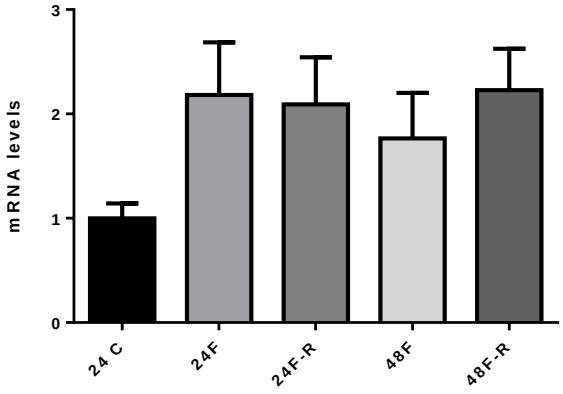

Figure 7.3. Dicer 1 gene expression in fasted quail leg muscles. Data are presented as mean ± SEM (n = 5-6/treatment). The birds were caged individually and subjected to two nutritional states: fasting for 24 or 48h and refeeding for 3h after each fasting period. *Ad libitum* fed group was used as a control. No significant difference was seen in gene expression of Dicer 1 in fasting quail muscles.

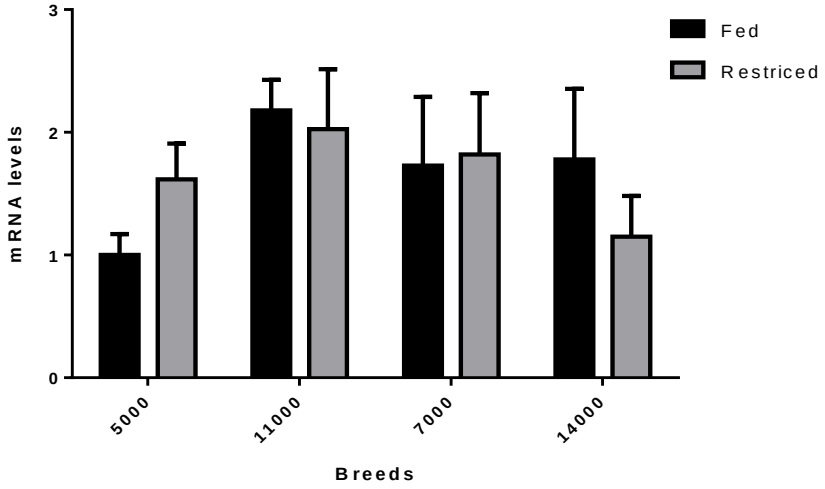

Figure 7.4. Dicer 1 expression in feed restricted chicken hypothalamus. Birds of four commercial broiler cross breeds followed feed restriction program of Cobb breeder management guide from 14 to 56 days of age. Then they were assigned to two feeding regimes equally and randomly by line: restricted or *ad libitum* finisher diet for next 2 weeks. Data are presented as mean ± SEM (n = 4-5/treatment for gene expression data). No significant difference in gene expression of Dicer 1 in chicken hypothalamus.

