5-2019

Hormone Signaling, Gene Expression, and Mitochondrial Hormone Receptor Expression in Avian Muscle (Cells)

Kentu Rushadd Lassiter
University of Arkansas, Fayetteville

Hormone Signaling, Gene Expression, and Mitochondrial Hormone Receptor Expression in Avian Muscle (Cells)

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

by

Kentu Lassiter
University of Arkansas
Bachelor of Science in Biology, 2003
University of Arkansas
Master of Science in Cell and Molecular Biology, 2005

May 2019
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Walter Bottje, Ph.D.
Dissertation Director

_____
Sami Dridi, Ph.D.
Committee Member

_____
Billy Hargis, Ph.D.
Committee Member

_____
Byung-Whi Kong, Ph.D.
Committee Member

_____
Tyrone Washington, Ph.D.
Committee Member

ABSTRACT

Mitochondria are vital to the proper growth and function of muscle cells since they're responsible for the majority of ATP production used for cellular energy. Previous studies have investigated how differences in mitochondrial function affects feed efficiency (FE) in broilers phenotyped for High and Low FE. Low FE broilers have been shown to have increased levels of reactive oxygen species (ROS), thus contributing to higher levels of oxidative stress and damage seen in these birds. Global gene and protein expression studies conducted on breast muscle of the High FE and Low FE phenotypes have suggested that differences in mitochondrial function and hormone signaling play a role in feed efficiency. In mammalian muscle cells, hormones such as the neuropeptide orexin are known to affect mitochondrial function. Therefore, the focus in this study was to determine whether hormones can affect mitochondrial dynamics in avian muscle cells, compare the expression of genes involved in muscle growth and insulin signaling in the High FE and Low FE phenotypes, and determine whether hormone receptors are present in the mitochondria of avian muscle cells. The actions of hormones and their receptors play an important role in the regulation of growth and metabolism. Investigation of orexin expression in avian muscle cells revealed that the hormone and its receptor are expressed in muscle. Orexin was also shown to be secreted by muscle cells and caused differential expression of a number of mitochondrial-related genes. Based on predictions generated by the results obtained from global expression studies, qRT-PCR analysis revealed several differentially expressed genes between the High and Low FE phenotype that are associated with muscle growth/development and the insulin signaling pathway. Lastly, due to the lack of scientific literature concerning the expression of hormone receptors in the mitochondria of avian muscle cells, studies were conducted that do indicate the presence of receptors in muscle mitochondria.

ACKNOWLEDGEMENTS

I would first like to thank my advisor Dr. Walter Bottje for his encouragement, patience, and friendship as I progressed through my graduate education. He has helped me to grow not only as a person but also as a researcher and professional. His support and mildly funny jokes have been and will always be invaluable to me. Additionally I would also like to thank my committee members: Dr. Billy Hargis and Dr. Sami Dridi for their words of advice and encouragement which has helped me more than they may know; Dr. Byungwhi Caleb Kong whose wealth of knowledge in molecular techniques and molecular pathways has been an important part of ensuring that my research remained on the right track; and Dr. Tyrone Washington who has served as a source of personal inspiration and motivation.

I would like to thank the faculty, staff, and graduate students I've had the chance to be acquainted with here in the Center of Excellence for Poultry Science through the years. There are so many people I could name here its unbelievable. This department has such a family-type atmosphere that I think is hard to find in the workplace these days, and I'm so appreciative of that. I mustn't forget to acknowledge the Arkansas Biosciences Institute and USDA-NIFA whose funding support made my research possible. Thank you all so much.

Most importantly I would like to thank my family. My mother (Helen), father (Leland), and sister (Yulanda) have sacrificed so much for me to be where I am today. Their love and support has been vital to anything that I've ever accomplished in my life, and the completion of this dissertation is no different. Please know that I love you all and I can never say thank you enough.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PUBLISHED PAPERS

All of Chapter 3 comes from the published paper:

Lassiter, K., E. Greene, A. Piekarski, O.B. Faulkner, B.M. Hargis, W. Bottje, and S. Dridi. 2015. Orexin system is expressed in avian muscle cells and regulates mitochondrial dynamics. Am. J. Physiol. Regul. Integr. Comp. Physiol. 308: R173-R187.

All of Chapter 5 comes from the published paper:

Lassiter, K., S. Dridi, E. Greene, B. C. Kong, and W. Bottje. 2018. Identification of mitochondrial hormone receptors in avian muscle cells. Poult. Sci. 97: 2926-2933.

1. INTRODUCTION

Mitochondria are essential to cellular growth, development, and function. These organelles account for ~90% of the ATP that is produced and used as energy to carry out numerous cellular actions (Lehninger et al., 1993). In addition to its role in energy production, mitochondria also function in regulating cell metabolism, antioxidant protection, heat production, and apoptosis among a number of other cellular processes. Therefore, the mitochondria are vital to the overall health and well-being of eukaryotic organisms. Mitochondrial dysfunction is typically caused by mutations in mitochondrial proteins that are encoded by either nuclear DNA or mitochondrial DNA (mtDNA), oxidative damage as a result of the production of reactive oxygen species, and the natural aging process. This can result in impaired growth and development, and a number of human diseases such as Parkinson's disease, diabetes, and Alzheimer's disease (Pieczenik and Neustadt, 2007).

One research area of interest is the stimulation and enhancement of mitochondrial function due to the effects of hormones and their respective receptors on the organelle. When hormones bind to their receptors the complex serves as a transcription factor that has the ability to bind to DNA and activate or repress specific genes and signaling pathways. The activation of genes by hormones and their receptors typically occurs through binding to hormone response elements (HREs), which are DNA sequences located in the promoter region of the gene (Wu et al., 2001; Scheller and Sekeris, 2003). The positive effects of steroid and thyroid hormones on mammalian mitochondrial function have been well documented, as evidenced by increases in mitochondrial respiration, gene expression, and biogenesis (Weber et al., 2002; Bassett et al., 2003; Dai et al., 2013; Liao et al., 2015) One particular hormone of interest is the neuropeptide orexin, which has been shown to regulate a variety of physiological processes in mammals,

including energy and glucose homeostasis, lipid metabolism, heart rate and blood pressure, and food and water intake (Sakurai, 1999; Zhang et al., 2005; Tsuneki et al., 2012; Shen et al., 2013). In recent studies the hormone orexin has also been shown to regulate mitochondrial biogenesis in addition to brown adipose tissue differentiation and thermogenesis in mammals (Sellayah et al., 2011; Swami, 2011).

Hormonal effects on muscle growth, development, and enhanced mitochondrial function have been well-illustrated in mammals. However, comparatively little is known about the inherent distribution and function of hormones and their respective receptors in the tissues of avian species, and how they affect mitochondrial dynamics. It is possible that a clearer understanding of the interactions between hormone signaling and mitochondrial function in birds reared specifically for meat production (i.e. broilers) could be beneficial currently and in the future. This type of information could be valuable to the poultry industry, where cost effective meat production is a cornerstone of maintaining the industry's viability.

One persistent issue facing the poultry industry is how to raise broilers to market weight for meat production in an efficient manner that does not incur excessive costs. The cost of feed that is required to raise birds to market weight generally accounts for 70% of the total costs (Willems et al., 2013). As a result, feed efficiency (FE), which is the amount of body weight gained over the amount of feed consumed (gain:feed), is very important when considering selection of animals with the desired production traits. Over the past several decades, vast improvements to FE and growth rate of birds within the poultry industry have been made due to improvements in genetic selection of animals and nutrition management, with as much as a 300% increase in FE being observed (Havenstein et al., 1994; 2007). However, despite these

improvments in poultry production, there are still issues with variation both within and between strains of broilers.

Prior research investigating broilers within the same genetic line that were phenotyped as having high or low FE showed that mitochondrial perturbations were a characteristic of the low FE phenotype. In studies conducted using isolated breast muscle and mitochondria isolated from duodenal tissue, low FE broilers displayed impaired mitochondrial function that occurred in the form of decreased respiratory chain coupling, increased electron leak from the electron transport chain, and increased hydrogen peroxide production (Bottje et al., 2002; Ojano-Dirain et al., 2004). This formation of hydrogen peroxide by the mitochondria leads to the generation of reactive oxygen species (ROS) such as superoxide and hydroxyl radicals which cause damage to DNA, lipids and proteins. Subsequent analysis of various tissues from high and low FE broilers (breast muscle, gut, leg muscle, heart, liver, and lymphocytes) indicated that the low FE broilers had elevated levels of protein carbonyls, which are an indicator of protein oxidation and damage (Iqbal et al., 2004, 2005; Ojano-Dirain et al., 2005, 2007; Lassiter et al., 2006; Tinsley et al., 2010). Based on these reports, the conclusion can be drawn that the low FE phenotype is more susceptible to the production of damaging oxygen radicals that occur as a result of defects in the mitochondrial electron transport chain.

Even though high levels of ROS are very damaging to cellular components, at low levels these molecules are an important component of signal transduction and the control of cell growth and proliferation through control of physiological processes such as mitochondrial biogenesis, growth factor signaling, and insulin sensitivity (Finkel, 2011). As a result, changes in the levels of ROS can have an impact on the expression of genes and proteins. Based on this observation, breast muscle from the high and low FE broilers were subjected to analysis of global gene (Kong

et al., 2011; Bottje et al., 2012) and protein expression (Kong et al., 2016) in order to develop a more comprehensive understanding of how FE is chararcterized at the cellular level. Interpretation of the data generated from these global expression studies was performed using the online software program Ingenuity Pathway Analysis (IPA; http://apps.ingenuity.com) as a way to generate predictions that illustrate the genomic and proteomic networks that are associated with high and low FE. In the microarray analysis of gene expression, multiple aspects of hormone signaling were determined to be involved in FE. A number of genes that take part in the insulin signaling pathway (i.e. AMPK, PI3K, PDK1, S6K, mTORC1) were predicted to be upregulated in the high FE phenotype (Bottje et al., 2014). Subsequent analysis of the same genomic dataset also suggests that progesterone signaling within avian mitochondria is associated with the divergence of the high and low FE broiler phenotype (Bottje et al., 2017). Shotgun proteomic analysis of high and low FE breast muscle also points to hormone signaling as being involved in the determination of FE. In addition to enhanced mitochondrial expression in the high FE broilers, Kong et al. (2016) also identified several molecules involved in hormone signaling, such as insulin receptor, insulin like growth receptor 1, progesterone, and triiodothyronine (T_3) as being activated in high FE broilers. The predictions based on these global expression datasets indicate that enhanced mitochondrial function and hormone signaling are characteristics of the high FE phenotype.

Based on the results obtained from the global expression data, it would be interesting to see whether hormone signaling has a direct action on the expression and function of mitochondria in chickens. This has been studied in mammals, where receptors for steroid and thyroid hormones have been identified in the mitochondria (Demonakos et al., 1993; Dai et al., 2013; Wrutniak-Cabello et al., 2001). Additionally, HREs for mammalian steroid and thyroid

hormones have also been identified within the mtDNA (Demonakos et al., 1995; Chen et al., 2005; Psarra et al., 2006). The presence of hormone receptors and HREs in the mitochondrial genome enhances gene and protein expression, thus enhancing energy production and mitochondrial function. However, searches of the current literature do not report the presence of hormone receptors in avian species. If these receptors are present in avian mitochondria, it would give further insight into how function of the organelle is controlled and whether it is related to genetic traits such as FE.

1.2 OBJECTIVES

The objectives of my research in this dissertation were to investigate if the presence of hormones and their respective receptors in avian muscle cells are involved in mitochondrial physiology, determine whether differences in FE can be partially attributed to differences in the expression of components of hormone signaling pathways, and to determine whether hormone receptors are present within the mitochondria of avian muscle cells.

Specific objectives for this dissertation are as follows:

1. To investigate whether the orexin system is expressed in avian muscle and its potential role in the regulation of muscle mitochondrial dynamics, biogenesis, and function.
2. To conduct targeted analysis of genes involved in muscle development, protein synthesis, and energy metabolism; particularly genes in part of the myostatin and insulin signaling pathways that are differentially expressed in breast muscle obtained from broilers exhibiting a high or low FE phenotype.
3. To determine whether the mitochondrial hormone receptors for progesterone, estrogen, glucocorticoid, thyroid, and insulin are present in both an avian muscle cell line and intact avian muscle cells.

1.3 REFERENCES

1. Bassett, J.H., C.B. Harvey, and G.R. Williams. 2003. Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol. Cell. Endocrinol. 213: 1-11.

2. Bottje, W., Z.X. Tang, M. Iqbal, D. Cawthon, R. Okimoto, T. Wing, and M. Cooper. 2002. Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult. Sci. 81: 546-555.

3. Bottje, W.G., B.-W. Kong, J.J. Song, J.Y. Lee, B.M. Hargis, K. Lassiter, T. Wing, and J. Hardiman. 2012. Gene expression in breast muscle associated with feed efficiency in a single male broiler line using a chicken 44K oligo microarray. II. Differentially expressed focus genes. Poult. Sci. 91: 2576-2587.

4. Bottje, W.G., B.-W. Kong, J.Y. Lee, T. Washington, J.I. Baum, S. Dridi, T. Wing, and J. Hardiman. 2014. Potential roles of mTOR and protein degradation pathways in the phenotypic expression of feed efficiency in broilers. Biochem. Physiol. 3: 125. doi:10.4172/2168-9652.1000125.

5. Bottje, W., B.-W. Kong, A. Reverter, A.J. Waardenberg, K. Lassiter, and N.J. Hudson. 2017. Progesterone signalling in broiler skeletal muscle is associated with divergent feed efficiency. BMC Syst. Biol. 11: 29. http://doi.org/10.1186/s12918-017-0396-2.

6. Chen, J.Q., J.D. Yager, and J. Russo. 2005. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. Biochim. Biophys. Acta. 1746: 1-17.

7. Dai, Q., A.A. Shah, R.V. Garde, B.A. Yonish, L. Zhang, N.A. Medvitz, S.E. Miller, E.L. Hansen, C.N. Dunn, and T.M. Price. 2013. A truncated progesterone receptor (PR-M) localizes to the mitochondrion and controls cellular respiration. Mol. Endo. 27:741-753.

8. Demonakos, C., N.C. Tsawdaroglou, R. Djordjevic-Markovic, M. Papalopoulou, V. Galanopolous, S. Papadogeorkagi, and C.E. Sekeris. 1993. Import of the glucocorticoid receptor into rat liver mitochondria *in vivo* and *in vitro*. J. Steroid. Biochem. Mol. Biol. 46: 401-413.

9. Demonakos, C., R. Djordjevic-Markovic, N. Tsawdaroglou, and C.E. Sekeris. 1995. The mitochondrion as a primary site of action of glucocorticoids: the interaction of the glucocorticoid receptor with mitochondrial DNA sequences showing partial similarity to the nuclear glucocorticoid responsive elements. J. Steroid Biochem. Mol. Biol. 55: 43-55.

10. Finkel, T. 2011. Signal transduction by reactive oxygen species. J. Cell Biol. 194: 7-15.
Havenstein, G.B., P.R. Ferket, S.E. Scheideler, and B.T. Larson. 1994. Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed “typical” 1957 and 1991 broiler diets. Poult. Sci. 73: 1785-1794.

11. Havenstein, G.B., P.R. Ferket, J.L. Grimes, M.A. Qureshi, and K.E. Nestor. 2007. Comparison of 1966- versus 2003-type turkeys when fed representative 1966 and 2003 turkey diets: growth rate, livability, and feed conversion. Poult. Sci. 86: 232-240.

12. Iqbal, M., N. Pumford, K. Lassiter, Z. Tang, T. Wing, M. Cooper, and W. Bottje. 2004. Low feed efficient broilers within a single genetic line exhibit higher oxidative stress and protein expression in breast muscle with lower mitochondrial complex activity. Poult. Sci. 83: 474-484.

13. Iqbal, M., N. Pumford, Z.X. Tang, K. Lassiter, C. Ojano-Dirain, T. Wing, M. Cooper, and W.G. Bottje. 2005. Compromised liver mitochondrial function and complex activity in low feed efficient broilers within a single genetic line associated with higher oxidative stress and differential protein expression. Poult. Sci. 84: 933-941.

14. Kong, B.-W., J.J. Song, J.Y. Lee, B.M. Hargis, T. Wing, K. Lassiter, and W. Bottje. 2011. Gene expression in breast muscle associated with feed efficiency in a single male broiler line using a chicken 44K oligo microarray. I. Top differentially expressed genes. Poult. Sci. 90: 2535-2547.

15. Kong, B.-W., K. Lassiter, A. Piekarski-Welsher, S. Dridi, A. Reverter, N.J. Hudson, and W.G. Bottje. 2016. Proteomics of Breast Muscle Tissue Associated with the Phenotypic Expression of Feed Efficiency within a Pedigree Male Broiler Line: I. Highlight on Mitochondria. PLoS ONE 11(7): e0159897. https://doi.org/10.1371/journal.pone.0159897.

16. Lassiter, K., C. Ojano-Dirain, M. Iqbal, N.R. Pumford, N. Tinsley, T.Wing, J. Lay, R. Liyanage, M. Cooper, and W. Bottje. 2006. Differential expression of mitochondrial and exta-mitochondrial proteins in lymphocytes of low and high feed efficient male broilers. Poult. Sci. 85: 2251-2259.

17. Lehninger, A.L., D.L. Nelson, and M.M. Cox. 1993. Principles of Biochemistry. 2nd ed. Worth Publishers, New York, NY.

18. Liao, T.L., C.R. Tzeng, C.L. Yu, Y.P. Wang, and S.H. Kao. 2015. Estrogen receptor-β in mitochondria: implications for mitochondrial bioenergetics and tumorigenesis. Ann. N.Y. Acad. Sci. 1350: 52-60.

19. Ojano-Dirain, C., M. Iqbal, D. Cawthon, S. Swonger, T. Wing, M. Cooper, and W. Bottje. 2004. Determination of mitochondrial function and site-specific defects in electron transport in duodenal mitochondria in broilers with low and high feed efficiency. Poult. Sci. 83: 1394-1403.

20. Ojano-Dirain, C., N.R. Pumford, M. Iqbal, T. Wing, M. Cooper, and W.G. Bottje. 2005. Biochemical evaluation of mitochondrial respiratory chain in duodenum of low and high feed efficient broilers. Poult. Sci. 84: 1926-1934.

21. Ojano-Dirain, C., M. Toyomizu, T. Wing, M. Cooper, and W.G. Bottje. 2007. Gene expression in breast muscle and duodenum from low and high feed efficient broilers. Poult. Sci. 86:372-381.

22. Pieczenik, S.R. and J. Neustadt. 2007. Mitochondrial dysfunction and molecular pathways of disease. Exp. Mol. Pathol. 83: 84–92.
Psarra, A.M., S. Solakidi, and C.E. Sekeris. 2006. The mitochondrion as a primary site of action of steroid and thyroid hormones: presence and action of steroid and thyroid hormone receptors in mitochondria of animal cells. Mol. Cell. Endocrinol. 246: 21-33.