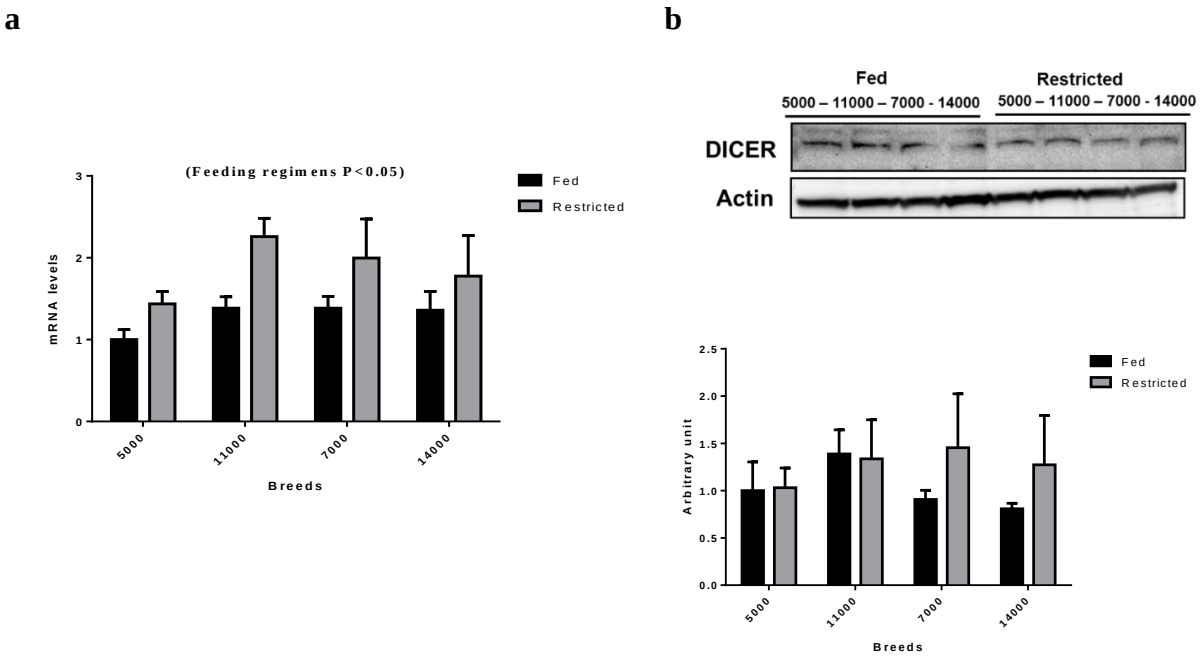

Figure 7.5. Dicer 1 expression in feed restricted chicken livers. Feed restriction markedly increased Dicer mRNA levels in chicken livers from different breeds (P<0.05) **(a)**. Western blot image and quantitative analysis of Dicer protein expression **(b)**. Birds of four commercial broiler cross breeds followed feed restriction program of Cobb breeder management guide from 14 to 56 days of age. Then they were assigned to two feeding regimes equally and randomly by line: restricted or *ad libitum* for next 2 weeks. Data are presented as mean ± SEM (n = 4-5/treatment for gene expression data; n=3/treatment for protein expression data). * P<0.05

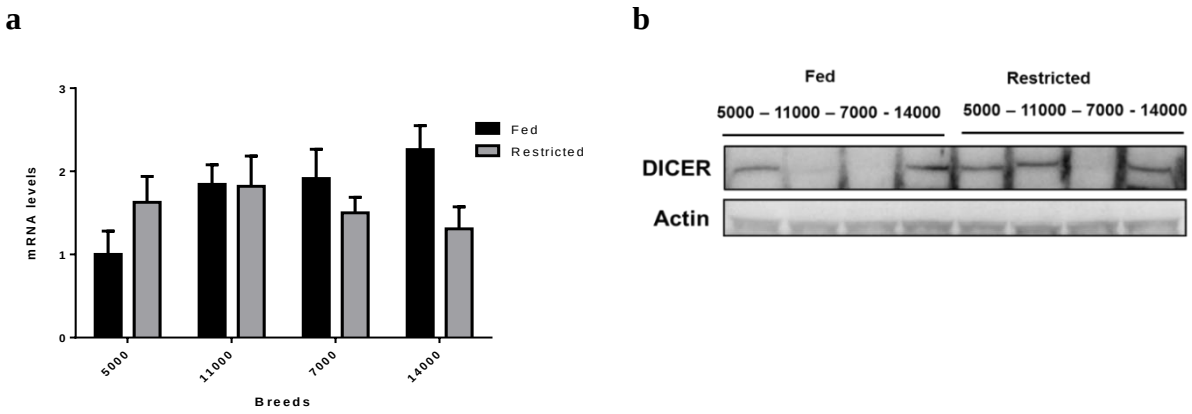

Figure 7.6. Dicer 1 expression in feed restricted chicken muscles. Dicer mRNA levels in chicken muscles from different breeds **(a)**. Western blot image of Dicer protein expression **(b)**. Birds of four commercial broiler cross breeds followed feed restriction program of Cobb breeder management guide from 14 to 56 days of age. Then they were assigned to two feeding regimes equally and randomly by line: restricted or *ad libitum* for next 2 weeks. Data are presented as mean ± SEM (n = 4-5/treatment for gene expression data; n=3/treatment for protein expression data).