23. Sakurai, T. 1999. Orexins and orexin receptors: implication in feeding behavior. Regul. Pept. 85: 25-30.

24. Scheller, K. and C.E. Sekeris. 2003. The effects of steroid hormones on the transcription of genes encoding enzymes of oxidative phosphorylation. Exp. Physiol. 88: 129-140.

25. Sellayah, D., P. Bharaj, and D. Sikder. 2011. Orexin is required for brown adipose tissue development, differentiation, and function. Cell Metab. 14: 478-490.

26. Shen, Y., Y. Zhao, D. Zheng, X. Chang, S. Ju, and L. Guo. 2013. Effects of orexin A on GLUT4 expression and lipid content via MAPK signaling in 3T3-L1 adipocytes. J. Steroid Biochem. Mol. Biol. 138: 376-383.

27. Swami, M. 2011. Metabolism: orexin acts on brown fat. Nat. Med. 17: 1356-1356.

28. Tinsley, N., M. Iqbal, N.R. Pumford, K. Lassiter, C. Ojano-Dirain, T. Wing, and W. Bottje. 2010. Investigation of mitochondrial protein expression and oxidation in heart muscle in low and high feed efficient male broilers in a single genetic line. Poult. Sci. 89: 349-352.

29. Tsuneki, H., T. Wada, and T. Sasaoka. 2012. Role of orexin in the central regulation of glucose and energy homeostasis. Endocr. J. 59: 365-374.

30. Weber, K., P. Brück, Z. Mikes, J. H. Küpper, M. Klingenspor, and R. J. Wiesner. 2002. Glucocorticoid hormone stimulates mitochondrial biogenesis specifically in skeletal muscle. Endocrinology, 143: 177-184.

31. Willems, O.W., S.P. Miller, and B.J. Wood. 2013. Aspects of selection for feed efficiency in meat producing poultry. Worlds Poult. Sci. J. 69: 77-88.

32. Wrutniak-Cabello, C., F. Casas, and G. Cabello. 2001. Thyroid hormone action in mitochondria. J. Mol. Endocrinol. 26: 67-77.

33. Wu, Y., B. Xu, and R.J. Koenig. 2001. Thyroid hormone response element sequence and the recruitment of retinoid X receptors for thyroid hormone responsiveness. J. Biol. Chem. 276: 3929-3936.

34. Zhang, W., Y. Fukuda, and T. Kuwaki. 2005. Respiratory and cardiovascular actions of orexin-A in mice. Neurosci. Lett. 385: 131-136.

2. REVIEW OF THE LITERATURE

2.1 OREXIN

Orexin, which regulates wakefulness, energy homeostasis, and appetite/feeding behavior based on nutritional status, is a neuropeptide hormone that was originally discovered in the hypothalamus of rats by investigating orphan G protein-coupled receptors (de Lecea et al., 1998; Sakurai et al., 1998). Orphan receptors are those whose ligand and physiological actions are unknown (Stadel et al., 1997). The term orexin originates from the Greek word "orexis", meaning appetite. There are two known orexin peptides (ORX-A and ORX-B), both of which are formed by proteolytic cleavage of the precursor prepro-orexin (Sakurai et al., 1998). The synonymous terms "hypocretin 1 and 2" were coined by de Lecea et al. (1998), where *hypo* refers to the peptide's origin in the hypothalamus and *cretin* refers to the similarity of the peptide's amino acid sequence with the gut hormone secretin. When initially discovered in rats, the precursor peptide prepro-orexin was shown to be a 130-residue polypeptide from which the mature peptides ORX-A and ORX-B were formed, with ORX-A containing 33 amino acids and a molecular weight 3.562 kDa and ORX-B containing 28 amino acids and a molecular weight of 2.937 kDa (Sakurai et al., 1998). When comparing the two peptides, ORX-B was shown to be forty-six percent identical in amino acid sequence to ORX-A. However, when comparing mammalian species (human, rat, mouse, pig, and cow), the sequence and structure of both peptides is highly conserved (Sakurai, 1998). A number of studies have also shown that the structures of ORX-A and ORX-B in chicken and certain types of fish are conserved when compared to their mammalian counterparts (Shibahara et al., 1999; Alvarez and Sutcliffe, 2002; Sakurai, 2005; Tsujino and Sakurai, 2009).

2.1.1 OREXIN RECEPTORS

ORX-A and ORX-B signal through the G protein-coupled receptors orexin receptor 1 (ORXR1) and orexin receptor 2 (ORXR2). These two ubiquitously expressed receptors were first identified in human brain tissue through expressed sequence tags combined with database searching using tBLASTn (Soppet et al., 1996; Sakurai et al., 1998). In humans it has been shown that the amino acid sequence for ORXR1 and ORXR2 is more than sixty percent identical, making them more similar to each other than to other G protein-coupled receptors (Sakurai et al., 1998). The same study also showed that both receptors are highly conserved between humans and rats, with the sequence identity being greater than ninety percent for both. The two orexin peptides have different binding affinities for the two orexin receptors. ORX-A is able to bind to both receptors but has a higher affinity for ORXR1, while ORX-B binds to ORXR2 with the same affinity as ORX-A (Tsujino and Sakurai, 2009). Several studies have indicated that the binding of orexins to orexin receptors activates multiple G proteins. In studies conducted using humans (Karteris et al., 2001; Randeva et al., 2001) and rats (Karteris et al., 2005), it was shown that the binding of ORXR2 activates G_i, G_s, $G_{o,}$ and G_q proteins in adrenal cortical tissue. It appears that the responses to orexin receptor signaling are highly diverse. The activation of the various G proteins can lead to a variety of cellular responses such as the regulation of protein/lipid kinases (Hepler and Gilman, 1992; Gautam et al., 1998). In the case of orexin stimulation activation of G proteins can lead to the excitation of neurons that affect the regulation of ion channels, the activation of signaling cascades that regulate the activity of adenylyl cyclase, phospholipases, and activation of cell death pathways (reviewed by Kukkonen, 2013; Kukkonen and Leonard, 2014).

2.1.2 OREXIN EXPRESSION IN MAMMALIAN PERIPHERAL TISSUES

As stated previously, orexin was initially discovered to be produced by neurons in the hypothalamus of rats. Additional studies have shown that the axons of these orexin-producing neurons are extensively distributed throughout the central nervous system (Peyron et al., 1998; Nambu et al., 1999; van den Pol, 1999), along with expression of both orexin receptors (Trivedi et al., 1998; Marcus et al., 2001). This indicates that the expression of orexin and its receptors is vital to the proper function of the central nervous system.

In addition to expression in the central nervous system, subsequent studies have pointed to the expression of orexins and orexin receptors in various mammalian peripheral tissues. Kirchgessner and Liu (1999) first reported the presence of ORX-A in human intestinal mucosa and pancreas. The presence of ORX-A in human plasma is another indicator of widespread orexin activity outside of the central nervous system (Arihara et al., 2001; Dalal et al., 2001). The distribution of ORX-A in human peripheral tissues was extensively studied by Nakabayashi et al. (2003) who showed that immunoreactivity for the peptide was present in several tissues that include ganglion cells of the thoracic sympathetic trunk, endocrine cells of the gastrointestinal tract, islet cells of the pancreas, and syncytiotrophoblasts and decidual cells of the placenta. Additionally, the expression of both orexin receptors (ORXR1 and ORXR2) has been identified in adipose tissue (Digby et al., 2006) as well as different areas of the male reproductive tract (Karteris et al., 2004) of humans.

Orexin and orexin receptors have also been detected in a number of peripheral rat and mouse tissues. Prepro-orexin mRNA was detected in rat testis (Sakurai et al., 1998) and duodenum (Naslund et al., 2002). Johren et al. (2001) investigated the presence of prepro-orexin and orexin receptor mRNA in peripheral rat tissues and was able to detect prepro-orexin mRNA in the testis,

low levels of ORXR1 mRNA in the kidney, adrenal, thyroid, testis, ovaries and jejunum, plus low levels of ORXR2 mRNA in the lung and pituitary. In addition, ORXR1 and ORXR2 mRNA have been detected in mouse adipose tissue (Skrzypski et al., 2011), and orexin produced in the placenta of mice is believed to be an important contributor to the prenatal development of brown adipose tissue (Sellayah et al., 2011). The presence of orexin and orexin receptors in mammalian peripheral tissues suggests that the system plays a part in other physiological roles in addition to the regulation of feeding behavior and wakefulness (Peyron et al., 1998).

2.1.3 EFFECTS OF OREXIN IN MAMMALS

Orexin has been shown to regulate a variety of processes in mammals. After the initial discovery of the peptide, one of the first observations made was that intracerebroventricular administration of ORX-A in rats causes an increase in food (Sakurai et al., 1998; Sakurai, 1999) and water (Kunii et al., 1999) consumption. Subsequent research by Edwards et al. (1999) indicated that ORX-A is a more potent stimulator of food intake than ORX-B in rats. In addition, the administration of an orexin receptor antagonist in rats and mice causes a decrease in food consumption, providing further evidence of orexin's stimulatory effect on food intake (Haynes et al., 2000, 2002). One of the other central effects of orexin is the control it exerts on sleep and wakefulness (Chemelli et al., 1999; Lin et al., 1999; Mignot and Thorsby, 2001). Studies have shown that the orexin-producing neurons in the hypothalamus are activated during the awake period (Tsujino and Sakurai, 2009). These neurons increase their activity/discharging during active waking and stop firing during periods of sleep (Lee et al., 2005; Mileykovskiy et al., 2005; Takahashi et al., 2008). In addition, orexin-producing neurons project to the majority of brain regions that are involved in regulating wakefulness (see review Alexandre et al., 2013). Specifically, cholinergic neurons located in the basal forebrain, whose involvement is also

important in regulating sleep and arousal, are directly excited by orexins. Evidence has shown that the central injection of ORX-A induces excitation of cholinergic neurons, which in turn promotes wakefulness (Eggermann et al., 2001; Xi et al., 2001 Takahashi et al., 2002;). Since orexin signaling is involved in controlling feeding behavior and sleep/wake cycles, it is inherently involved in control of circadian rhythms in mammals (Mieda et al., 2004; Kantor et al., 2009). Neurons located in the suprachiasmatic nucleus (SCN) of the brain serve as the control center of the circadian clock. It appears that input from neurons in the SCN to orexin neurons exerts regulation over the control of arousal and appetitive states in circadian rhythms (Inutsuka and Yamanaka, 2013; Belle et al., 2014).

Another central effect of orexin in mammals is the ability to regulate glucose homeostasis and energy balance when hypothalamic neurons sense changes in the circulating levels of glucose (Tsuneki et al., 2010; 2012). There are several lines of evidence to support the relationship between orexin expression and glucose levels. Energy balance in animals has a profound input on the orexin system. For example, studies have shown that the extracellular presence of glucose and leptin inhibits the excitability of orexin neurons; whereas the presence of ghrelin along with decreased glucose and energy levels causes stimulation of orexin neurons (Yamanaka et al., 2003; Burdakov et al., 2005). Burdakov et al. (2006) provides further evidence of this by showing that the physiological changes in glucose levels that occur between meals is reflected in the variations of the firing rate of orexin neurons. Also, the increase in mRNA levels of prepro-orexin seen under hypoglycemic conditions supports the idea that orexin is glucose-responsive (Griffond et al., 1999). The effect of orexin expression in the brain on glucose homeostasis and energy balance can be seen in mammals affected with the orexin-deficient condition narcolepsy. Humans identified with the condition are known to have decreased energy

intake, yet there is an increase in body mass index (BMI), leading to an increase in the occurrence of type 2 diabetes (Honda et al., 1986; Schuld et al., 2000). Similarly, transgenic mice that were deficient in orexin neurons displayed late-onset obesity even though they consumed less food than their non-transgenic counterparts (Hara et al., 2001).

It has been discovered that orexins are also involved in other physiological processes. A number of studies have shown that the orexin system is involved in lipid metabolism. Kukkonen (2014) reports that one of the significant ways of signaling for orexin receptors is through lipid cascades such as phospholipase C and phospholipase D pathways. In other studies, administration of ORX-A, but not ORX-B has the greatest effect on lipid metabolism. ORX-A has been shown to decrease levels of lipid peroxidation and apoptosis in rat hypothalamic cells (Butterick et al., 2012). ORX-A administration also inhibits lipolysis and stimulates lipogenesis in the adipocytes of rats (Skrzypski et al., 2011; Shen et al., 2013) and pigs (Pruszynska-Oszmalek et al., 2018). Orexins have also been implicated in exerting some control over cardiovascular regulation of heart rate and blood pressure in mammals. Central injection of orexin in rats causes an increase in sympathetic nerve activity (Shirasaka et al., 1999; Ciriello et al., 2003) as well as an increase in heart rate and mean arterial blood pressure (Samson et al., 1999; Zhang et al., 2005). In addition to changes in sympathetic nerve activity, orexin is also involved in the neuroendocrine stress response and the secretion of stress hormones such as adrenocorticotropic hormone (ACTH) and corticosterone (Samson et al., 2002, 2007). In mice it has been shown that the binding and activation of ORXR2 is more prominent in inducing a stress response as opposed to activation of ORXR1 (Yun et al., 2017). Under stress-inducing conditions, mobilization of the orexin system causes a stress response that includes associated anxiety behavior as well as other endocrine and cardiorespiratory responses (see review Johnson

et al., 2012). As a consequence, abnormal function of the orexin system is a factor in psychiatric and behavioral disorders. Hyperactivity of the orexin system contributes to anxiety and panic disorders, whereas hypoactivity of the system is associated with decreased motivational behavior and depression (James et al., 2017).

2.1.4 EFFECTS OF OREXIN IN MAMMALIAN MITOCHONDRIA

In mammals, orexin has been shown to induce differentiation of brown adipose tissue (BAT), subsequently leading to thermogenesis (Sellayah et al., 2011; Swami, 2011). One of the effects orexin has in this process is the regulation of genes involved in mitochondrial biogenesis. The study by Sellayah et al. (2011) revealed several changes in the mitochondrial dynamics of mouse preadipocytes treated with ORX-A. The expression of a number of genes involved in mitochondrial biogenesis (i.e. PGC-1α, PGC-1β, PPARγ1, and UCP1) were up-regulated following treatment. These findings were further supported when subsequent immunofluorescence staining revealed an increase in mitochondrial abundance of the treated cells. Studies using other cell types treated with ORX-A have also shown effects on mitochondrial function. Human neuroblastoma cells treated with ORX-A had increased mitochondrial membrane potential (Pasban-Aliabadi, et al., 2017). Additionally, in studies using human hepatoma cells (Wan et al., 2017) and human embryonic kidney cells (Sikder and Kodadek, 2007), treatment with ORX-A resulted in increased ATP production that shifted from glycolysis in the cytoplasm to oxidative phosphorylation in the mitochondria. Taken all together, these studies indicate that orexin is able to enhance mitochondrial function, biogenesis, and ATP production.

2.1.5 OREXIN SYSTEM IN AVIAN SPECIES

Significantly fewer studies concerning the orexin system have been conducted in avian species when compared to mammals. Chicken prepro-orexin was first cloned, sequenced, and characterized by Ohkubo et al (2002). In that study, chicken orexin cDNA was shown to be expressed in the periventricular and lateral hypothalamic areas and consisting of 658 bp that encode 148 amino acids. Also, chicken ORX-A and ORX-B are evolutionary conserved with their mammalian counterparts, showing approximately 85% and 65% similarity at the amino acid level (Ohkubo et al., 2002). Characterization of the chicken orexin receptor shows that its cDNA has a length of 1869 bp that encode 501 amino acids, which corresponds to mammalian ORXR2 with an 80% homology (Ohkubo et al., 2003). Studies looking at tissue distribution of orexin and orexin receptors in chickens show that the peptides are expressed in the brain (Ohkubo et al., 2002; Ohkubo et al., 2003; Miranda et al., 2013; Godden et al., 2014), pituitary gland, adrenal gland, testis and ovary (Ohkubo et al., 2003), and the stomach and intestine (Arcamone et al., 2014).

Orexin does not appear to elicit the same responses in birds as it does in mammals. One of the most noted actions that centrally administered orexin has in mammals is that it stimulates feeding/food intake (Sakurai et al., 1998; Edwards et al., 1999; Sakurai, 1999; reviewed by Tsujino and Sakurai, 2009). However, central administration of ORX-A or ORX-B did not stimulate feed intake in neonatal broiler and layer chicks (Furuse et al., 1999; Katayama et al., 2010) or adult pigeons (da Silva et al., 2008). Studies examining mRNA expression of prepro-orexin in the hypothalamus of chicken (Ohkubo et al., 2002) and quail (Phillips-Singh et al., 2003) following 24h fasting showed no increase in expression, providing further evidence for the lack of a stimulatory effect on feeding behavior in birds. The study conducted by Song et al.

(2012) did show an increase in prepro-orexin mRNA, but this was measured after 48h fasting, which would be an extreme fasting condition for broiler chickens.

As stated previously, another hallmark of orexin function in mammals is its effects on the regulation of sleep/wakefulness, where a dysfunction in the orexin system is associated with the sleep condition narcolepsy (Chemelli et al., 1999). Studies investigating the effects of orexin on arousal in birds have been conducted with mixed results. It has been concluded that either hypothalamic orexin does not play a role in arousal of the sleep/wake cycle (Miranda et al., 2013), or that only ORX-A in conjunction with the enzyme monoamine oxidase-A (MAO-A) increases arousal in layer chicks only and not broiler chicks (Katayama et al., 2010; Katayama et al., 2011). Multiple studies investigating orexin in avian species theorize that the peptide appears to be more involved in the regulation of energy balance than feed intake and sleep/wake cycles (Miranda et al., 2013; Song et al., 2013; Godden et al., 2014).

2.2 FEED EFFICIENCY IN POULTRY PRODUCTION

Feed efficiency (FE) is one of the most important traits in domestic poultry when selecting animals for commercial breeding programs and consumer use. FE is defined as the ratio of body weight gained to the amount of feed consumed (gain:feed). Feed conversion ratio (FCR) is the inverse of FE (feed:gain) and is the most commonly used term in commercial animal production. One of the primary reasons that FE is so important is that feed accounts for up to 70% of the costs needed to rear an animal to market weight (Willems et al., 2013). Studies investigating changes in FE in poultry production have shown that there has been significant improvements over the last several decades. The work conducted by Havenstein et al. (1994) is one of the hallmark studies illustrating this, where the comparison of broiler strains from 1957 and 1991 showed that the 1991 strain had a 250 to 300% increase in FE and body weight. A

subsequent study showed that 2001 commercial broilers had improved FE when compared to 1957 broilers regardless of whether they were fed either one of the diets that were representative of the two time periods (Havenstein et al., 2003). The increase in growth rates and FE seen over the years can be attributed to genetic selection by commercial breeding programs and improvements in nutrition and management of nutrition (Havenstein et al., 2007). However, despite the improvements seen in growth rate and FE, variations still remain both within and between strains of broilers (Emmerson, 1997). Therefore, additional research using modern techniques to investigate molecular interactions with genomic and proteomic expression and how it affects FE is still needed.