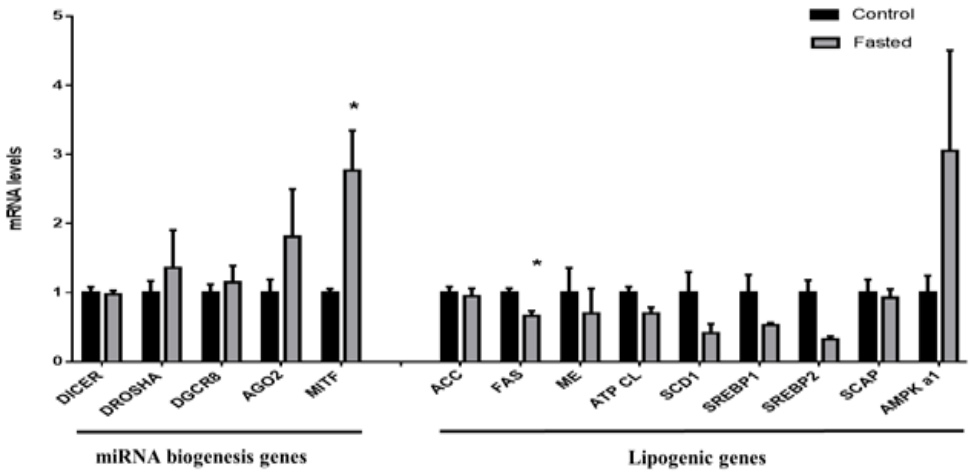

Figure 7.7 Serum fasting for 24 hours significantly increased mRNA level of MITF, Dicer transcriptional factor, and reduced FAS mRNA level in CEL-im cell line ($P<0.05$). CEL-im cell line was treated with standard growth media (Control) and no FBS - DMEM media (Fast) for 24 hours. After treatment, gene expression of miRNA biogenesis genes and lipogenic genes were examined by qPCR method. Data are presented as mean ± SEM. * $P<0.05$

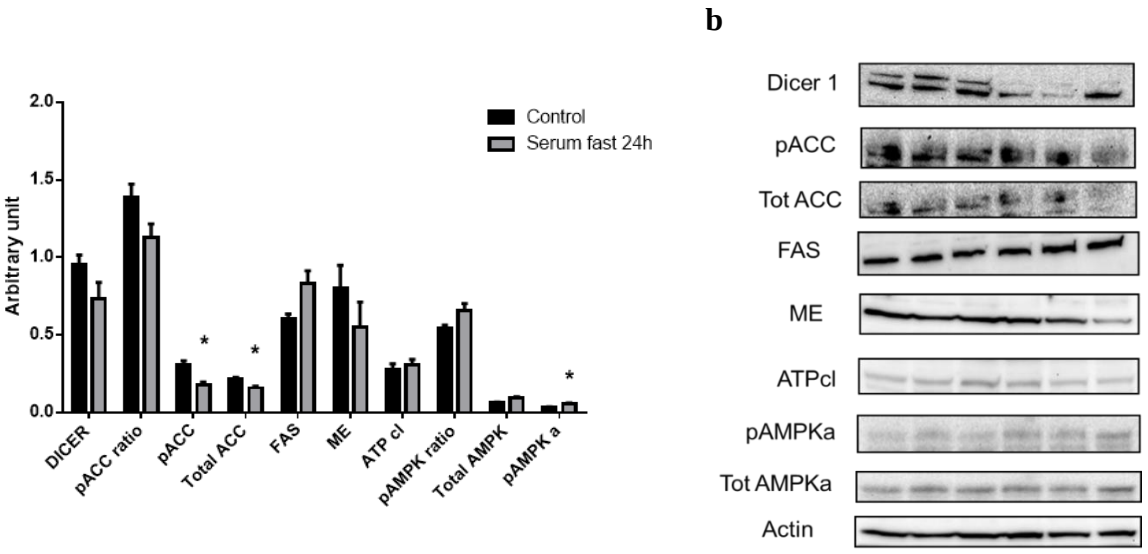

Figure 7.8. Dicer 1 and lipogenic protein expression in 24h fasted Cel-im cells. CEL-im cell line was treated with standard growth media (Control) and no FBS - DMEM media (Fast) for 24 hours. After treatment, protein expression of Dicer protein were examined by Western Blot method. Western blot quantitative analysis of Dicer and lipogenic protein expression **(a)** Dicer and lipogenic protein expression image **(b)**. Data are presented as mean ± SEM (n=3). * $P<0.05$