In addition to FE and FCR, there are also other methods used to evaluate efficiency in commercial animal production. Another common method of evaluating efficiency is a measurement termed residual feed intake (RFI). The basic definition of RFI is the difference between actual feed intake and predicted feed intake which is based on the regression of requirements for production (body weight gain) and the maintenance of body weight (Van Der Werf, 2004). The original concept of RFI was introduced by Byerly (1941) and used by Luiting (1990) in the production of poultry eggs. Since FE and FCR are ratio-based traits, it is possible that while their use selects for larger animals, it can also lead to increased feed costs. On the other hand RFI is phenotypically independent of body weight and weight gain and can be used to select for animals that have reduced feed intake yet are able to still meet production goals (Bottje and Carstens, 2009: Aggrey et al., 2010). A number of alternative methods for measuring efficiency have also been reported in recent studies (Willems et al., 2013). These include residual maintenance energy (RME_M) which measures energetic efficiency without including feed intake, residual gain (RG) that is calculated from linear regression of average daily gain on feed intake

and body weight, and residual intake and gain (RIG) which is calculated using both RFI and RG (Romero et al., 2009; Berry and Crowley, 2012).

2.2.1 MITOCHONDRIA, OXIDATIVE STRESS, AND FEED EFFICIENCY IN POULTRY

Mitochondria are the major source of cellular energy production and generate about 90% of the ATP needed to meet the cell's demands (Lehninger et al., 1993). In addition to cellular energy production, mitochondria are also involved in apoptosis, thermogenesis, and the maintenance of calcium homeostasis in the cell (Rossignol et al., 2000). ATP is produced by the electron transport chain (ETC) located on the inner mitochondrial membrane. The ETC consists of five multi-subunit protein complexes (complex I-V). Electrons from energy substrates move down the ETC from complex I to complex IV where they are transferred to oxygen (O_2), the final electron acceptor. This movement of electrons is coupled to the movement of protons into the intermembrane space. The accumulation of protons sets up a proton motive force that drives ATP synthesis as the protons are pumped through complex V into the mitochondrial matrix (Lehninger et al., 1993).

In addition to its involvement in ATP production, the ETC is a major source of reactive oxygen species (ROS) production and endogenous oxidative stress (Yu, 1994). ROS include compounds such as superoxide, hydrogen peroxide, and hydroxyl radicals. ROS are formed in the mitochondria due to electrons leaking from the ETC before they are able to reach the terminal electron acceptor (O_2). Instead of being completely reduced to water, 2 to 4% of the O_2 used by mitochondria may only be partially reduced to superoxide (Boveris and Chance, 1973; Chance et al., 1979). Complex I and complex III are considered to be the primary producers of superoxide within the ETC (Chen et al., 2003; Drose and Brandt, 2012). Superoxide is typically converted to the less reactive hydrogen peroxide by the enzyme superoxide dismutase. However,

when hydrogen peroxide is in the presence of iron (Fe^{2+}) and copper (Cu^{2+}) it can be converted into highly reactive hydroxyl radicals. The accumulation of ROS can lead to the oxidative damage of DNA, lipids, and proteins that exacerbate inefficiencies in the mitochondria, thus initiating a continuous cycle of cellular damage and ROS production.

Several studies have been conducted to investigate the relationship between mitochondrial function and feed efficiency in broilers. Bottje et al. (2002) was the first study to show that in the comparison of breast muscle isolated from broilers with low FE and high FE, the low FE broilers had impaired mitochondrial function. The impaired function was observed in the form of lower respiratory chain coupling that was possibly due to decreased activity of Complexes I and II in the ETC, greater electron leak from the ETC, and increased hydrogen peroxide production. This study was significant because the broilers came from the same genetic line and were fed the same diet. Additional studies by Iqbal et al. (2004, 2005) indicated that ETC complex activities were significantly lower in muscle and liver mitochondria of low FE broilers. In a subsequent study by the same group, mitochondria were isolated from the duodenum of broilers phenotyped for low and high FE and similar to the observations in breast muscle, the production of oxygen radicals was higher in low FE mitochondria (Ojano-Dirain et al., 2004). Since increased levels of hydrogen peroxide were consistently observed in mitochondria from low FE broilers, it would be logical to hypothesize that oxidation of proteins would also be higher. One method to determine the presence of protein oxidation is to quantify the levels of protein carbonyls, which serve as an indicator (Stadtman and Levine, 2000). In a number of studies, breast muscle mitochondria as well as homogenate from gut, leg, heart, liver, and lymphocytes of low FE broilers displayed increased amounts of protein carbonyls when compared to high FE broilers, further supporting the evidence that the mitochondria and tissue of

low FE birds are more susceptible to protein oxidation/damage (Iqbal et al., 2004, 2005; Ojano-Dirain et al., 2005, 2007; Lassiter et al., 2006; Tinsley et al., 2010).

Despite the oxidative damage that is caused by high levels of ROS, low levels of these molecules are actually important in signal transduction and control various physiological processes (e.g. growth factor signaling, mitochondrial biogenesis, insulin sensitivity) that affect cell growth and proliferation (see review Finkel, 2011). The mitochondria in particular are thought to play a role in the control of these processes through redox signaling, since they possess both a system for generating ROS and for antioxidant protection (Chandel, 2010; Finkel, 2012). As mentioned earlier, the generation of superoxide occurs primarily in complex I and complex III. It is believed that the superoxide produced by complex I is released into the mitochondrial matrix where ROS are the most damaging since they can easily react with mitochondrial DNA and other molecules easily affected by oxidative damage; whereas ROS generated via complex III is released into the intermembrane space where it is converted into hydrogen peroxide that diffuses into the cytosol where it can act as a second messenger in cellular signaling (Bleier and Rose, 2013; Bleier et al., 2015). Since ROS can function in signal transduction and secondary messaging, it is possible that the low FE phenotype broilers, characterized by increased ROS levels, may show differences in gene and protein expression of key molecules when compared to the high FE phenotype.

2.2.2 GENOMIC AND PROTEOMIC EXPRESSION ANALYSIS OF FEED EFFICIENCY IN POULTRY

Since the previously cited studies have shown that low FE broilers are more susceptible to elevated levels of ROS production and oxidative stress due to defects in mitochondrial function, and that changes in the levels of ROS can have an impact on genomic and proteomic

expression, global expression studies have also been conducted to determine which genes and proteins are affected in order to develop a comprehensive understanding of the cellular basis of FE. Analysis of global gene (Kong et al., 2011; Bottje et al., 2012; Zhou et al., 2015) and protein (Kong et al., 2016) expression was conducted on breast muscle acquired from high and low FE broilers. The online software program Ingenuity Pathway Analysis (IPA; http://apps.ingenuity.com) was used in these studies to interpret the data and generate predictions that provide a visualization of the networks of genes and proteins that are associated with FE. In the initial microarray analysis of gene expression, the findings from Kong et al. (2011) and Bottje et al. (2012) indicated that the high FE phenotype is derived from the upregulation of genes associated with growth-promoting anabolic processes, signal transduction pathways, and enhanced energy sensing and energy production; whereas genes upregulated in the low FE phenotype were associated with muscle fiber development and function, organization of cytoskeletal architecture, fatty acid oxidation, growth factors, and genes induced in response to oxidative stress. Subsequent analysis of genomic data obtained from the same group of high and low FE broilers also indicated that a number of genes involved in insulin signaling (i.e. AMPK, PI3K, PDK1, S6K, mTORC1) were predicted to be upregulated in the high FE phenotype (Bottje et al., 2014). In the same study, myostatin, which is a well-known inhibitor of muscle growth and development (McPherron et al., 1997; Kollias and McDermott, 2008) was predicted to be upregulated in the low FE phenotype. Interestingly, progesterone signaling within avian mitochondria also appears to be associated with the divergence seen in the broiler FE phenotype (Bottje et al., 2017).

Shotgun proteomic analysis conducted on the high and low FE phenotype breast muscle samples by Kong et al. (2016) revealed some interesting results. A number of mitochondrial

proteins were identified as being upregulated in the high FE phenotype, indicating that mitochondrial expression was greater in this group. Also, based on the differential expression of proteins it was predicted that molecules involved in hormone signaling (i.e. insulin receptor, insulin like growth receptor 1, progesterone, triiodothyronine) would be activated in the high FE phenotype. Subsequent analysis of the dataset revealed that a mitochondrial isoform of creatine kinase as well as proteins involved in energy production and transfer (i.e. ANT, VDAC, ETC proteins) were upregulated in the high FE phenotype (Bottje et al., 2017a). Furthermore, it was revealed that the enrichment of mitochondrial ribosomal proteins and proteins involved in ribosomal assembly were enhanced in the high FE phenotype (Bottje et al., 2017b). The collective results obtained from the proteomic analysis of breast muscle from the high and low FE phenotype suggests that the high FE phenotype is characterized by enhanced mitochondrial expression in breast muscle, an enhanced ability to maintain the energy requirements needed in skeletal muscle mitochondria, and enhanced translation of proteins.

2.3 MYOSTATIN AND INSULIN SIGNALING IN MUSCLE GROWTH AND DEVELOPMENT

Myostatin (MSTN), initially referred to as growth/differentiation factor-8 (GDF-8), is an inhibitor of muscle growth and development that was first reported by McPherron et al. (1997) where myostatin-null mice showed an increase in muscle mass due to both hyperplasia and hypertrophy of muscle fibers. Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily. TGF-β members are involved in growth and differentiation and play an important role in regulating development in embryos, and maintenance of tissue homeostasis in adult animals (McPherron and Lee, 1996). In mice, the myostatin protein was characterized as being 376 amino acids in length and containing a signal sequence for secretion, a proteolytic

processing site, and a carboxy-terminal region of conserved cysteine residues, which is characteristic of TGF-β proteins (McPherron et al., 1997). In the same article it was also reported that myostatin is highly conserved across species, including mammals as well as chickens.

Myostatin's role as a negative regulator of muscle growth has been illustrated in a number of species, including cattle, humans, sheep, and dogs, where mutations in the myostatin gene that lead to a loss of function have resulted in a significant increase of muscle mass (Kambadur et al., 1997; Schuelke et al., 2004; Clop et al., 2006; Mosher et al., 2007). It has been shown that myostatin's negative effect on muscle growth is caused by its ability to prevent the proliferation and differentiation of myoblasts into myotubes (Thomas et al., 2000). The mechanism by which this happens appears to be dependent on more than one signaling pathway in the cell. A good illustration of initial myostatin signaling is provided by Lee and Glass (2011). Myostatin first binds to either one of two activin type-II receptors (ActRIIA, ActRIIB), and subsequently one of the two type-I receptors (ALK4, ALK5) which leads to phosphorylation/activation of one or both of the transcription factors SMAD2 and SMAD3. The activation of SMAD2, SMAD3 ultimately leads to the blockage of the transcription factor MyoD, thereby inhibiting differentiation and proliferation of myoblasts (Zhu et al., 2004; Allen and Unterman, 2007). Inhibition of this signaling pathway occurs when myostatin binds to either the protein follistatin (FSTN) or the soluble form of the ActRIIB receptor (ActRIIB-Fc) instead of the true membrane-bound receptors (Lee and McPherron, 2001; Lee and Glass, 2011).

The regulation of muscle growth by myostatin is also linked to components of the insulin signaling pathway, where it is intricately involved in signaling via the protein kinase Akt (see reviews Elkina et al., 2011; Elliott et al., 2012). Akt is able to influence either protein synthesis or protein degradation through multiple routes during insulin signaling that can be affected by

the expression of myostatin. Typically, Akt promotes myoblast differentiation and myotube hypertrophy through the subsequent activation of mTOR and p70s6K. However, signaling by myostatin through Akt can activate the transcription factor Forkhead box O (FoxO), which leads to an increase in expression of the proteasome ubiquitin ligase atrogin-1 that induces protein degradation and the loss of muscle mass. The regulation of muscle growth and development through signal transduction involving myostatin and the insulin pathway is complex since it involves multiple pathways and is not yet fully understood, particularly in avian species.

2.4 MECHANISMS OF HORMONE ACTIONS IN MAMMALIAN MITOCHONDRIA

Steroid and thyroid hormones influence a number cellular processes in mammals such as growth, development, and metabolism. Once a hormone binds to its respective receptor it functions as a transcription factor whose influence on the activation or repression of certain genes is mediated by hormone response elements (HREs). HREs are short DNA sequences typically located in the promoter region of genes, where binding of the hormone/receptor complex to the HRE initiates activation of the gene (Wu et al., 2001; Scheller and Sekeris, 2003). In addition to the classical activation of nuclear-encoded genes, evidence also indicates that hormones may act on mitochondria as well by 2 mechanisms, either an indirect or a direct mechanism. In the indirect mechanism, once a hormone binds to its respective receptor, it is translocated to the nucleus where binding to HREs leads to the activation of nuclear-encoded mitochondrial transcription factors (e.g. peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α); mitochondrial transcription factor A (mtTFA)), and nuclear-encoded mitochondrial proteins of the electron transport chain (see reviews by Wrutniak-Cabello et al., 2001; Chen et al., 2005; Psarra et al., 2006). The activation of these nuclear-encoded genes

that function in the mitochondria exerts a positive effect on mitochondrial biogenesis, as well as metabolism, growth, and development.

In the direct mechanism, instead of initially signaling through the nucleus, hormones bind directly to receptors located on or within the mitochondria. Studies show that mammalian mitochondria contain receptors for progesterone (Dai et al., 2013; Feng et al., 2014), glucocorticoid (Demonakos et al., 1993; Moutsatsou et al., 2001; Du et al., 2009), thyroid (Wrutniak et al., 1995; Wrutniak-Cabello et al., 2001), and estrogen (Chen et al., 2004a,b). The hormone/receptor complex then binds to mitochondrial DNA (mtDNA), where it has been shown that mtDNA also contains HREs similar to their nuclear counterparts. HREs located within mtDNA have been identified for steroid hormones such as estrogen, progesterone, and glucocorticoids, as well as thyroid hormones (Demonakos et al., 1995; reviewed by Chen et al., 2005 and Psarra et al., 2006). Since mtDNA encodes for a number of the protein subunits located in the electron transport chain, the presence of hormone receptors and HREs within the mitochondrial genome provides another avenue for the translation of mitochondrial-encoded proteins, thereby enhancing energy production and mitochondrial function.

2.4.1 EFFECTS OF HORMONES ON MAMMALIAN MITOCHONDRIAL FUNCTION

Studies have been conducted in various mammalian tissue types to further understand the effects that hormone/receptor signaling have on mitochondrial physiology. For instance, progesterone signaling is typically associated with the reproductive process in females, however research has shown that the hormone and its receptor is influential in the function of mitochondria. A truncated progesterone receptor localized to the outer mitochondrial membrane was first recognized by Dai et al. (2013) in human heart tissue. In the same study, expression of this mitochondrial receptor was accompanied by an increase in cellular respiration (increased

membrane potential and O_2 consumption). A similar study using human uterine cells also showed an increase in mitochondrial membrane potential following treatment with progestin (Feng et al., 2014). Also, mitochondria isolated from rat brain and treated with progesterone and estrogen showed enhanced respiratory function and reduced oxidative damage (Irwin et al., 2008). The estrogen receptor is also known to be localized to the mitochondria in human and rat tissues (Yang et al., 2004) and is beneficial to mitochondria through the modulation of mitochondrial metabolism and gene expression (Liao et al., 2015; Chmielewska et al., 2017). Overall these studies indicate that the actions of progesterone and estrogen signaling positively affect mitochondrial function.

Glucocorticoid receptors have been identified in mammalian mitochondria using immunoblotting, immunofluorescence, and immunogold electron microscopy (Demonakos et al., 1993; Scheller et al., 2000). The receptor is normally located in the cytoplasm and upon binding with the hormone is translocated to the mitochondria (Scheller et al., 2002). Translocation of the receptor to the mitochondria is chaperoned by heat shock proteins and Bcl-2-associated athanogene (Bag-1) (Du et al., 2009). This association of glucocorticoid receptors with Bcl-2 proteins plays a role in regulating apoptosis in the mitochondria (Prenek et al., 2017). Reports have also illustrated how glucocorticoid receptors present in the mitochondria affect energy production and activity. Application of the synthetic glucocorticoid dexamethasone stimulated mitochondrial biogenesis in rat skeletal muscle and cultures of C2C12 cells (Weber et al., 2002). Transfected HepG2 that overexpress mitochondrial-targeted glucocorticoid receptors showed an increase in mitochondrial ATP production, RNA synthesis, and expression of the electron transport chain protein cytochrome oxidase subunit I (Psarra and Sekeris, 2011). There has even been evidence presented that the presence of a mitochondria-specific isoform of the

glucocorticoid receptor causes an increase in mitochondrial mass, oxygen consumption, and ATP production (Morgan et al., 2016).

Thyroid hormones are an important component in normal growth, development, and the regulation of metabolism (Yen, 2001). Similar to steroid hormone receptors, thyroid hormone receptors function as ligand-activated transcription factors by interacting with DNA-binding sites that regulate gene and protein expression (Psarra et al., 2006). It has been discovered that mammalian mitochondria contain two mitochondria-specific isoforms of the thyroid alpha receptor. These two truncated thyroid receptor alpha isoforms (p28 and p43) have been shown to increase mitochondrial gene expression, oxidative phosphorylation, thermogenesis (Bassett et al., 2003), and influence cell differentiation and apoptosis (Wrutniak-Cabello et al., 2017). The p43 isoform, which is located in the mitochondrial matrix and has direct contact with the HREs of the mitochondrial DNA appears to be the primary effector on mitochondrial function (Wrutniak et al., 1995).

Currently, there is no scientific literature published indicating that the insulin receptor is present in mammalian mitochondria. However reports do show that insulin signaling affects mitochondrial function. Studies in human neuronal cells show that the binding of insulin to its receptor induces the production of ROS, specifically H_2O_2, by mitochondria (Pomytkin, 2012). This H_2O_2 is generated primarily by the oxidation of succinate at complex II within the ETC and leads to the autophosphorylation and subsequent activation of the insulin receptor. The subsequent phosphorylation cascade within the insulin signaling pathway affects cellular mechanisms that help to regulate proliferation, metabolism, and differentiation in the cell. Additionally, studies in human skeletal muscle have shown that the infusion of insulin increases mitochondrial protein expression, ETC activity, and mitochondrial ATP synthesis (Stump et al.,

2003; Asmann et al., 2006). Recent reports have shown that multiple members of the family of receptor tyrosine kinases (RTKs) translocate to the mitochondria where they phosphorylate mitochondrial proteins and regulate mitochondrial bioenergetics (Ding et al., 2012; Salvi et al., 2013). The insulin receptor is a RTK and it is possible that it may translocate to the mitochondria, but to date has not been identified as such.

2.5 REFERENCES

1. Aggrey, S.E., A.B. Karnuah, B. Sebastian, and N.B. Anthony. 2010. Genetic properties of feed efficiency parameters in meat-type chickens. Genet. Sel. Evol. 42: 25-29.

2. Alexandre, C., M.L. Andermann, and T.E. Scammell. 2013. Control of arousal by the orexin neurons. Curr. Opin. Neurobiol. 23: 752-759.

3. Allen, D.L. and T.G. Unterman. 2007. Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors. Am. J. Physiol. Cell Physiol. 292: C188-C199.

4. Alvarez, C.E., J.G. Sutcliffe. 2002. Hypocretin is an early member of the incretin gene family. Neurosci. Lett. 324: 169-172.

5. Arcamone, N., L. D'Angelo, P. de Girolamo, C. Lucini, A. Pelagalli, and L. Castaldo. 2014. Orexin and orexin receptor like peptides in the gastroenteric tract of gallus domesticus: an immunohistochemical survey on presence and distribution. Res. Vet. Sci. 96: 234-240.

6. Arihara, Z., K. Takahashi, O. Murakami, K. Totsune, M. Sone, F. Satoh, S. Ito, and T. Mouri. 2001. Immunoreactive orexin-A in human plasma. Peptides. 22: 139-142.

7. Asmann, Y.W., C.S. Stump, K.R. Short, J.M. Coenen-Schimke, Z. Guo, M.L. Bigelow, and K.S. Nair. 2006. Skeletal muscle mitochondrial functions, mitochondrial DNA copy numbers, and gene transcript profiles in type 2 diabetic and nondiabetic subjects at equal levels of low or high insulin and euglycemia. Diabetes. 55: 3309-3319.

8. Bassett, J.H., C.B. Harvey, and G.R. Williams. 2003. Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol. Cell. Endocrinol. 213: 1-11.

9. Belle, M.D., A.T. Hughes, D.A. Bechtold, P. Cunningham, M. Pierucci, D. Burdakov, and H.D. Piggins. 2014. Acute suppressive and long-term phase modulation actions of orexin on the mammalian circadian clock. J. Neurosci. 34: 3607-3621.

10. Berry, D. and J. Crowley. 2012. Residual intake and body weight gain: a new measure of efficiency in growing cattle. J. Anim. Sci. 90: 109-115.

11. Bleier, L. and S. Drose. 2013. Superoxide generation by complex III: from mechanistic rationales to functional consequences. Biochim. Biophys. Acta. 1827: 1320-1331.

12. Bleier, L., I. Wittig, H. Heide, M. Steger, U. Brandt, and S. Drose. 2015. Generator-specific targets of mitochondrial reactive oxygen species. Free Rad. Biol. Med. 78: 1-10.

13. Bottje, W., Z.X. Tang, M. Iqbal, D. Cawthon, R. Okimoto, T. Wing, and M. Cooper. 2002. Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult. Sci. 81: 546-555.

14. Bottje, W.G. and G.E. Carstens. 2009. Association of mitochondrial function and feed efficiency in poultry and livestock species. J. Anim. Sci. 87: E-48-E63.

15. Bottje, W.G., B.-W. Kong, J.J. Song, J.Y. Lee, B.M. Hargis, K. Lassiter, T. Wing, and J. Hardiman. 2012. Gene expression in breast muscle associated with feed efficiency in a single male broiler line using a chicken 44K oligo microarray. II. Differentially expressed focus genes. Poult. Sci. 91: 2576-2587.

16. Bottje, W.G., B.-W. Kong, J.Y. Lee, T. Washington, J.I. Baum, S. Dridi, T. Wing, and J. Hardiman. 2014. Potential roles of mTOR and protein degradation pathways in the phenotypic expression of feed efficiency in broilers. Biochem. Physiol. 3: 125. doi:10.4172/2168-9652.1000125.

17. Bottje, W., B.-W. Kong, A. Reverter, A.J. Waardenberg, K. Lassiter, and N.J. Hudson. 2017. Progesterone signalling in broiler skeletal muscle is associated with divergent feed efficiency. BMC Syst. Biol. 11: 29. http://doi.org/10.1186/s12918-017-0396-2.

18. Bottje, W.G., K. Lassiter, S. Dridi, N. Hudson, and B.-W. Kong. 2017a. Enhanced expression of proteins involved in energy production and transfer in breast muscle of pedigree male broilers exhibiting high feed efficiency. Poult. Sci. 96: 2454-2458.

19. Bottje, W.G., K. Lassiter, A. Piekarski-Welsher, S. Dridi, A. Reverter, N.J. Hudson, and B.-W. Kong. 2017b. Proteogenomics reveals enriched ribosome assembly and protein translation in *Pectoralis major* of high feed efficiency pedigree broiler males. Front. Physiol. 8: 306. http://doi.org/10.3389/fphys.2017.00306.

20. Boveris, A. and B. Chance. 1973. The mitochondrial generation of hydrogen peroxide. Biochem. J. 134: 707-711.

21. Burdakov, D., O. Gerasimenko, and A. Verkhratsky. 2005. Physiological changes in glucose differentially modulate the excitability of hypothalamic melanin-concentrating hormone and orexin neurons in situ. J. Neurosci. 25: 2429-2433.

22. Burdakov, D., L.T. Jensen, H. Alexopoulos, R.H. Williams, I.M. Fearon, I. O'Kelly, O. Gerasimenko, L. Fugger, and A. Verkhratsky. 2006. Tandem-pore K+ channels mediate inhibition of orexin neurons by glucose. Neuron. 50: 711-722.

23. Butterick, T.A., J.P. Nixon, C.J. Billington, and C.M. Kotz. 2012. Orexin A decreases lipid peroxidation and apoptosis in a novel hypothalamic cell model. Neurosci. Lett. 524: 30-34.

24. Byerly, T.C. 1941. Feed and other costs of producing market eggs (Vol. A1). Maryland: University of Maryland, Agricultural Experiment Station.

25. Chance, B., H. Sies, and A. Boveris. 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59: 527-605.

26. Chemelli, R.M., J.T. Willie, C.M. Sinton, J.K. Elmquist, T. Scammell, C. Lee, J.A. Richardson, S.C. Williams, Y. Xiong, Y. Kisanuki, T.E. Fitch, M. Nakazato, R.E. Hammer, C.B. Saper, and M. Yanagisawa. 1999. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell. 98: 437-451.

27. Chen, Q., E.J. Vazquez, S. Moghaddas, C.L. Hoppel, and E.J. Lesnefsky. 2003. Production of reactive oxygen species by mitochondria: central role of complex III. J. Biol. Chem. 278: 36027-36031.

28. Chen, J.Q., M. Delannoy, C. Cooke, and J.D. Yager. 2004a. Mitochondrial localization of ERalpha and ERbeta in human mcf-7 cells. Am. J. Physiol.: Endocrinol. Metab. 286: E1011-E1022.

29. Chen, J.Q., M. Eshete, W.L. Alworth, and J.D. Yager. 2004b. Binding of mcf-7 cell mitochondrial proteins and recombinant human estrogen receptors alpha and beta to human mitochondrial DNA estrogen response elements. J. Cell. Biochem. 93: 358.

30. Chen, J.Q., J.D. Yager, and J. Russo. 2005. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. Biochim. Biophys. Acta. 1746: 1-17.

31. Chmielewska, M., I. Skibinska, and M. Kotwicka. 2017. Mitochondria: target organelles for estrogen action. Postepy. Hig. Med. Dosw. 71: 454-465.

32. Clop, A., F. Marcq, H. Takeda, D. Pirottin, X. Tordoir, B. Bibe, J. Bouix, F. Caiment, J.M. Elsen, F. Eychenne, C. Larzul, E. Laville, F. Meish, D. Milenkovic, J. Tobin, C. Charlier, and M. Georges. 2006. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nature Genet. 38: 813-818.

33. Ciriello, J., Z. Li, and C.V. de Oliveira. 2003. Cardioacceleratory responses to hypocretin-1 injections into rostral ventromedial medulla. Brain Res. 991: 84-95.

34. Dai, Q., A.A. Shah, R.V. Garde, B.A. Yonish, L. Zhang, N.A. Medvitz, S.E. Miller, E.L. Hansen, C.N. Dunn, and T.M. Price. 2013. A truncated progesterone receptor (PR-M) localizes to the mitochondrion and controls cellular respiration. Mol. Endo. 27:741-753.

35. Dalal, M.A., A. Schuld, M. Haack, M. Uhr, P. Geisler, I. Eisensehr, S. Noachtar, and T. Pollmacher. 2001. Normal plasma levels of orexin A (hypocretin-I) in narcoleptic patients. Neurology. 56: 1749-1751.

36. da Silva, E.S., T.V. dos Santos, A.A. Hoeller, T.S. dos Santos, G.V. Pereira, C. Meneghelli, A.I. Penzlin, M.M. dos Santos, M.S. Faria, M.A. Paschoalini, and J. Marino-Neto. 2008. Behavioral and metabolic effects of central injections or orexins/hypocretins in pigeons (Columba livia). Regul. Pept. 147: 9-18.

37. de Lecea, L., T.S. Kilduff, C. Peyron, X-B. Gao, P.E. Foye, P.E. Danielson, C. Fukuhara, E.L.F. Battenberg, V.T. Gautvik, F.S. Bartlett II, W.N. Frankel, A.N. van den Pol, F.E. Bloom, K.M. Gautvik, and J.G. Sutcliffe. 1998. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. USA 95:322-327.

38. Demonakos, C., N.C. Tsawdaroglou, R. Djordjevic-Markovic, M. Papalopoulou, V. Galanopolous, S. Papadogeorkagi, and C.E. Sekeris. 1993. Import of the glucocorticoid receptor into rat liver mitochondria *in vivo* and *in vitro*. J. Steroid. Biochem. Mol. Biol. 46: 401-413.

39. Demonakos, C., R. Djordjevic-Markovic, N. Tsawdaroglou, and C.E. Sekeris. 1995. The mitochondrion as a primary site of action of glucocorticoids: the interaction of the glucocorticoid receptor with mitochondrial DNA sequences showing partial similarity to the nuclear glucocorticoid responsive elements. J. Steroid Biochem. Mol. Biol. 55: 43-55.

40. Digby, J.E., J. Chen, J.Y. Tang, H. Lehnert, R.N. Matthews, and H.S. Randeva. 2006. Orexin receptor expression in human adipose tissue: effects of orexin-A and orexin-B. J. Endocrinol. 191: 129-136.

41. Ding, Y., Z. Liu, S. Desai, Y. Zhao, H. Liu, L. K. Pannell, H. Yi, E. R. Wright, L. B. Owen, W. Dean-Colomb, O. Fodstad, J. Lu, S. P. LeDoux, G. L. Wilson, and M. Tan. 2012. Receptor tyrosine kinase ErbB2 translocates into mitochondria and regulates cellular metabolism. Nat. Comm. 3: 1271.

42. Drose, S. and U. Brandt. 2012. Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. Adv. Exp. Med. Biol. 748: 145-169.

43. Du, J., B. McEwen, and H. K. Manji. 2009. Glucocorticoid receptors modulate mitochondrial function. Comm. Integrat. Biol. 2(4): 350-352.

44. Edwards, C.M., S. Abusnana, D. Sunter, K.G. Murphy, M.A. Ghatei, and S.R. Bloom. 1999. The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin. J. Endocrinol. 160: R7-R12.

45. Eggermann, E., M. Serafin, L. Bayer, D. Machard, B. Saint-Mleux, B.E. Jones, and M. Muhlethaler. 2001. Orexins/hypocretins excite basal forebrain cholinergic neurones. Neuroscience. 108: 177-181.

46. Elkina, Y., S. von Haehling, S.D. Anker, and J. Springer. 2011. The role of myostatin in muscle wasting: an overview. J. Cachexia Sarcopenia Muscle. 2: 143-151.

47. Elliot, B., D. Renshaw, S. Getting, and R. Mackenzie. 2012. The central role of myostatin in skeletal muscle and whole body homeostasis. Acta. Physiol. 205: 324-340.

48. Emmerson, D.A. 1997. Commercial approaches to genetic selection for growth and feed conversion in domestic poultry. Poult. Sci. 76: 1121-1125.

49. Feng, Q., J. R. Crochet, Q. Dai, P. C. Leppert, and T. M. Price. 2014. Expression of a mitochondria progesterone receptor (PR-M) in leiomyomata and association with increased mitochondrial membrane potential. J. Clin. Endo. Metab. 99: E390-E399.

50. Finkel, T. 2011. Signal transduction by reactive oxygen species. J. Cell Biol. 194: 7-15.

51. Furuse, M., R. Ando, T. Bungo, R. Ao, M. Shimojo, and Y. Masuda. 1999. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. Br. Poult. Sci. 40: 698-700.

52. Gautam, N., G.B. Downes, K. Yan, and O. Kisselev. 1998. The g-protein betagamma complex. Cell Signal. 10: 447-455.

53. Godden, K.E., J.P. Landry, N. Slepneva, P.V. Migues, and M. Pompeiano. 2014. Early expression of hypocretin/orexin in the chick embryo brain. PLoS One. 9(9): e106977. http://doi.org/10.1371/journal.pone.0106977.

54. Griffond, B., P.Y. Risold, C. Jacquemard, C. Colard, and D. Fellmann. 1999. Insulin-induced hypoglycemia increases preprohypocretin (orexin) mRNA in the rat lateral hypothalamic area. Neurosci. Lett. 262: 77-80.

55. Hara, J., C.T. Beuckmann, T. Nambu, J.T. Willie, R.M. Chemelli, C.M. Sinton, F. Sugiyama, K. Yagami, K. Goto, M. Yanagisawa, and T. Sakurai. 2001. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron. 30: 345-354.

56. Havenstein, G.B., P.R. Ferket, S.E. Scheideler, and B.T. Larson. 1994. Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed "typical" 1957 and 1991 broiler diets. Poult. Sci. 73: 1785-1794.

57. Havenstein, G.B., P.R. Ferket, and M.A. Qureshi. 2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82: 1500-1508.

58. Havenstein, G.B., P.R. Ferket, J.L. Grimes, M.A. Qureshi, and K.E. Nestor. 2007. Comparison of 1966- versus 2003-type turkeys when fed representative 1966 and 2003 turkey diets: growth rate, livability, and feed conversion. Poult. Sci. 86: 232-240.

59. Haynes A.C., B. Jackson, H. Chapman, M. Tadayyon, A. Johns, R.A. Porter, and J.R. Arch. 2000. A selective orexin-1 receptor antagonist reduces food consumption in male and female rats. Regul. Pept. 96: 45-51.

60. Haynes, A.C., H. Chapman, C. Taylor, G.B. Moore, M.A. Cawthorne, M. Tadayyon, J.C. Clapham, and J.R. Arch. 2002. Anorectic, thermogenic, and anti-obesity activity of a selective orexin-1 receptor antagonist in ob/ob mice. Regul. Pept. 104: 153-159.

61. Hepler, J.R. and A.G. Gilman. 1992. G proteins. Trends Biochem. Sci. 17: 383-387.

62. Honda, Y., Y. Doi, R. Ninomiya, and C. Ninomiya. 1986. Increased frequency of non-insulin-dependent diabetes mellitus among narcoleptic patients. Sleep. 9: 254-259.

63. Inutsuka, A. and A. Yamanaka. 2013. The physiological role of orexin/hypocretin neurons in the regulation of sleep/wakefulness and neuroendocrine functions. Front. Endocrinol. 4: 18. http://doi.org/10.3389/fendo.2013.00018.

64. Iqbal, M., N. Pumford, K. Lassiter, Z. Tang, T. Wing, M. Cooper, and W. Bottje. 2004. Low feed efficient broilers within a single genetic line exhibit higher oxidative stress and protein expression in breast muscle with lower mitochondrial complex activity. Poult. Sci. 83: 474-484.

65. Iqbal, M., N. Pumford, Z.X. Tang, K. Lassiter, C. Ojano-Dirain, T. Wing, M. Cooper, and W.G. Bottje. 2005. Compromised liver mitochondrial function and complex activity in low feed efficient broilers within a single genetic line associated with higher oxidative stress and differential protein expression. Poult. Sci. 84: 933-941.

66. Irwin, R. W., J. Yao, R.T. Hamilton, E. Cadenas, R.D. Brinton, and J. Nilsen. 2008. Progesterone and estrogen regulate oxidative metabolism in brain mitochondria. Endocrinology. 149: 3167-3175.

67. James, M.H., E.J. Campbell, and C.V. Dayas. 2017. Role of the orexin/hypocretin system in stress-related psychiatric disorders. Curr. Top. Behav. Neurosci. 33: 197-219.

68. Johnson, P.L, A. Molosh, W.A. Truitt, S.D. Fitz, and A. Shekhar. 2012. Orexin, stress and anxiety/panic states. Prog. Brain Res. 198: 133-161.

69. Johren, O., S.J. Neidert, M. Kummer, A. Dendorfer, and P. Dominiak. 2001. Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. Endocrinology. 142: 3324-3331.

70. Kambadur, R., M. Sharma, T.P.L. Smith, and J.J. Bass. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res. 7: 910-916.

71. Kantor, S., T. Mochizuki, A.M. Janisiewicz, E. Clark, S. Nishino, T.E. Scammell. 2009. Orexin neurons are necessary for the circadian control of REM sleep. Sleep. 32: 1127-1134.

72. Karteris, E., H.S. Randeva, D.K. Grammatopoulos, R.B. Jaffe, and E.W. Hillhouse. 2001. Expression and coupling characteristics of the crh and orexin type 2 receptors in human fetal adrenals. J. Clin. Endocrinol. Metab. 86: 4512-4519.

73. Karteris, E., J. Chen, and H.S. Randeva. 2004. Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. J. Clin. Endocrinol. Metab. 89: 1957-1962.

74. Karteris, E., R.J. Machado, J. Chen, S. Zervou, E.W. Hillhouse, and H.S. Randeva. 2005. Food deprivation differentially modulates orexin receptor expression and signaling in the rat hypothalamus and adrenal cortex. Am. J. Physiol. Endocrinol. Metab. 288: E1089-E-1100.

75. Katayama, S., K. Hamasu, K. Shigemi, M.A. Cline, and M. Furuse. 2010. Intracerebroventricular injection of orexin-A, but not orexin-B, induces arousal of layer-type neonatal chicks. Comp. Biochem. Physiol. A. 157: 132-135.

76. Katayama, S., K. Shigemi, M.A. Cline, and M. Furuse. 2011. Clorgyline inhibits orexin-A-induced arousal in layer-type chicks. J. Vet. Med. Sci. 73: 471-474.

77. Kirchgessner, A.L., and M. Liu. 1999. Orexin synthesis and response in the gut. Neuron. 24: 941-951.

78. Kollias, H.D. and J.C. McDermott. 2008. Transforming growth factor-β and myostatin signaling in skeletal muscle. J. Appl. Physiol. 104: 579-587.

79. Kong, B.-W., J.J. Song, J.Y. Lee, B.M. Hargis, T. Wing, K. Lassiter, and W. Bottje. 2011. Gene expression in breast muscle associated with feed efficiency in a single male broiler line using a chicken 44K oligo microarray. I. Top differentially expressed genes. Poult. Sci. 90: 2535-2547.

80. Kong, B.-W., K. Lassiter, A. Piekarski-Welsher, S. Dridi, A. Reverter, N.J. Hudson, and W.G. Bottje. 2016. Proteomics of Breast Muscle Tissue Associated with the Phenotypic Expression of Feed Efficiency within a Pedigree Male Broiler Line: I. Highlight on Mitochondria. PLoS ONE 11(7): e0159897. https://doi.org/10.1371/journal.pone.0159897.