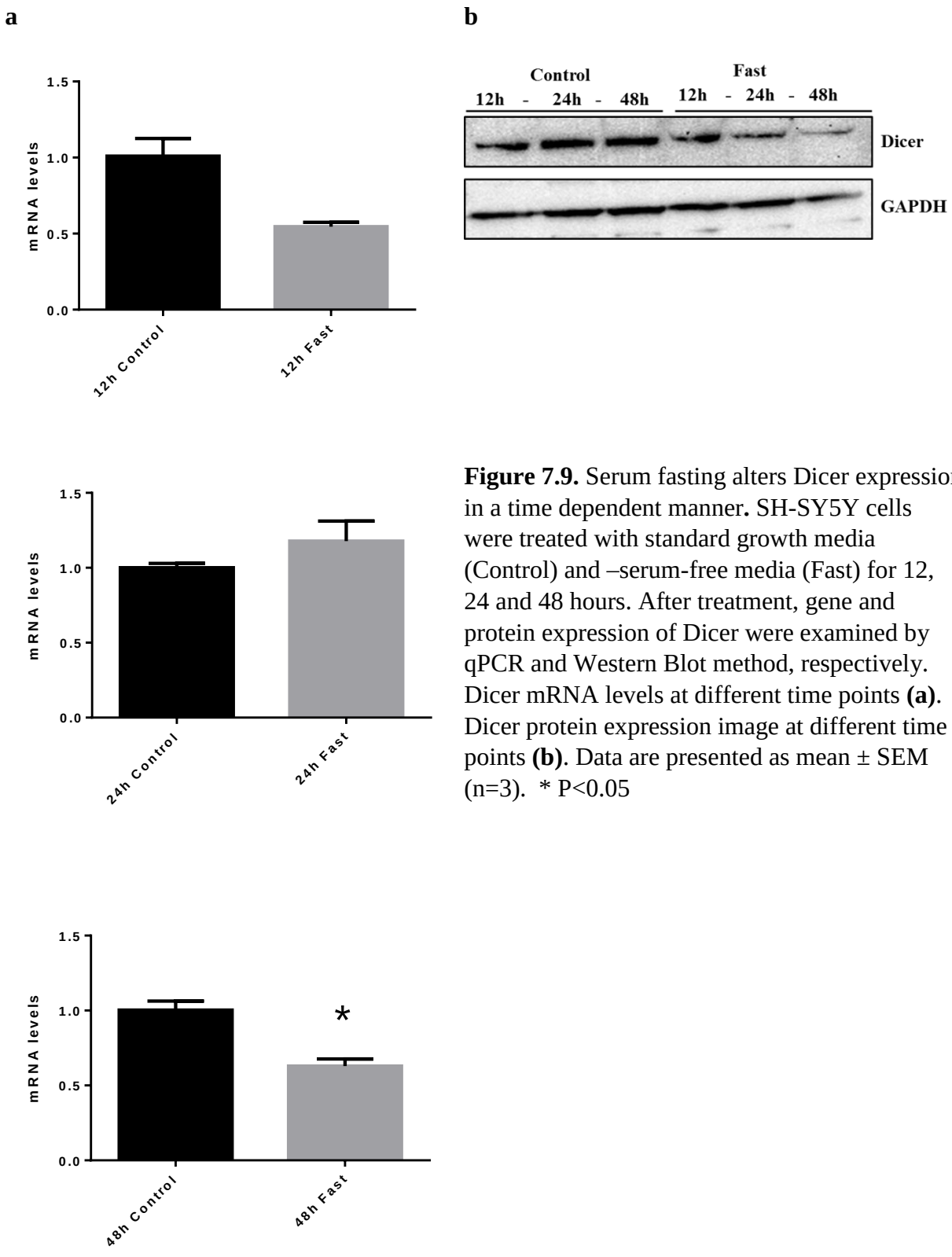

Figure 7.9. Serum fasting alters Dicer expression in a time dependent manner**.** SH-SY5Y cells were treated with standard growth media (Control) and –serum-free media (Fast) for 12, 24 and 48 hours. After treatment, gene and protein expression of Dicer were examined by qPCR and Western Blot method, respectively. Dicer mRNA levels at different time points **(a)**. Dicer protein expression image at different time points **(b)**. Data are presented as mean ± SEM (n=3). * P<0.05

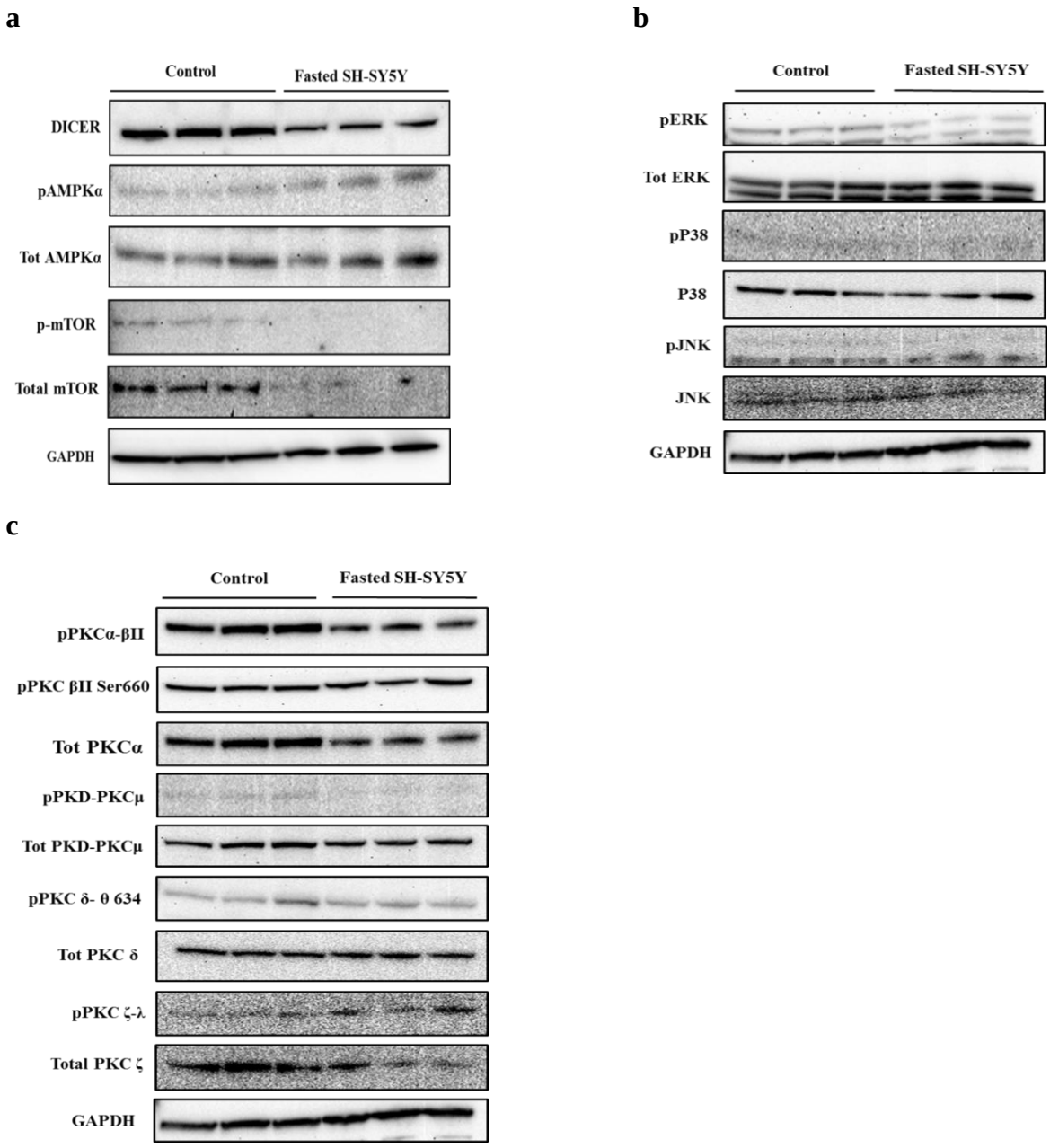

Figure 7.10. Serum fasting for 24 hours alters Dicer and energy homeostasis related protein expression**.** SH-SY5Y cells were treated with standard growth media (Control) and no FBS - DMEM media (Fast) for 24 hours. After treatment, protein expression of Dicer and energy homeostasis related proteins were examined by Western Blot method. Dicer and AMPK and mTOR protein levels Western Blot images **(a)** ERK, P38 and JNK protein levels Western Blot images **(b)** PKC isoform protein levels Western Blot images **(c)**.