81. Kukkonen, J.P. 2013. Physiology of the orexinergic/hypocretinergic system: a revisit in 2012. Am. J. Physiol. Cell Physiol. 304(1): C2-C32.

82. Kukkonen, J.P. 2014. Lipid signaling cascades of orexin/hypocretin receptors. Biochimie. 96: 158-165.

83. Kukkonen, J.P. and C.S. Leonard. 2014. Orexin/hypocretin receptor signaling cascades. Br. J. Pharmacol. 171(2): 314-331.

84. Lassiter, K., C. Ojano-Dirain, M. Iqbal, N.R. Pumford, N. Tinsley, T.Wing, J. Lay, R. Liyanage, M. Cooper, and W. Bottje. 2006. Differential expression of mitochondrial and exta-mitochondrial proteins in lymphocytes of low and high feed efficient male broilers. Poult. Sci. 85: 2251-2259.

85. Lee, S.J. and A.C. McPherron. 2001. Regulation of myostatin activity and muscle growth. Proc. Natl. Acad. Sci. U.S.A. 98: 9306-9311.

86. Lee, M.G., O.K. Hassani, and B.E. Jones. 2005. Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. J. Neurosci. 25: 6716-6720.

87. Lee, S. and D.J. Glass. 2011. Treating cancer cachexia to treat cancer. Skel. Musc. 1: 1-5.
Lehninger, A.L., D.L. Nelson, and M.M. Cox. 1993. Principles of Biochemistry. 2nd ed. Worth Publishers, New York, NY.

88. Liao, T.L., C.R. Tzeng, C.L. Yu, Y.P. Wang, and S.H. Kao. 2015. Estrogen receptor-β in mitochondria: implications for mitochondrial bioenergetics and tumorigenesis. Ann. N.Y. Acad. Sci. 1350: 52-60.

89. Lin, L., J. Faraco, R. Li, H. Kadotani, W. Rogers, X. Lin, X. Qiu, P.J. de Jong, S. Nishino, and E. Mignot. 1999. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell. 98: 365-376.

90. Luiting, P. 1990. Genetic variation of energy partitioning in laying hens: causes of variation in residual feed consumption. World's Poult. Sci. J. 46: 133-152.

91. Marcus, J.N., C.J. Aschkenasi, C.E. Lee, R.M. Chemelli, C.B. Saper, M. Yanagisawa, and J.K. Elmquist. 2001. Differential expression of orexin receptors 1 and 2 in the rat brain. J. Comp. Neurol. 435: 6-25.

92. McPherron, A.C. and S.J. Lee. 1996. Growth factors and cytokines in health and disease, Vol 1B. (eds. D. LeRoith and C. Bondy). JAI, Greenwich. 357-393.

93. McPherron, A.C., A.M. Lawler, and S.J. Lee. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature. 387: 83-90.

94. Mieda, M., S.C. Williams, C.M. Sinton, J.A. Richardson, T. Sakurai, and M. Yanagisawa. 2004. Orexin neurons function in an efferent pathway of a food-entrainable circadian oscillator in eliciting food-anticipatory activity and wakefulness. J. Neurosci. 24: 10493-10501.

95. Mignot, E. and E. Thorsby. 2001. Narcolepsy and the HLA system. 2001. N. Engl. J. Med. 344: 692.

96. Mileykovskiy, B.Y., L. Kiyashchenko, and J.M. Siegel. 2005. Behavioral correlates of activity in identified hypocretin/orexin neurons. Neuron. 46: 787-798.

97. Miranda, B., V. Esposito, P. de Girolamo, P.J. Sharp, P.W. Wilson, and I.C. Dunn. 2013. Orexin in the chicken hypothalamus: immunocytochemical localization and comparison of mRNA concentrations during the day and night, and after chronic food restriction. Brain Res. 1513: 34-40.

98. Morgan, D. J., T. M. Poolman, A. J. Williamson, Z. Wang, N. R. Clark, A. Ma'ayan, A. D. Whetton, A. Brass, L. C. Matthews, and D. W. Ray. 2016. Glucocorticoid receptor isoforms direct distinct mitochondrial programs to regulate ATP production. Sci. Rep. 6: 26419.

99. Mosher, D.S., P. Quignon, C.D. Bustamante, N.B. Sutter, C.S. Mellersh, H.G. Parker, and E.A. Ostrander. 2007. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. PLoS Genetics. 3: 779-786.
Moutsatsou, P., A.-M. Psarra, A. Tsiapara, H. Paraskevakou, P. Davaris, and C.E. Sekeris. 2001. Localization of the glucocorticoid receptor in rat brain mitochondria. Arch. Biochem. Biophys. 386: 69-78.

100. Nakabayashi, M., T. Suzuki, K. Takahashi, K. Totsune, Y. Muramatsu, C. Kaneko, F. Date, J. Takeyama, A.D. Darnel, T. Moriya, and H. Sasano. 2003. Orexin-A expression in human peripheral tissues. Mol. Cell. Endocrinol. 205: 43-50.

101. Nambu, T., T. Sakurai, K. Mizukami, Y. Hosoya, M. Yanagisawa, and K. Goto. 1999. Distribution of orexin neurons in the adult rat brain. Brain Res. 827: 243-260.

102. Naslund, E., M. Ehrstrom, J. Ma, P.M. Hellstrom, and A.L. Kirchgessner. 2002. Localization and effects of orexinon fasting motility in the rat duodenum. Am. J. Physiol. Gastrointest. Liver Physiol. 282: G470-G479.

103. Ohkubo, T., T. Boswell, and S. Lumineau. 2002. Molecular cloning of chicken prepro-orexin cDNA and preferential expression in the chicken hypothalamus. Biochim. Biophys. Acta. 1577: 476-480.

104. Ohkubo, T., A. Tsukada, and K. Shamoto. 2003. cDNA cloning of chicken orexin receptor and tissue distribution: sexually dimorphic expression in chicken gonads. J. Mol. Endocrinol. 31: 499-508.

105. Ojano-Dirain, C., M. Iqbal, D. Cawthon, S. Swonger, T. Wing, M. Cooper, and W. Bottje. 2004. Determination of mitochondrial function and site-specific defects in electron transport in duodenal mitochondria in broilers with low and high feed efficiency. Poult. Sci. 83: 1394-1403.

106. Ojano-Dirain, C., N.R. Pumford, M. Iqbal, T. Wing, M. Cooper, and W.G. Bottje. 2005. Biochemical evaluation of mitochondrial respiratory chain in duodenum of low and high feed efficient broilers. Poult. Sci. 84: 1926-1934.

107. Ojano-Dirain, C., M. Toyomizu, T. Wing, M. Cooper, and W.G. Bottje. 2007. Gene expression in breast muscle and duodenum from low and high feed efficient broilers. Poult. Sci. 86:372-381.

108. Pasban-Aliabadi, H., S. Esmaeili-Mahani, M. Abbasnejad. 2017. Orexin-A protects human neuroblastoma SH-SY5Y cells against 6-hydroxydopamine-induced neurotoxicity: involvement of PKC and PI3K signaling pathways. Rejuvenation Res. 20: 125-133.

109. Peyron, C., D.K. Tighe, A.N. van den Pol, L. de Lecea, H.C. Heller, J.G. Sutcliffe, and T.S. Kilduff. 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J. Neurosci. 9996-10015.

110. Phillips-Singh, D., Q. Li, S. Takeuchi, T. Ohkubo, P.J. Sharp, and T. Boswell. 2003. Fasting differentially regulates expression of agouti-related peptide, pro-opiomelanocortin, prepro-orexin, and vasoactive intestinal polypeptide mRNAs in the hypothalamus of Japanese quail. Cell Tissue Res. 313: 217-225.

111. Pomytkin, I. A. 2012. H_2O_2 Signaling pathway: a possible bridge between insulin receptor and mitochondria. Curr. Neuropharm. 10: 311-320.
Prenek, L., F. Boldizsar, R. Kugyelka, E. Ugor, G. Berta, P. Nemeth, and T. Berki. 2017. The regulation of the mitochondrial apoptotic pathway by glucocorticoid receptor in collaboration with Bcl-2 family proteins in developing T cells. Apoptosis. 22: 239-253.

112. Pruszynska-Oszmalek, E., P.A. Kolodziejski, P. Kaczmarek, M. Sassek, D. Szczepankiewicz, R. Mikula, and K.W. Nowak. 2018. Orexin A but not orexin B regulates lipid metabolism and leptin secretion in isolated porcine adipocytes. Domest. Anim. Endocrinol. 63: 59-68.

113. Psarra, A.M., S. Solakidi, and C.E. Sekeris. 2006. The mitochondrion as a primary site of action of steroid and thyroid hormones: presence and action of steroid and thyroid hormone receptors in mitochondria of animal cells. Mol. Cell. Endocrinol. 246: 21-33.

114. Psarra, A. M. G., and C. E. Sekeris. 2011. Glucocorticoids induce mitochondrial gene transcription in HepG2 cells: role of the mitochondrial glucocorticoid receptor. Biochim. Biophys. Acta. 1813(10): 1814-1821.

115. Randeva, H.S., E. Karteris, D.K. Grammatopoulos, and E.W. Hillhouse. 2001. Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. J. Clin. Endocrinol. Metab. 86: 4808-4813.

116. Romero, L.F., M.J. Zuidhof, R.A. Renema, A.N. Naeima, and F.E. Robinson. 2009. Effects of maternal energetic efficiency on egg traits, chick traits, broiler growth, yield, and meat quality. Poult. Sci. 88: 236-245.

117. Rossignol, R., T. Letellier, M. Malgat, C. Rocher, and J.P. Mazat. 2000. Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial disease. Biochem. J. 347: 45-53.

118. Sakurai, T., A. Amemiya, M. Ishii, I. Matsuzaki, R.M. Chemelli, H. Tanaka, S.C. Williams, J.A. Richardson, G.P. Kozlowski, S. Wilson, J.R.S. Arch, R.E. Buckingham, A.C. Haynes, S.A. Carr, R.S. Annan, D.E. McNulty, W-S. Liu, J.A. Terrett, N.A. Elshourbagy, D.J. Bergsma, and M. Yanagisawa. 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and g protein-coupled receptors that regulate feeding behavior. Cell 92:573-585.

119. Sakurai, T. 1999. Orexins and orexin receptors: implication in feeding behavior. Regul. Pept. 85: 25-30.

120. Sakurai, T. 2005. Reverse pharmacology of orexin: from an orphan GPCR to integrative physiology. Regul. Pept. 126: 3-10.
Salvi, M. 2013. Receptor tyrosine kinases take a direct route to mitochondria: an overview. Curr. Protein Peptide Sci. 14(7): 635-640.

121. Samson, W.K., B. Gosnell, J.K. Chang, Z.T. Resch, and T.C. Murphy. 1999. Cardiovascular regulatory actions of the hypocretins in brain. Brain Res. 831: 248-253.
Samson, W.K., M.M. Taylor, M. Follwell, and A.V. Ferguson. 2002. Orexin actions in hypothalamic paraventricular nucleus: physiological consequences and cellular correlates. Regul. Pept. 104: 97-103.

122. Samson, W.K., S.L. Bagley, A.V. Ferguson, M.M. White. 2007. Hypocretin/orexin type 1 receptor in brain: role in cardiovascular control and the neuroendocrine response to immobilization stress. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292: R382-R387.

123. Scheller, K., C. E. Sekeris, G. Krohne, R. Hock, I. A. Hansen, and U. Scheer. 2000. Localization of glucocorticoid hormone receptors in mitochondria of human cells. Eur. J. Cell Biol. 79(5): 299-307.

124. Scheller, K., P. Seibel, and C.E. Sekeris. 2002. Glucocorticoid and thyroid hormone receptors in animal cells. Int. Rev. Cytol. 222: 1-61.

125. Scheller, K. and C.E. Sekeris. 2003. The effects of steroid hormones on the transcription of genes encoding enzymes of oxidative phosphorylation. Exp. Physiol. 88: 129-140.

126. Schuelke, M., K.R. Wagner, L.E. Stolz, C. Hubner, T. Riebel, W. Komen, T. Braun, J.F. Tobin, and S.-J. Lee. 2004. Myostatin mutation associated with gross muscle hypertrophy in a child. N. Engl. J. Med. 350: 2682-2688.

127. Schuld, A., J. Hebebrand, F. Geller, and T. Pollmacher. 2000. Increased body-mass index in patients with narcolepsy. Lancet. 355: 1274-1275.

128. Sellayah, D., P. Bharaj, and D. Sikder. 2011. Orexin is required for brown adipose tissue development, differentiation, and function. Cell Metab. 14: 478-490.

129. Shen, Y., Y. Zhao, D. Zheng, X. Chang, S. Ju, and L. Guo. 2013. Effects of orexin A on GLUT4 expression and lipid content via MAPK signaling in 3T3-L1 adipocytes. J. Steroid Biochem. Mol. Biol. 138: 376-383.

130. Shibahara, M., T. Sakurai, T. Nambu, T. Takenouchi, H. Iwaasa, S.I. Egashira, M. Ihara, and K. Goto. 1999. Structure, tissue distribution, and pharmacological characterization of Xenopus orexins. Peptides 20: 1169-1176.

131. Shirasaka, T., M. Nakazato, S. Matsukura, M. Takasaki, and H. Kannan. 1999. Sympathetic and cardiovascular actions of orexins in conscious rats. Am. J. Physiol. 6 Pt. 2: R1780-R1785.

132. Sikder, D. and T. Kodadek. 2007. The neurohormone orexin stimulates hypoxia-inducible factor-1 activity. Genes Dev. 21: 2995-3005.

133. Skrzypski, M., T.T. Le, P. Kaczmarek, E. Pruszynska-Oszmalek, P. Pietrzak, D. Szczepankiewicz, P.A. Kolodziejski, M. Sassek, A. Arafat, B. Wiedenmann, K.W. Nowak, and M.Z. Strowski. 2011. Orexin A stimulates glucose uptake, lipid accumulation and adiponectin secretion from 3T3-L1 adipocytes and isolated primary rat adipocytes. Diabetologia. 54: 1841-1852.

134. Song, Z., L. Liu, Y. Yue, H. Jiao, H. Lin, A. Sheikhahmadi, N. Everaert, E. Decuypere, and J. Buyse. 2012. Fasting alters protein expression of AMP-activated protein kinase in the hypothalamus of broiler chicks (Gallus gallus domesticus). Gen. Comp. Endocrinol. 178: 546-555.

135. Song, Z., N. Everaert, Y. Wang, E. Decuypere, and J. Buyse. 2013. The endocrine control of energy homeostasis in chickens. Gen. Comp. Endocrinol. 190: 112-117.

136. Soppet, D.R., Y. Li, and C.A. Rosen. 1996. Human genome sciences inc. Human neuropeptide receptor. World patent no. WO9634877.

137. Stadel, J.M., S. Wilson, and D. Bergsma. 1997. Orphan g protein-coupled receptors: a neglected opportunity for pioneer drug discovery. Trends Pharmacol. Sci. 18: 430-437.

138. Stadtman, E.R., and R.L. Levine. 2000. Protein oxidation. Ann. N. Y. Acad. Sci. 899:191-208.

139. Stump, C.S., K.R. Short, M.L. Bigelow, J.M. Schimke, and K.S. Nair. 2003. Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts. Proc. Natl. Acad. Sci. U.S.A. 100: 7996-8001.

140. Swami, M. 2011. Metabolism: orexin acts on brown fat. Nat. Med. 17: 1356-1356.
Takahashi, K., Y. Koyama, Y. Kayama, and M. Yamamoto. 2002. Effects of orexin on the laterodorsal tegmental neurones. Psychiatry Clin. Neurosci. 56: 335-336.

141. Takahashi, K. J.S. Lin, and K. Sakai. 2008. Neuronal activity of orexin and non-orexin waking-active neurons during wake-sleep states in the mouse. Neuroscience. 153: 860-870.

142. Thomas, M., B. Langley, C. Berry, M. Sharma, S. Kirk, J. Bass, and R. Kambadur. 2000. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J. Biol. Chem. 275: 40235-40243.

143. Tinsley, N., M. Iqbal, N.R. Pumford, K. Lassiter, C. Ojano-Dirain, T. Wing, and W. Bottje. 2010. Investigation of mitochondrial protein expression and oxidation in heart muscle in low and high feed efficient male broilers in a single genetic line. Poult. Sci. 89: 349-352.

144. Trivedi, P., H. Yu, D.J. MacNeil, L.H. Van der Ploeg, and X.M. Guan. 1998. Distribution of orexin receptor mRNA in the rat brain. FEBS Lett. 438: 71-75.
Tsujino, N. and T. Sakurai. 2009. Orexin/hypocretin: a neuropeptide at the interface of sleep, energy homeostasis, and reward system. Pharmacol. Rev. 61: 162-176.

145. Tsuneki, H., T. Wada, and T. Sasaoka. 2010. Role of orexin in the regulation of glucose homeostasis. Acta. Physiol. 198: 335-348.

146. Tsuneki, H., T. Wada, and T. Sasaoka. 2012. Role of orexin in the central regulation of glucose and energy homeostasis. Endocr. J. 59: 365-374.

147. van den Pol, A.N. 1999. Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. J. Neurosci. 19: 3171-3182.

148. Van Der Werf, J.H.J. 2004. Is it useful to define residual feed intake as a trait in animal breeding programmes? Aus. J. Exp. Agri. 44: 405-409.

149. Wan, X., Y. Liu, Y. Zhao, X. Sun, D. Fan, and L. Guo. 2017. Orexin A affects HepG2 human hepatocellular carcinoma cells glucose metabolism via HIF-1α-dependent and –independent mechanism. PLoS One. 12(9): e0184213. http://doi.org/10.1371/journal.pone.0184213.

150. Weber, K., P. Brück, Z. Mikes, J. H. Küpper, M. Klingenspor, and R. J. Wiesner. 2002. Glucocorticoid hormone stimulates mitochondrial biogenesis specifically in skeletal muscle. Endocrinology, 143: 177-184.

151. Willems, O.W., S.P. Miller, and B.J. Wood. 2013. Aspects of selection for feed efficiency in meat producing poultry. Worlds Poult. Sci. J. 69: 77-88.

152. Wrutniak, C., I. Cassar-Malek, S. Marchal, A. Rascle, S. Heusser, J.M. Keller, J. Flechon, M. Dauca, J. Samarut, J. Ghysdael, and G. Cabello. 1995. A 43-kDa proein related to c-Erb A alpha 1 is located in the mitochondrial matrix of rat liver. J. Biol. Chem. 270: 16347-16354.

153. Wrutniak-Cabello, C., F. Casas, and G. Cabello. 2001. Thyroid hormone action in mitochondria. J. Mol. Endocrinol. 26: 67-77.

154. Wrutniak-Cabello, C., F. Casas, and G. Cabello. 2017. Mitochondrial T3 receptor and targets. Mol. Cell. Endocrinol. 458: 112-120.

155. Wu, Y., B. Xu, and R.J. Koenig. 2001. Thyroid hormone response element sequence and the recruitment of retinoid X receptors for thyroid hormone responsiveness. J. Biol. Chem. 276: 3929-3936.