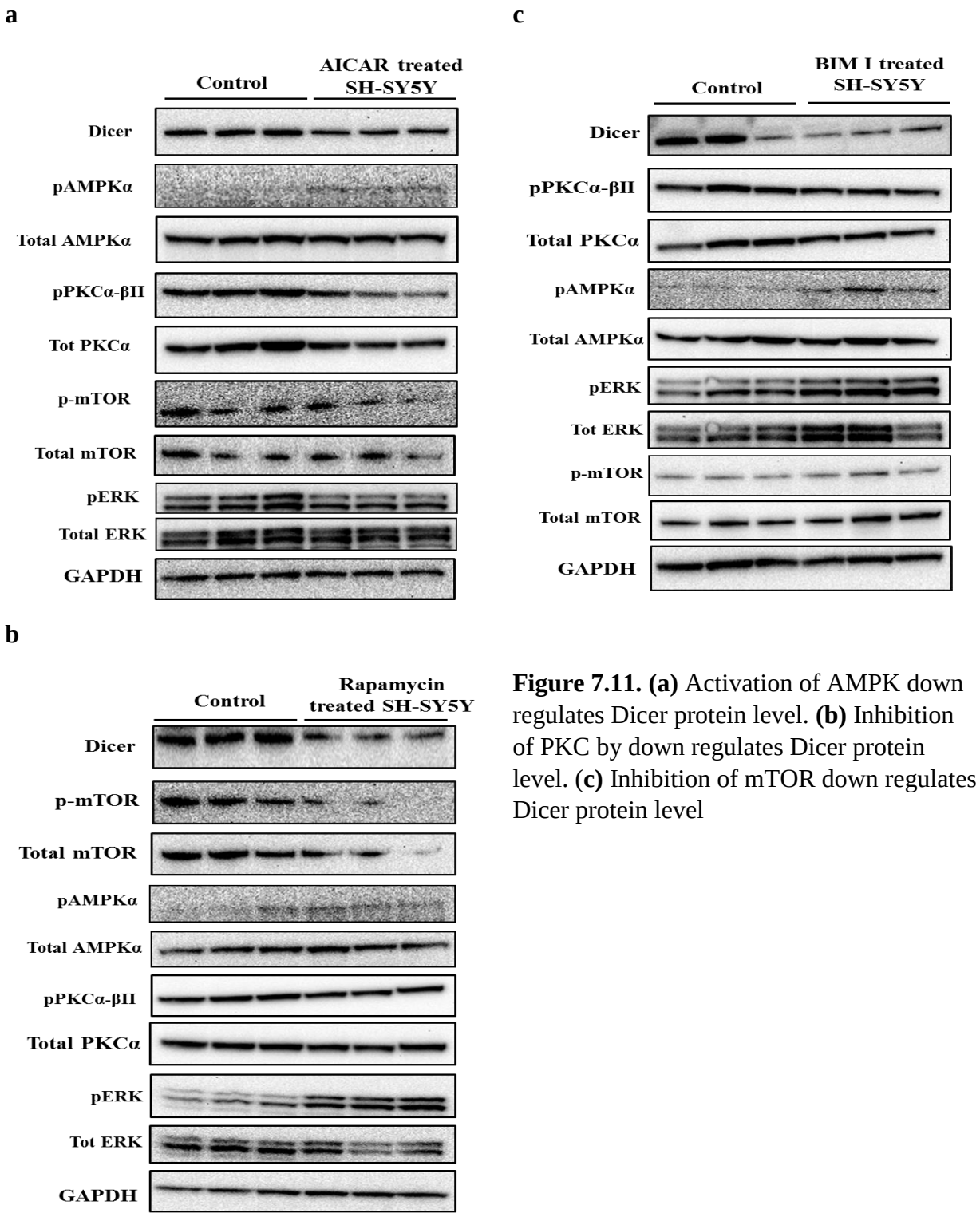

Figure 7.11. (a) Activation of AMPK down regulates Dicer protein level. **(b)** Inhibition of PKC by down regulates Dicer protein level. (**c)** Inhibition of mTOR down regulates Dicer protein level