156. Xi, M.C., F.R. Morales, and M.H. Chase. 2001. Effects on sleep and wakefulness of the injection of hypocretin-1 (orexin-A) into the laterodorsal tegmental nucleus of the cat. Brain Res. 90: 259-264.

157. Yamanaka, A., C.T. Beuckmann, J.T. Willie, J. Hara, N. Tsujino, M. Mieda, M. Tominaga, K. Yagami, F. Sugiyama, K. Goto, M. Yanagisawa, and T. Sakurai. 2003. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron. 38: 701-713.

158. Yang, S.H., R. Liu, E.J. Perez, Y. Wen, S.M. Stevens Jr., T. Valencia, A.M. Brun-Zinkernagel, L. Prokai, Y. Will, J. Dykens, P. Koulen, and J.W. Simpkins. 2004. Mitochondrial localization of estrogen receptor beta. Proc. Natl. Acad. Sci. U.S.A. 101: 4130-4135.

159. Yen, P.M. 2001. Physiological and molecular basis of thyroid hormone action. Physiol. Rev. 81: 1097-1142.

160. Yu, B.P. 1994. Cellular defenses against damage from reactive oxygen species. Physiol. Rev. 74: 139-162.

161. Yun, S., M. Wennerholm, J.E. Shelton, P. Bonaventure, M.A. Letavic, B.T. Shireman, T.W. Lovenberg, C. Dugovic. 2017. Selective inhibition of orexin-2 receptors prevents stress-induced ACTH release in mice. Front. Behav. Neurosci. 11:83. http://doi.org/10.3389/fnbeh.2017.00083.

162. Zhang, W., Y. Fukuda, and T. Kuwaki. 2005. Respiratory and cardiovascular actions of orexin-A in mice. Neurosci. Lett. 385: 131-136.

163. Zhou, N., W.R. Lee, and B. Abasht. 2015. Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens' feed efficiency. BMC Genomics. 16(1): 195. http://doi.org/10.1186/s12864-015-1364-0.

164. Zhu, X., S. Topouzis, L.F. Liang, and R.L. Stotish. 2004. Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. Cytokine. 26: 262-272.

CHAPTER 3

Orexin system is expressed in avian muscle cells and regulates mitochondrial dynamics

Kentu Lassiter, Elizabeth Greene, Alissa Piekarski, Olivia B. Faulkner, Billy M. Hargis, Walter Bottje, and Sami Dridi

Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, Arkansas, United States of America

Published:

Lassiter, K., E. Greene, A. Piekarski, O.B. Faulkner, B.M. Hargis, W. Bottje, and S. Dridi. 2015. Orexin system is expressed in avian muscle cells and regulates mitochondrial dynamics. Am. J. Physiol. Regul. Integr. Comp. Physiol. 308: R173-R187.

3.1 ABSTRACT

Orexin A and B, orexigenic peptides produced primarily by the lateral hypothalamus that signal through two G-proteins coupled receptors, orexin receptors 1/2, have been implicated in the regulation of several physiological processes in mammals. In avian (non-mammalian vertebrates) species, however, the physiological roles of orexin are not well defined. Here we provide novel evidence that not only is orexin and its related receptors 1/2 (ORXR1/2) expressed in chicken muscle tissue and quail muscle (QM7) cell line, orexin appears to be a secretory protein in QM7 cells. *In vitro* administration of recombinant orexin A and B (rORX-A and B) differentially regulated prepro-orexin expression in a dose-dependent manner with up-regulation for rORX-A ($P<0.05$) and down-regulation for rORX-B ($P<0.05$) in QM7 cells. While both peptides up-regulated ORXR1 expression, only high dose of rORX-B decreased the expression of ORXR2 ($P<0.05$). The presence of orexin and its related receptors and the regulation of its own system in avian muscle cells indicate that orexin may have autocrine, paracrine and/or endocrine roles. rORXs differentially regulated mitochondrial dynamics network. While rORX-A significantly induced the expression of mitochondrial fission-related genes (DNM1, MTFP1, MTFR1), rORX-B increased the expression of mitofusin2, OPA1 and OMA1 genes that are involved in mitochondrial fusion. Concomitant with these changes, rORXs differentially regulated the expression of several mitochondrial metabolic genes (av-UCP, av-ANT, Ski and NRF-1) and their related transcriptional regulators (PPARγ, PPARα, PGC-1α, PGC-1β and FoxO-1) without affecting ATP synthesis. Taken together, our data represent the first evidence of the presence and secretion of orexin system in the muscle of non-mammalian species and its role in mitochondrial fusion and fission, probably through mitochondrial-related genes and their related transcription factors.

***Key words*:** orexin system, muscle, mitochondrial dynamics, fusion, fission, cellular bioenergetics, gene expression

3.2 INTRODUCTION

Orexins (ORX-A and ORX-B) (56), also referred to as hypocretin 1 and 2 (21), are two peptides proteolytically derived from a single precursor (prepro-orexin) produced mainly in the dorsal and lateral hypothalamic areas and perifornical nucleus in rat and humans (21, 56). Prepro-orexin mRNA was detected also in rat testis (56) and duodenum (47). Recently, immunoreactivity for ORX-A was detected in several human peripheral tissues including ganglion cells of the thoracic sympathetic trunk, endocrine cells of the gastrointestinal tract, islets cells of the pancreas and syncytiotrophoblasts and decidual cells of the placenta (46).

Orexins signal through two ubiquitously expressed G-proteins coupled receptors: orexin receptor 1 (ORXR1) and orexin receptor 2 (ORXR2) (8, 25, 31, 35, 42, 56) and regulate several physiological processes in mammals. Consistent with this, central administration of orexins increases food and water intake in mammals (55). Other central effects of orexins include control of wakefulness (14, 41, 44), circadian clock (6), energy and glucose homeostasis (71-73), lipid metabolism (62, 63), heart rate and blood pressure (19, 78), and neuroendocrine response to stress (57) have been reported. As orexins and its receptors are not restricted to the hypothalamus, these aforementioned pleiotropic actions of orexins might be also mediated through a direct interaction with peripheral target tissues (20, 26, 31).

In contrast to mammals, little is known about orexin system distribution and functions in avian (non-mammalian vertebrates) species and such studies are very limited. Ohkubo et al. (48) were the first to clone the chicken prepro-orexin, which is highly conserved among vertebrates and found that it was expressed in periventricular and lateral hypothalamic areas. In a subsequent study, the same group characterized the chicken orexin receptor and found that it corresponded to the type 2 mammalian orexin receptor (49). Both orexin and its receptor were expressed in

chicken brain and gonads (testis and ovary) (49). Central administration of orexin did not affect feed intake in neonatal chickens (24) and its effects on sleep/wakefulness cycle is still controversial (33, 34, 45). The localization of orexin and its receptors in chicken peripheral tissues such as testis, ovary, stomach, and intestine (3, 49), indicate that orexin system may have other physiological functions rather than the regulation of feeding behavior.

Orexin has been recently shown to regulate mitochondrial biogenesis and induce brown adipose tissue (BAT) differentiation and thermogenesis in mammals (61, 66). Mitochondria are dynamic organelles that constantly fuse and divide, and an imbalance of these two processes dramatically alters mitochondrial morphology and function (15). The molecular mechanism that controls mitochondrial dynamics and biogenesis is a complex network requiring the participation and coordination of the nuclear and mitochondrial genomes. This network has been partially unraveled in mammals after the identification of some of the genes responsible for mitochondrial fusion [mitofusins (MFN1 and MFN2), and optic atrophy 1 (OPA1)], fission [dyanamin-related protein 1 (Drp1 or DNM1), fission 1 (FIS1), and mitochondrial protein 18 kDa], and biogenesis [peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and nuclear respiratory factor 1 (NRF1)]. Such mitochondrial network and its integration are still unknown in avian species.

As chickens do not have functional BAT, we undertook the present study to investigate whether orexin system is expressed in the avian muscle, the main site for thermogenesis, and its potential role in the regulation of muscle mitochondrial dynamics, biogenesis and function.

3.3 MATERIALS AND METHODS

3.3.1 ANIMALS

One-day-old male and female Cobb-500 broiler chickens were reared on floor pen in controlled environment room with *ad libitum* access to food (12.6 MJ kg^{-1}, 22% protein) and clean water until 3 weeks of age. The ambient temperature was reduced gradually from 32 to 26°C at 21 days of age and the relative humidity was 55 ± 5%. Birds (n=4 for each gender) were killed by cervical dislocation, and leg muscles, whole brain, hypothalamus, liver, ovary and testis were quickly removed and snap frozen in liquid nitrogen and stored at -80°C until use. Animal care and housing and all procedures were approved by the University of Arkansas Animal Care and Use Committee under protocol (no. 13039).

3.3.2 QUAIL MUSCLE (QM7) CELL CULTURE AND TREATMENTS

QM7 cells were grown in M199 medium (Life technologies, grand Island, NY) complemented with 10% FBS (Life technologies, grand Island, NY), 10% tryptose phosphate (Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. At 80-90% confluence, cells were synchronized overnight in serum free medium and treated with human recombinant orexin A or B (0, 10 and 100 nM) (Interchim, Montlucon, France) for 24h. Untreated cells were used as control. The dose and duration of treatments were chosen based on pilot and previous published experiments (12, 62, 63). QM7 cells were washed twice with phosphate buffered saline (PBS 1X) and incubated for 12h in medium with brefeldin A (0.3 µg/mL) and lysates and medium were subjected to immunoblot analysis.

3.3.3 RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Nuclear, cytoplasmic, and total RNA were isolated, as previously described (30), with some modifications. Briefly, cells were rinsed twice and harvested in 5 ml of ice-cold PBS and centrifuged [1,000 relative centrifugal force (RCF), 4°C, 5 min]. Cells were resuspended in lysis buffer A [10 mM Tris (pH 8.0), 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 2 mM vanadyl ribonucleoside complex (VRC; Sigma-Aldrich)] and incubated on ice for 5 min. One-fifth of the lysate was used for total RNA isolation. The rest was centrifuged (1,000 RCF, 4°C, 3 min) to pellet the nuclei. The nuclear pellet was washed twice and then resuspended in the lysis buffer A. All RNA fractions (total, cytoplasmic, and nuclear) were extracted by TriZOL reagent (Life Technologies) according to the manufacturer's recommendations, DNase-treated, and reverse-transcribed (Quanta Biosciences, Gaithersburg, MD). RNA integrity and quality were assessed using 1% agarose gel electrophoresis, and RNA concentrations and purity were determined for each sample by Take 3 microvolume plate using Synergy HT multimode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems, 7500 real-time PCR system) with Power SYBR Green Master Mix.

Oligonucleotide primers used for chicken prepro-orexin (ORX), ORXR1, ORXR2, uncoupling protein (av-UCP), adenosine nucleotide translocator 1(ANT1), the nuclear sarcoma viral oncogene homolog (Ski), NRF-1, peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ), forkhead box protein O1 (FoxO-1), peroxisome proliferator-activated receptor gamma coactivator 1α and β (PGC-1α and PGC-1β), OxPhos Complex IV subunit I, Cox 5a, mitochondrial single-stranded DNA binding protein 1 (mtSSBP1), mitochondrial transcription factor A (TFAM), MFN1 and MFN2, dynamin-related protein 1 (DNM1 or Drp1), OPA1, OMA1 zinc metallopeptidase (OMA1), mitochondrial fission process 1 (MTFP1),

mitochondrial fission regulator 1 (MTFR1), mitochondrial fission factor 1, and the housekeeping gene ribosomal 18S are summarized in Table 1. The quantitative PCR (qPCR) cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (60).

3.3.4 REVERSE TRANSCRIPTION AND CONVENTIONAL PCR

Total RNA was reverse transcribed as described in the previous section. Long fragments of ORX (411 bp), ORXR1 (832 bp), and 18S (515 bp) were amplified by PCR using oligonucleotide primers specific for chicken ORX, ORXR1, and 18S (Table 1). PCR was performed in 50 µl containing 5 µl of the RT reaction, 1 µl of forward and reverse primer, and 43 µl of platinium PCR SuperMix (Life Technologies). Thermal cycling parameters were as follows: 1 cycle of 94°C for 4 min, followed by 39 cycles of 94°C for 30 s, 50–55°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified fragments were separated on a low-melting point agarose gel (1%), and the appropriate bands were cut out, purified by using spin-column DNA gel extraction kit (Biobasic, Amherst, NY), and stored at -20°C.

3.3.5 NORTHERN BLOT ANALYSIS

For probe preparation, the long PCR fragments (obtained as described above) were cloned using the TOPO PCR cloning kit (Life Technologies) and automatically sequenced using an Applied Biosystems automated sequencer. Then 100 ng of cloned probes were labeled with biotin using North2South Biotin Random Prime Labeling kit, according to manufacturer recommendations (Pierce Thermo Scientific, Rockford, IL). As previously described (32), total RNA (10 µg) was separated by size on 1% agarose, 0.7 M formaldehyde gels and visualized on an ultraviolet transilluminator to ensure consistent loading between different groups and to record the distance of migration of the 18S and 28S rRNA bands. RNA ladder (0.1–10 kb; Life Technologies) was used as markers. Gels were then transferred to a positively charged Nylon membrane (Hybond-N+, GE Healthcare Bio-Sciences, Buckinghamshire, UK) by a vacuum blotting apparatus (VacuGene XL Vacuum Blotting System, GE Healthcare Bio-Sciences). The RNAs were crosslinked to the membranes by ultraviolet irradiation and baked at 80°C for 20–30 min. Membranes were hybridized with biotin-labeled DNA probes (prepro-orexin or 18S) at 42°C overnight. On the following day, the membranes were rinsed twice with 1X SSC, 0.1% SDS at 55°C. Each wash was for 20 min, and then the signals were detected by using the chemiluminescent nucleic acid detection kit (Pierce Thermo Scientific, Rockford, IL) and the FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA).

3.3.6 WESTERN BLOT ANALYSIS

Muscle tissues and QM7 cells were homogenized in lysis buffer (10 mM Tris base, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 0.5% NP-40, protease, and phosphatase inhibitor cocktail). Protein concentrations were determined using Synergy HT

multimode microplate reader (BioTek, Winooski, VT) and a Bradford assay kit (Bio-Rad, Hercules, CA) with BSA as a standard. Proteins (70 µg) were run on 4–12% Novex

Bis-Tris gels (Life Technologies). The transferred membranes were blocked for 1 h at room temperature, and incubated with primary antibodies (diluted 1:500 –1:1,000) at 4°C overnight. The polyclonal antibodies used were as follows: rabbit anti-mouse ORX, rabbit anti-rat ORXR1 and ORXR2, rabbit anti-ANT1, rabbit anti-PGC-1α, rabbit anti-MFN1, rabbit anti-MFN2, and rabbit anti-OPA1. Protein loading was assessed by immunobloting using rabbit anti-β actin or rabbit anti-vinculin. Prestained molecular weight marker (precision plus protein Dual color) was used as a standard (Bio-Rad, Hercules, CA). All of the primary antibodies were purchased from (Interchim, Montlucon, France) except for anti-β actin from Cell Signaling Technology (Danvers, MA), anti-vinculin from Sigma-Aldrich and anti-ANT1 from Pierce Thermo Scientific (Rockford, IL). Anti-PGC-1α, anti-MFN1, anti-MFN2, and anti-OPA1 were from Dr. Nicholas

Greene (University of Arkansas). The secondary antibodies were used (1:5,000) 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 (Proteinsimple).

Image acquisition and analysis were performed by AlphaView software (version 3.4.0, 1993–2011; Proteinsimple).

3.3.7 IMMUNOFLUORESCENCE

Immunofluorescence was performed as previously described (23). Briefly, cells were grown to 50–60% confluence in chamber slides (Lab-Tek, Hatfield, PA) and fixed in methanol for 10 min at -20°C. Cells were blocked with protein block serum-free blocking buffer (Dako, Carpinteria, CA), and incubated with rabbit anti-ORX, anti-ORXR1, or anti-ORXR2 antibody

(1:200; Interchim, Montlucon France) overnight at 4°C and visualized with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Life Technologies). After DAPI counterstaining, slides were cover-slipped in Vectashield (Vector Laboratories, Burlingame, CA). Images were obtained using the Zeiss Imager M2 with a 20X Plan-APOCHROMAT 20X/0.8 objective and a 100X EC PLANNEOFLUOR 100X/1.3 oil objective. The Alexa Fluor 488 fluorophore was observed through filter set 38 1031–346 with an excitation of BP 470/40, beamsplitter of FT 495, and emission spectrum of BP 525/50. Differential interference contrast images were collected using DIC M27 condensers. The Alexa Fluor 488 fluorophore was excited for 500 ms prior to capturing each image using an Axio Cam MR3 camera. All analysis was performed using AxioVision SE64 4.9.1 SP1 software (Carl Zeiss Microscopy 2006–2013).

3.3.8 ANALYSIS OF CELLULAR BIOENERGETICS

Analysis of cellular bioenergetics was conducted using the XF24 extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). Cells were plated into each well of a 24-well Seahorse cell culture plate and allowed to attach. Once the cells were attached, the growth media listed in the previous section were exchanged for fasting (no FBS) media for overnight incubation. Cells were treated then with rORX-A or rORX-B (10 and 100 nM, Interchim, Montlucon, France) for 24 h. Untreated cells were used as a control. The following day, the cell culture media were changed to XF Assay Media (unbuffered DMEM containing 25 mM glucose and 10 mM sodium pyruvate; Seahorse Biosciences, North Billerica, MA) for the XF bioenergetics analysis. Cellular bioenergetics analysis was conducted by first measuring the baseline oxygen consumption rate (OCR), followed by measuring the OCR after sequential addition of chemical mitochondrial effectors; oligomycin (inhibitor of ATP synthesis by blocking the proton channel of the F_0 portion ATP synthase, complex V), carbonyl cyanide-p-

trifluoromethoxyphenylhydrazone, FCCP (uncoupler,), and antimycin A (blocker of O_2 consumption and inhibitor of complex III). All three chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Pilot studies were conducted to optimize QM7 cell seeding density (25 X 10^3 cells/well) and concentrations of oligomycin (1.5 µM), FCCP (250 nM), and antimycin A (10 µM) prior to assessing bioenergetics response to orexin treatments. Nonmitochondrial OCR values for each well were subtracted from basal OCR, OCR following oligomycin, and OCR following FCCP in the same well and bioenergetics components, including oxygen consumption due to ATP synthesis, mitochondrial oxygen reserve capacity, and proton leak were determined, as previously described (28, 39).

3.3.9 MITOCHONDRIAL BIOGENESIS AND DNA QUANTITATION

After orexin treatments for 24 h, mitochondrial content of QM7 cells was determined by measuring mitochondrial DNA levels and mitochondrial mass with MitoTracker Red CMXRos (Life Technologies). Mito-Tracker Red CMXRos is a cell-permeable mitochondrion-selective dye that has been used for mitochondrial mass measurement (61). QM7 cells were washed twice with PBS and stained with 75 nM MitoTracker Red CMXRos for 15 min at 37°C, and fluorescence intensity was detected following the protocol provided by the manufacturer.

DNA was extracted using the EZ-10 spin column genomic DNA kit (Biobasic, Amherst, NY), and the expression of the chicken mtDNA was measured by real-time qPCR in the presence of the oligonucleotide primers summarized in Table 1. The qPCR conditions and parameters are described above.

3.3.10 STATISTICAL ANALYSES

Data were analyzed by one-factor ANOVA. Significant differences among individual group means were determined with Student-Newman-Keuls (SNK)'s multiple range test using the GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla, CA). Significance was set at $P < 0.05$. Data are expressed as the means ± SE.

3.4 RESULTS

3.4.1 BOTH OREXIN AND ITS RELATED RECEPTORS ARE EXPRESSED IN AVIAN MUSCLE TISSUE AND CELLS

Using RT-PCR, a 411 bp and 832 bp partial ORX and ORXR1 cDNA corresponding to nucleotides 51–462 (GenBank accession no. AB056748) and 335–1145 (GenBank accession no. NM_001024584), respectively, were detected in the total RNA extracted from 3 wk-old male broiler chicken leg muscle (Fig. 1A) and quail muscle (QM7) cell lines (Fig. 2A). The same bands (size and sequences) were observed in the positive controls (brain, testis and ovary) (Figs. 1A and 2A). The use of negative control (water instead of cDNA or RNA that was not reverse transcribed) did not produce any PCR product confirming the absence of genomic DNA contamination (Figs. 1A and 2A). Northern blot analysis revealed a single prepro-orexin mRNA signal (3.1 kb) in both brain and QM7 cell (Fig. 2C). Next, using a rabbit anti-mouse prepro-orexin and a rabbit anti-rat orexin receptor 1 and 2 antibodies, Western blot analyses showed bands of 16, 48, and 51 kDa in chicken muscle and QM7 cells corresponding to prepro-orexin, ORXR1, and ORXR2, respectively (Figs. 1B and 2B). These molecular weights were observed in the positive controls (whole brain and hypothalamus) and correspond to those of prepro-orexin and ORXR1/2 found in mammalian brain and chicken gastroenteric tract. Immunofluorescence

staining demonstrated abundant immunoreactivity for orexin and its receptors in the cytoplasmic compartment of QM7 cells, which has been confirmed by Western blot using cell fractions (Fig. 2, D–F). When the primary antibodies were replaced by isotype antibodies or nonimmune PBS solution, no reactivity could be observed (data not shown).

3.4.2 SECRETION OF OREXIN IN QM7 CELLS

The first 33 amino acids of prepro-orexin exhibit characteristics of a secretory signal sequence (hydrophobic core followed by residues with small polar side chains) in combination with its presence in the circulation indicate that orexin is a secretory protein (4). To ascertain whether orexin is, in fact, secreted, QM7 cell monolayers were incubated in serum-free medium for 24 h, after which orexin levels in lysates (Fig. 2B) and medium (Fig. 3A) were assessed by SDS-PAGE and immunoblot analysis with antibody against prepro-orexin. Orexin was detected in both the cell lysate (Fig. 2B) and the medium from untreated or orexin-treated cells (positive control) (Fig. 3A). Consistent with the predicted molecular weight of orexin, the immunoreactive protein exhibited a mobility by SDS-PAGE corresponding to molecular mass of 16 KDa (Fig. 3A). It is, however, noteworthy that a second band with higher molecular weight (~230 kDa) was detected in the medium and not in the cell lysate. Furthermore, the time course study of orexin secretion in QM7 cells showed that orexin steadily accumulated in the medium over 48 h period (Fig. 3B). Treatment of cells with brefeldin A, an inhibitor of translocation of secretory proteins from the endoplasmic reticulum to the Golgi apparatus (36), for 12 h blocked secretion of orexin into the medium leading to increased intracellular expression (Fig. 3C).

3.4.3 OREXINS REGULATE THE EXPRESSION OF ITS OWN SYSTEM IN QM7 CELLS

The effects of 10 and 100 nM of recombinant human orexin A (rORX-A) or B (rORX-B) on the expression (mRNA and protein) of ORX and its related receptors (ORXR1 and ORXR2) in QM7 cells are shown in Fig. 4. Treatment of QM7 cells for 24 h with either 10 or 100 nM rORX-A upregulated ORX and ORXR1, but not ORXR2, gene expression ($P < 0.05$; Fig. 4A). Treating cells with rORX-B downregulated ORX and ORXR2 and increased ORXR1 mRNA levels ($P < 0.05$ Fig. 4C). ORX, ORXR1, and ORXR2 proteins showed the same expression patterns as their corresponding genes (Fig. 4, B and D). These effects seemed to be dose-dependent.

3.4.4 OREXINS DIFFERENTIALLY REGULATE MITOCHONDRIAL-RELATED GENES AND THEIR TRANSCRIPTIONAL REGULATORS IN QM7 CELLS

The rORX-A and rORX-B treatments had differential effects on av-UCP, av-ANT, Ski, and NRF-1 expression, as shown in Fig. 5. Whereas rORX-A had no effect on av-UCP mRNA abundance (Fig. 5A), it downregulated the expression of av-ANT (mRNA and protein levels), Ski, and NRF-1 in a dose-dependent manner (Fig. 5, B–D). rORX-B, however, downregulated the expression of av-UCP and increased the expression of Ski and NRF-1 without altering the expression of av-ANT (Fig. 5, A–D). These changes coincided with a significant downregulation of PPARγ, PPARα, and FoxO-1 expression by both doses of rORX-A (Fig. 6, A, B, and E). A high dose of rORX-A significantly downregulated the expression of PGC-1β, and both doses did not alter PGC-1α mRNA abundance (Fig. 6, C and D). rORX-B, however, induced the expression of these transcription factors in a dose-dependent manner, but the effects were

statistically significant only for PGC-1β, PGC-1α, and FoxO-1 with the high dose (Fig. 6, C, D–E).

3.4.5 OREXINS REGULATE MITOCHONDRIAL BIOENERGETICS IN QM7 CELLS

Bioenergetics in QM7 cells treated with 10 and 100 nM of rORX-A and rORX-B were assessed by monitoring basal oxygen consumption rate (OCR) followed by sequential treatment of cells with oligomycin, FCCP, and antimycin A as shown in Fig. 7A. As described previously (27), the decrease in OCR following oligomycin (which blocks ATP synthase) reveals OCR attributed to ATP synthesis activity. Maximal OCR is revealed in response to the uncoupling compound FCCP, and the difference between maximal OCR and basal OCR (prior to oligomycin) represents mitochondrial oxygen reserve capacity that cells can draw upon when increased energy production is needed. Oxygen consumption that remains following treatment with antimycin A is attributed to nonmitochondrial OCR (i.e., OCR due to activities other than nonmitochondrial c oxidase activity, such as mitochondrial reactive oxygen species production, oxidase activities, etc.). The amount of OCR attributed to proton leak is determined by the difference between oligomycin and antimycin A-inhibited OCR. When the nonmitochondrial component of cellular OCR was subtracted and by setting maximal OCR following FCCP at 100%, the effects of ORX-A and ORX-B on ATP synthesis, reserve capacity, and proton leak were determined and are presented in Fig. 7, B–D. ATP synthesis was slightly elevated by both orexins, but the effect was not statistically discernable (Fig. 7B). Analysis of reserve capacity indicated no effect of both doses of rORX-A and rORX-B (Fig. 7C); however, proton leak was decreased by 10 nM of rORX-A, and by 100 nM of rORX-B ($P < 0.05$, Fig. 7D).

3.4.6 OREXINS DIFFERENTIALLY REGULATE MITOCHONDRIAL BIOGENESIS IN QM7 CELLS

Mitochondrial DNA (mtDNA) replication and quantitation are a necessary component of mitochondrial biogenesis. MtDNA and mtSSBP1 expressions were measured in QM7 cells treated with orexins as shown in Fig. 9. In contrast to rORX-A, in which both doses significantly downregulated mtDNA and upregulated mtSSBP1 expression, rORX-B (high dose) significantly increased mtDNA expression without affecting mtSSBP1 levels (Fig. 9, A and B). Consistent with these observations and in contrast to rORX-A, rORX-B increased mitochondrial content as visualized with MitoTracker Red probe (Fig. 8). Neither rORX-A nor rORX-B affected the expression of the mitochondrial transcription factor TFAM (data not shown). The expression of Cox IV and Cox 5a genes, commonly used markers for mitochondrial mass and biogenesis, was determined. The high dose (100 nM) of rORX-A decreased Cox IV gene expression; however, the high-dose of rORX-B increased Cox IV and Cox 5a mRNA levels compared with untreated cells ($P < 0.05$, Fig. 9, C and D).

3.4.7 OREXINS DIFFERENTIALLY REGULATE MITOCHONDRIAL DYNAMICS IN QM7 CELLS

The expression of four genes related to mitochondrial fusion and three genes related to mitochondrial fission were measured as shown in Fig. 10. Recombinant ORX-B at high dose significantly induced the expression of MFN2, OPA1, and OMA1, but decreased the mRNA levels of MFN1 (Fig. 10, A–D). The same effect was observed at the protein levels (Fig. 10E). However, rORX-A significantly downregulated the expression of MFN1 and OMA1 with both doses, and OPA1 with the high dose, but did not affect that of MNF2 (Fig. 10, A–D). Interestingly, and in contrast to rORX-B, where no significant effects were observed, rORX-A

upregulated the expression of mitofission-related genes MTFP1, DNM1, and MTFR1 ($P < 0.05$, Fig. 10, F–H).

3.5 DISCUSSION

The growing obesity epidemic has sparked numerous studies on the identification and the roles of feeding-related hypothalamic neuropeptides in the regulation of energy homeostasis. Orexins (A and B) or hypocretins (1 and 2) are multifunctional neuropeptides that bind to two receptors, ORXR1 and ORXR2, to regulate feeding behavior (56), sleep-wake cycle (41), circadian clock (6), and glucose and lipid metabolism in mammals (62). Such physiological roles in avian (nonmammalian vertebrate) species are not well defined yet, although orexins are expressed in several tissues, including hypothalamus (48), testis, ovary (49), and gastroenteric tract (3). Intracerebroventricular injection of orexin did not affect feeding behavior in neonatal chickens (24). Katayama et al. (33) reported that intracerebroventricular administration of orexin A induced arousal in layer-type chickens; however, Miranda et al. (45) could not find evidence that hypothalamic orexin plays a similar role. This suggests that orexins may have other physiological roles in avian species.

Because birds lack functional BAT, and avian muscle is the main site for thermogenesis (43), the recently discovered function of orexin in mammalian BAT development, differentiation, and thermogenesis (61) prompted our group to investigate whether orexin system is expressed in chicken muscle.

The present study is the first to report the presence of orexin system in chicken skeletal muscle and quail QM7 cells. We provided novel evidence that avian skeletal muscle expresses both prepro-orexin and its related receptors (ORXR1 and ORXR2). In birds, one type of orexin receptor (ORXR1) has been previously identified (49). In the present study, we used specific

primers for the predicted 2,170 nucleotide chicken ORXR2 that has 43% homology with the previous identified 1,869 nucleotide chicken ORXR1 and amplified only one band corresponding to the expected size and sequence by real-time PCR. Additionally, by using orexin and orexin receptor polyclonal antisera raised against the well-conserved NH2 terminal region of mouse ORX and the COOH and NH2 terminus of the rat ORXR1/2, we detected immunoreactivity to ORX, ORXR1, and ORXR2 in chicken muscle tissue and QM7 cells by Western blot and immunofluorescence staining, corroborating previous data in chicken intestine, stomach, and pancreas (3). Orexin receptor ORXR1 and two isoforms of ORXR2 (ORX2αR and ORX2βR) have been also shown to be expressed in mouse muscle (17).

The expression of orexin and its related receptors (ORXR1 and ORXR2) in avian muscle and the detection of orexin in culture media suggest that avian muscle might be a source for orexin production and secretion and indicate possible autocrine, paracrine, and/or endocrine roles. In support of this, rORX-A and rORX-B differentially regulate its own gene and protein expression. Although the underlying mechanism is still unknown, the divergent effects of orexin A and orexin B on orexin expression might be related to their structure (presence of disulfide bonds in orexin A and not in orexin B) and their different binding affinity to ORXR2, since they had similar effects on ORXR1 expression. In mammals, indeed, the ORXR1 preferentially binds orexin-A, whereas ORXR2 binds both peptides (orexin A and B), apparently with similar affinity (56). Interestingly, it is likely that ORXR2 and not ORXR1 mediated the activation of the dorsomedial and lateral hypothalamic neurons leading to the adaptive response (thermogenesis and physical activity) to diet restriction in rat (59). As we used here recombinant human orexins and chicken orexin A and B that showed approximately only 85% and 65% identity with the corresponding mammalian sequences (48), further binding studies are warranted. Moreover, the

potential involvement of the structure, the function of the cytoplasmic tails, and the desensitization of ORXR1/2 in the divergent effects of orexins are not ruled out.

In line with the aforementioned divergent effects, orexins differentially regulated the expression of mitochondrial-related genes (av-UCP, av-ANT, Ski, NRF-1) and their related transcription factors and coactivators (PPARα, PPARγ, PGC-1β, PGC-1α, and FoxO-1) with downregulation for orexin A and upregulation for orexin B. Such divergent effects of orexins on gastric acid secretion and arousal have been previously reported in rats and chickens, respectively (33, 52). In contrast to the well-known role of UCP1 in BAT thermogenesis, the function of av-UCP that is homologous to mammalian UCP2 and UCP3 is still under debate. We previously hypothesized that av-UCP might be involved in avian muscle adaptive thermogenesis and energy dissipation as heat based on its upregulation during cold exposure and after chronic treatment with glucagon (53). Teulier et al. (69), however, suggested that av-UCP might not be involved in heat production through mitochondrial uncoupling. Two other independent groups reported a possible role for av-UCP in mitochondrial reactive oxygen species (ROS) production (1, 54). As in mammals, the avian mitochondrial anion carrier ANT (also known as avATP/ADP carrier) has been reported to be involved in thermogenesis and mitochondrial ROS control (11, 67, 70).

Mitochondria are responsible for producing over 90% of the ATP for the cell by oxidative phosphorylation associated with the electron transport chain (37), and mitochondrial dysfunction in skeletal muscle is closely associated with insulin resistance in mammals (50) and feed inefficiency in chickens (10). The alteration of mitochondrial-related genes and their transcriptional regulators indicated that orexin might control avian muscle mitochondrial dynamics and respiratory function. Mitochondria are constantly undergoing both fusion

(biogenesis) and fission (15). Fusion involves complete mtDNA replication; however, fission occurs when the existing copies of mtDNA are simply divided between the new fissioned mitochondria (15). In support of our above-mentioned hypothesis, orexins differently regulated mitochondrial dynamics-related genes. While rORX-B increased mitofusion, as indicated by the upregulation of MFN2, OPA1, and OMA1 gene expression, rORX-A promoted mitofission as reflected in the increased expression of MTFP1, DNM1, and MTFR1 genes. Despite the unchanged expression of TFAM (the direct regulator of mtDNA replication/transcription), the increased levels of mtDNA and CoxIV and Cox5a mRNA indicated that rORX-B might induce mitochondrial content and mass through other TFAM isoforms (22), TFAM posttranslational modifications (58), and/or other transcription factors (9). An increase in mitochondrial content following rORX-B treatment is also supported by the increased MitoTracker staining.

The effects of orexin on avian muscle mitochondrial dynamics are very likely mediated through NRF-1, Ski, and their related transcription regulators (PPARγ, PGC-1β, PGC-1α, and FoxO-1). Although their roles are not well defined in birds, the NRF-1 and Ski play a key role in mammalian mitochondrial biogenesis and respiratory function (75, 76). Mammalian NRF-1 is a direct target for PPARγ and PGC-1 (75). PGC-1 stimulates induction of NRF-1 gene expression and coactivates its transcriptional function on TFAM promoter (75). MFN2 expression has been shown to be controlled by two distinct regulatory mechanisms, including PGC-1α/estrogen-related receptor (ERR) α- and PGC-1β/ERRα-dependent signaling pathways (13, 40, 64). FoxO-1 has been shown to interact with heme oxygenase 1 (HMOX1) and regulate mitochondrial biogenesis and function through sirtuin 1 (SIRT1) and PGC-1 pathways (18).

To gain better insight into the physiological roles of orexins in avian muscle and to evaluate whether the alteration of mitochondrial dynamics impairs mitochondrial respiratory

function, we measured bioenergetics components in orexin-treated and untreated (control) QM7 cells using extracellular XF-24 Flux analyzer. On the basis of our findings, orexin-induced mitochondrial fusion/fission seemed not to affect ATP synthesis. In mammals, the impact of mitochondrial dynamics on mitochondrial energization and function varied upon the experimental model (cell line) and conditions. For instance, inhibition of mitochondrial fusion by manipulation of MFN2/ OPA1 expression caused a reduction in mitochondrial membrane potential and activity of respiratory complexes. Overexpression of a truncated form of MFN2 (Δ602–757) enhances mitochondrial metabolism independent of fusion activity (51); however, other MFN2 mutants did not induce metabolic alterations (68). Some OPA1 mutants showed impaired ATP synthesis driven by complex I substrates and decreased rates of mitofusion (77); however, other mutants showed normal mitochondrial activity and bioenergetics (65). One study (16) reported that overexpression of OPA1 did not modify mitochondrial metabolism in MEF cells. Emerging evidence indicated that alterations in mitofission-related proteins such as DNM1 and FIS1 produced similar effects on mitochondrial metabolism (7). Together, these data indicated that specific mutations in mitochondrial dynamics-related genes alter mitochondrial metabolism.

Although we did not see any effect on ATP synthesis in the present study, we anticipate the possibility that mitochondrial dynamics might regulate mitochondrial metabolism via different pathways, including a direct “physical” effect of fusion/fission and/or downstream signaling cascades of dynamics-related genes that cause direct changes in the expression of oxidative phosphorylation subunits, as previously shown (51). Therefore, compensatory or feedback mechanisms between fusion-fission events or between fusion- and fission-related genes might be expected to maintain mitochondrial homeostasis and might explain the absence of

metabolic alteration after orexins treatments. In addition, it is possible that some mitochondria might be only tethered or connected but not completely fissioned or fusioned; thus, an additional level of complexity in the regulation of mitochondrial dynamics is likely to occur (74). Finally, we measured only a few genes, and further analysis of additional genes and proteins involved in mitochondrial network is required, and a demonstration of how they are regulated in vivo conditions is mandatory.

The findings of the present study are the first evidence of orexin system expression and secretion in muscle and unveil its important role in mitochondrial network.

3.5.1 PERSPECTIVES AND SIGNIFICANCE

Orexins are originally identified as feeding-related hypothalamic neuropeptides that have effects on diverse processes, including obesity and diabetes. A major goal is to identify and unravel the mode of orexin action in these processes using different experimental models. In the present study, we used chickens as a model of choice because they are naturally hyperglycemic compared with mammals (38), insulin-resistant (2), lack of functional BAT (5), and prone to obesity (29). We provided evidence that orexin system is expressed in chicken muscle and plays a key role in mitochondrial dynamics. Because chicken muscle is the main site for thermogenesis and because mitochondrial functions are implicated in many (patho)-physiological processes, our findings open new vistas on the role of orexin in muscle energy metabolism. As genetic selection for rapid growth, driven by economic demands, have resulted in dramatic increase in body weight of broiler chickens arising from increased skeletal muscle mass, further studies investigating the role of orexin in muscle development, myogenesis, insulin sensitivity, and glucose uptake are warranted. Insights into the molecular mechanisms and physiological role of orexin in skeletal muscle are of uppermost interest not only in animal biology for health and feed

efficiency improvement, but also in molecular medicine for pathophysiological understanding and therapeutic perspectives.