8. CONCLUSION

In this study, we identified several molecular signatures that are responsive to environmental and nutritional stress in avian species. In a cold stress study, we found that chronic mild conditioning cold can improve growth performance in young chicks via metabolic-related genes. Two molecular signatures AMPK and mTOR may be involved in orchestrating cellular fatty acid utilization to satisfy energy requirement in avian cold stress.

In addition, orexin and its related receptors expression were found to be altered by heat and oxidative stress in avian muscle tissues and cells. Therefore, they may have potential as bio-markers for these stressors. Further understanding their regulation can help alleviate heat stress and oxidative stress effects in birds.

HSPA9 gene, a heat shock protein family member, is differently distributed in various tissues of avian species and it is expressed in the manner of gender and genotype specific. It is expressed abundantly in intracellular of different cell types including Human neuroblastoma (SH-SY5Y), immortalized chicken embryo liver (CEl-im) and Quail muscle clone 7 (QM7) cells. HSPA9 expression is not affected by conditioning chronic cold stress in young chicks. However, acute heat stress alters its gene expression in S quail livers, SH-SY5Y cells and Cel-im cells. Moreover, acute heat stress also increases HSPA9 protein levels in QM7 cells. There is a need to examine further on the mechanisms of HSPA9 expression in avian species to use this gene as a molecular marker for thermo-tolerance improvement in poultry production in the future.

By conducting feed deprivation study in avian species and different cell types, it is observed that DICER1 is responsive to the nutritional state (fasting) and it may be involved in the regulation of energy homeostasis in avian species. Results from cells indicates that the

regulation of Dicer 1 in fasted cells may involve in AMPK/Dicer 1 pathway. There should be further investigation to understand more the mechanistic of Dicer 1 regulation in fasted cells.

APPENDIX

1. IACUC approval for cold stress study

UNIVERSITY OF ARKANSAS

Research Compliance
Office of the Director

MEMORANDUM

TO: Gerry Huff

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

DATE: June 29, 2012

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date : **June 28, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#12050- "THE ROLE OF STRESS IN THE PERSISTENCE OF FOODBORNE PATHOGENS IN POULTRY"**. You may begin this study immediately.

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

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing [Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **06-28-2015,** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

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

cnc/car

cc: Animal Welfare Veterinarian

120 Ozark Hall • University of Arkansas • Fayetteville AR 72701
Voice (479) 575-3845 • Fax (479) 575-3846

2. IACUC approval for heat stress in quail and feed restriction in quail studies

UNIVERSITY OF ARKANSAS

Office of Research Complian

MEMORANDUM

TO: Dr. Sam Dridi

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee (IACUC)

DATE: November 7, 2014

SUBJECT: IACUC APPROVAL
Expiration date: June 30, 2016

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your modification (to add quail) to protocol 13039 *Regulation of energy homeostasis and fat metabolism in avian species.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing(via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond June 30, 2016, you must submit a new protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

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

CNC/aem

cc: Animal Welfare Veterinarian

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UNIVERSITY OF ARKANSAS

Office of Research Compliar

MEMORANDUM

To: Sam Dridi

From: Craig Coon, IACUC Chair

Date: July 08, 2016

Subject: IACUC Approval

Expiration Date: July 7, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 3 16084 "Regulation of energy homeostasis and fat metabolism in avian species."

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond July 7, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

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

CNC/aem
cc: Animal Welfare Veterinarian

Button

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3. IACUC approval for feed restriction in chicken study

UNIVERSITY OF ARKANSAS

Office of Research Complian

MEMORANDUM

TO: Nicholas Anthony

FROM: Craig N. Coon, Chairman

DATE: Apr 3, 2015

SUBJECT: IACUC Approval

Expiration Date: Apr 5, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Protocol: 15039 "General Rearing of Selected Chicken and Quail Populations" to begin April 6, 2015

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Apr 5, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

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

CNC/aem

cc: Animal Welfare Veterinarian

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