3.6 REFERENCES

1. Abe T, Mujahid A, Sato K, Akiba Y, Toyomizu M. Possible role of avian uncoupling protein in down-regulating mitochondrial superoxide production in skeletal muscle of fasted chickens. *FEBS Lett* 580: 4815–4822, 2006.

2. Akiba Y, Chida Y, Takahashi T, Ohtomo Y, Sato K, Takahashi K. Persistent hypoglycemia induced by continuous insulin infusion in broiler chickens. *Br Poult Sci* 40: 701–705, 1999.

3. Arcamone N, D'Angelo L, de Girolamo P, Lucini C, Pelagalli A, Castaldo L. Orexin and orexin receptor like peptides in the gastroenteric tract of *Gallus domesticus:* An immunohistochemical survey on presence and distribution. *Res Vet Sci* 96: 234–240, 2014.

4. Arihara Z, Takahashi K, Murakami O, Totsune K, Sone M, Satoh F, Ito S, Mouri T. Immunoreactive orexin-A in human plasma. *Peptides* 22: 139–142, 2001.

5. Barre H, Cohen-Adad F, Duchamp C, Rouanet JL. Multilocular adipocytes from Muscovy ducklings differentiated in response to cold acclimation. *J Physiol* 375: 27–38, 1986.

6. Belle MD, Hughes AT, Bechtold DA, Cunningham P, Pierucci M, Burdakov D, Piggins HD. Acute suppressive and long-term phase modulation actions of orexin on the mammalian circadian clock. *J Neurosci* 34: 3607–3621, 2014.

7. Benard G, Bellance N, James D, Parrone P, Fernandez H, Letellier T, Rossignol R. Mitochondrial bioenergetics and structural network organization. *J Cell Sci* 120: 838–848, 2007.

8. Blanco M, Lopez M, Garcia-Caballero T, Gallego R, Vazquez-Boquete A, Morel G, Senaris R, Casanueva F, Dieguez C, Beiras A. Cellular localization of orexin receptors in human pituitary. *J Clin Endocrinol Metab* 86: 1616–1619, 2001.

9. Blumberg A, Sri Sailaja B, Kundaje A, Levin L, Dadon S, Shmorak S, Shaulian E, Meshorer E, Mishmar D. Transcription factors bind negatively selected sites within human mtDNA genes. *Genome Biol Evol* 6: 2634–2646, 2014.

10. Bottje WG, Carstens GE. Association of mitochondrial function and feed efficiency in poultry and livestock species. *J Anim Sci* 87: E48–E63, 2009.

11. Brand MD, Pakay JL, Ocloo A, Kokoszka J, Wallace DC, Brookes PS, Cornwall EJ. The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *Biochem J* 392: 353–362, 2005.

12. Butterick TA, Nixon JP, Billington CJ, Kotz CM. Orexin A decreases lipid peroxidation and apoptosis in a novel hypothalamic cell model. *Neurosci Lett* 524: 30–34, 2012.

13. Cartoni R, Leger B, Hock MB, Praz M, Crettenand A, Pich S, Ziltener JL, Luthi F, Deriaz O, Zorzano A, Gobelet C, Kralli A, Russell AP. Mitofusins 1/2 and ERRα expression are increased in human skeletal muscle after physical exercise. *J Physiol* 567: 349–358, 2005.

14. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 98: 437–451, 1999.

15. Chen H, Chan DC. Emerging functions of mammalian mitochondrial fusion and, fission. *Hum Mol Genet 14 Spec No 2*: R283–R289, 2005.

16. Chen H, Chomyn A, Chan DC. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem* 280: 26185–26192, 2005.

17. Chen J, Randeva HS. Genomic organization of mouse orexin receptors: characterization of two novel tissue-specific splice variants. *Mol Endocrinol* 18: 2790–2804, 2004.

18. Cheng Z, Tseng Y, White MF. Insulin signaling meets mitochondria in metabolism. *Trends Endocrinol Metab* 21: 589–598, 2010.

19. Ciriello J, Li Z, de Oliveira CV. Cardioacceleratory responses to hypocretin-1 injections into rostral ventromedial medulla. *Brain Res* 991: 84–95, 2003.

20. Dalal MA, Schuld A, Haack M, Uhr M, Geisler P, Eisensehr I, Noachtar S, Pollmacher T. Normal plasma levels of orexin A (hypocretin-1) in narcoleptic patients. *Neurology* 56: 1749–1751, 2001.

21. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, 2nd Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA* 95: 322–327, 1998.

22. De Virgilio C, Pousis C, Bruno S, Gadaleta G. New isoforms of human mitochondrial transcription factor A detected in normal and tumoral cells. *Mitochondrion* 11: 287–295, 2011.

23. Dridi S, Hirano Y, Tarallo V, Kim Y, Fowler BJ, Ambati BK, Bogdanovich S, Chiodo VA, Hauswirth WW, Kugel JF, Goodrich JA, Ponicsan SL, Hinton DR, Kleinman ME, Baffi JZ, Gelfand BD, Ambati J. ERK1/2 activation is a therapeutic target in age-related macular degeneration. *Proc Natl Acad Sci USA* 109: 13,781–13,786, 2012.

24. Furuse M, Ando R, Bungo T, Ao R, Shimojo M, Masuda Y. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. *Br Poult Sci* 40: 698–700, 1999.

25. Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. *Neuroscience* 103: 777–797, 2001.

26. Higuchi S, Usui A, Murasaki M, Matsushita S, Nishioka N, Yoshino A, Matsui T, Muraoka H, Ishizuka Y, Kanba S, Sakurai T. Plasma orexin-A is lower in patients with narcolepsy. *Neurosci Lett* 318: 61–64, 2002.

27. Hill BG, Awe SO, Vladykovskaya E, Ahmed Y, Liu SQ, Bhatnagar A, Srivastava S. Myocardial ischaemia inhibits mitochondrial metabolism of 4-hydroxy-trans-2-nonenal. *Biochem J* 417: 513–524, 2009.

28. Hill BG, Dranka BP, Zou L, Chatham JC, Darley-Usmar VM. Importance of the bioenergetic reserve capacity in response to cardiomyocyte stress induced by 4-hydroxynonenal. *Biochem J* 424: 99–107, 2009.

29. Hood RL. The cellular basis for growth of the abdominal fat pad in broiler-type chickens. *Poult Sci* 61: 117–121, 1982.

30. Hwang HW, Wentzel EA, Mendell JT. A hexanucleotide element directs microRNA nuclear import. *Science* 315: 97–100, 2007.

31. Johren O, Neidert SJ, Kummer M, Dendorfer A, Dominiak P. Preproorexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. *Endocrinology* 142: 3324–3331, 2001.

32. Kaneko H, Dridi S, Tarallo V, Gelfand BD, Fowler BJ, Cho WG, Kleinman ME, Ponicsan SL, Hauswirth WW, Chiodo VA, Kariko K, Yoo JW, Lee DK, Hadziahmetovic M, Song Y, Misra S, Chaudhuri G, Buaas FW, Braun RE, Hinton DR, Zhang Q, Grossniklaus HE, Provis JM, Madigan MC, Milam AH, Justice NL, Albuquerque RJ, Blandford AD, Bogdanovich S, Hirano Y, Witta J, Fuchs E, Littman DR, Ambati BK, Rudin CM, Chong MM, Provost P, Kugel JF, Goodrich JA, Dunaief JL, Baffi JZ, Ambati J. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature* 471: 325–330, 2011.

33. Katayama S, Hamasu K, Shigemi K, Cline MA, Furuse M. Intracerebroventricular injection of orexin-A, but not orexin-B, induces arousal of layer-type neonatal chicks. *Comp Biochem Physiol A* 157: 132–135, 2010.

34. Katayama S, Shigemi K, Cline MA, Furuse M. Clorgyline inhibits orexin-A-induced arousal in layer-type chicks. *J Vet Med Sci* 73: 471–474, 2011.

35. Kirchgessner AL, Liu M. Orexin synthesis and response in the gut. *Neuron* 24: 941–951, 1999.

36. Klausner RD, Donaldson JG, Lippincott-Schwartz J. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol* 116: 1071–1080, 1992.

37. Knopp A, Thierfelder S, Doepner B, Benndorf K. Mitochondria are the main ATP source for a cytosolic pool controlling the activity of ATP-sensitive K^+ channels in mouse cardiac myocytes. *Cardiovasc Res* 52: 236–245, 2001.

38. Krzysik-Walker SM, Ocon-Grove OM, Maddineni SR, Hendricks GL, 3rd, Ramachandran R. Is visfatin an adipokine or myokine? Evidence for greater visfatin expression in skeletal muscle than visceral fat in chickens. *Endocrinology* 149: 1543–1550, 2008.

39. Lassiter K, Dridi S, Piekarski A, Greene E, Hargis B, Kong BW, Bottje W. Bioenergetics in chicken embryo fibroblast cells: evidence of lower proton leak in spontaneously immortalized chicken embryo fibroblasts compared with young and senescent primary chicken embryo fibroblast cells. *Comp Biochem Physiol A* 175: 115–123, 2014.

40. Liesa M, Borda-d'Agua B, Medina-Gomez G, Lelliott CJ, Paz JC, Rojo M, Palacin M, Vidal-Puig A, Zorzano A. Mitochondrial fusion is increased by the nuclear coactivator PGC-1β. *PloS One* 3: e3613, 2008.

41. Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98: 365–376, 1999.

42. Lopez M, Senaris R, Gallego R, Garcia-Caballero T, Lago F, Seoane L, Casanueva F, Dieguez C. Orexin receptors are expressed in the adrenal medulla of the rat. *Endocrinology* 140: 5991–5994, 1999.

43. Marjoniemi K, Hohtola E. Shivering thermogenesis in leg and breast muscles of galliform chicks and nestlings of the domestic pigeon. *Physiol Biochem Zool* 72: 484–492, 1999.

44. Mignot E, Thorsby E. Narcolepsy and the HLA system. *N Engl J Med* 344: 692, 2001.

45. Miranda B, Esposito V, de Girolamo P, Sharp PJ, Wilson PW, Dunn IC. Orexin in the chicken hypothalamus: immunocytochemical localization and comparison of mRNA concentrations during the day and night, and after chronic food restriction. *Brain Res* 1513: 34–40, 2013.

46. Nakabayashi M, Suzuki T, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Date F, Takeyama J, Darnel AD, Moriya T, Sasano H. Orexin-A expression in human peripheral tissues. *Mol Cell Endocrinol* 205: 43–50, 2003.

47. Naslund E, Ehrstrom M, Ma J, Hellstrom PM, Kirchgessner AL. Localization and effects of orexin on fasting motility in the rat duodenum. *Am J Physiol Gastrointest Liver Physiol* 282: G470–G479, 2002.

48. Ohkubo T, Boswell T, Lumineau S. Molecular cloning of chicken prepro-orexin cDNA and preferential expression in the chicken hypothalamus. *Biochim Biophys Acta* 1577: 476–480, 2002.

49. Ohkubo T, Tsukada A, Shamoto K. cDNA cloning of chicken orexin receptor and tissue distribution: sexually dimorphic expression in chicken gonads. *J Mol Endocrinol* 31: 499–508, 2003.

50. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300: 1140–1142, 2003.

51. Pich S, Bach D, Briones P, Liesa M, Camps M, Testar X, Palacin M, Zorzano A. The Charcot-Marie-Tooth type 2A gene product, Mfn2, up-regulates fuel oxidation through expression of OXPHOS system. *Hum Mol Genet* 14: 1405–1415, 2005.

52. Piper DC, Upton N, Smith MI, Hunter AJ. The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. *Eur J Neurosci* 12: 726–730, 2000.

53. Raimbault S, Dridi S, Denjean F, Lachuer J, Couplan E, Bouillaud F, Bordas A, Duchamp C, Taouis M, Ricquier D. An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. *Biochem J* 353: 441–444, 2001.

54. Rey B, Spee M, Belouze M, Girard A, Prost J, Roussel D, Duchamp C. Oxygen recovery up-regulates avian UCP and ANT in newly hatched ducklings. *J Comp Physiol B* 180: 239–246, 2010.

55. Sakurai T. Orexins and orexin receptors: implication in feeding behavior. *Regul Pept* 85: 25–30, 1999.

56. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92: 573–585, 1998.

57. Samson WK, Bagley SL, Ferguson AV, White MM. Hypocretin/orexin type 1 receptor in brain: role in cardiovascular control and the neuroendocrine response to immobilization stress. *Am J Physiol Regul Integr Comp Physiol* 292: R382–R387, 2007.

58. Santos JM, Mishra M, Kowluru RA. Posttranslational modification of mitochondrial transcription factor A in impaired mitochondria biogenesis: implications in diabetic retinopathy and metabolic memory phenomenon. *Exp Eye Res* 121: 168–177, 2014.

59. Satoh A, Brace CS, Ben-Josef G, West T, Wozniak DF, Holtzman DM, Herzog ED, Imai S. SIRT1 promotes the central adaptive response to diet restriction through activation of the dorsomedial and lateral nuclei of the hypothalamus. *J Neurosci* 30: 10,220 –10,232, 2010.

60. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3: 1101–1108, 2008.

61. Sellayah D, Bharaj P, Sikder D. Orexin is required for brown adipose tissue development, differentiation, and function. *Cell Metab* 14: 478–490, 2011.

62. Shen Y, Zhao Y, Zheng D, Chang X, Ju S, Guo L. Effects of orexin A on GLUT4 expression and lipid content via MAPK signaling in 3T3–L1 adipocytes. *J Steroid Biochem Mol Biol* 138: 376–383, 2013.

63. Skrzypski M, TTL, Kaczmarek P, Pruszynska-Oszmalek E, Pietrzak P, Szczepankiewicz D, Kolodziejski PA, Sassek M, Arafat A, Wiedenmann B, Nowak KW, Strowski MZ. Orexin A stimulates glucose uptake, lipid accumulation and adiponectin secretion from 3T3–L1 adipocytes and isolated primary rat adipocytes. *Diabetologia* 54: 1841–1852, 2011.

64. Soriano FX, Liesa M, Bach D, Chan DC, Palacin M, Zorzano A. Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1α, estrogen-related receptor-α, and mitofusin 2. *Diabetes* 55: 1783–1791, 2006.

65. Spinazzi M, Cazzola S, Bortolozzi M, Baracca A, Loro E, Casarin A, Solaini G, Sgarbi G, Casalena G, Cenacchi G, Malena A, Frezza C, Carrara F, Angelini C, Scorrano L, Salviati L, Vergani L. A novel deletion in the GTPase domain of OPA1 causes defects in mitochondrial morphology and distribution, but not in function. *Hum Mol Genet* 17: 3291–3302, 2008.

66. Swami M. Metabolism: Orexin acts on brown fat. *Nat Med* 17: 1356– 1356, 2011.

67. Talbot DA, Duchamp C, Rey B, Hanuise N, Rouanet JL, Sibille B, Brand MD. Uncoupling protein and ATP/ADP carrier increase mitochondrial proton conductance after cold adaptation of king penguins. *J Physiol* 558: 123–135, 2004.

68. Tamai S, Iida H, Yokota S, Sayano T, Kiguchiya S, Ishihara N, Hayashi J, Mihara K, Oka T. Characterization of the mitochondrial protein LETM1, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. *J Cell Sci* 121: 2588–2600, 2008.

69. Teulier L, Rouanet JL, Letexier D, Romestaing C, Belouze M, Rey B, Duchamp C, Roussel D. Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency. *J Exp Biol* 213: 2476–2482, 2010.

70. Toyomizu M, Ueda M, Sato S, Seki Y, Sato K, Akiba Y. Cold-induced mitochondrial uncoupling and expression of chicken UCP and ANT mRNA in chicken skeletal muscle. *FEBS Lett* 529: 313–318, 2002.

71. Tsuneki H, Murata S, Anzawa Y, Soeda Y, Tokai E, Wada T, Kimura I, Yanagisawa M, Sakurai T, Sasaoka T. Age-related insulin resistance in hypothalamus and peripheral tissues of orexin knockout mice. *Diabetologia* 51: 657–667, 2008.

72. Tsuneki H, Wada T, Sasaoka T. Role of orexin in the central regulation of glucose and energy homeostasis. *Endocr J* 59: 365–374, 2012.

73. Tsuneki H, Wada T, Sasaoka T. Role of orexin in the regulation of glucose homeostasis. *Acta Physiol (Oxf)* 198: 335–348, 2010.

74. Twig G, Graf SA, Wikstrom JD, Mohamed H, Haigh SE, Elorza A, Deutsch M, Zurgil N, Reynolds N, Shirihai OS. Tagging and tracking individual networks within a complex mitochondrial web with photoactivatable GFP. *Am J Physiol Cell Physiol* 291: C176–C184, 2006.

75. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98: 115–124, 1999.

76. Ye F, Lemieux H, Hoppel CL, Hanson RW, Hakimi P, Croniger CM, Puchowicz M, Anderson VE, Fujioka H, Stavnezer E. Peroxisome proliferator-activated receptor gamma (PPARγ) mediates a Ski oncogeneinduced shift from glycolysis to oxidative energy metabolism. *J Biol Chem* 286: 40,013–40,024, 2011.

77. Zanna C, Ghelli A, Porcelli AM, Karbowski M, Youle RJ, Schimpf S, Wissinger B, Pinti M, Cossarizza A, Vidoni S, Valentino ML, Rugolo M, Carelli V. OPA1 mutations associated with dominant optic atrophy impair oxidative phosphorylation and mitochondrial fusion. *Brain* 131: 352–367, 2008.

78. Zhang W, Fukuda Y, Kuwaki T. Respiratory and cardiovascular actions of orexin-A in mice. *Neurosci Lett* 385: 131–136, 2005.

3.7 TABLES AND FIGURES

Table 3.1 Oligonucleotide primers used for the detection of target genes.

Gene	Accession Number[a]	Primer Sequence (5' --> 3')	Orientation	Product Size, bp
PCR				
ORX	AB056748	ATGGAGGTGCCCAACGCCAAGCTGC	Forward	411
		CAGGTCCTTCTCAGCGTGCTCCTGG	Reverse	
ORXR1	AB110634	AACCACCACATGAGGACAGTCACC	Forward	832
		CGCAGCACTATTGGCATATACAA	Reverse	
18S	AF173612	CTGCCCTATCAACTTTCG	Forward	515
		CATTATTCCTAGCTGGAG	Reverse	
QPCR				
ORX	AB056748	CCAGGAGCACGCTGAGAAG	Forward	67
		CCCATCTCAGTAAAAGCTCTTTGC	Reverse	
ORXR1	AB110634	TGCGCTACCTCTGGAAGGA	Forward	58
		GCGATCAGCGCCCATTC	Reverse	
ORXR2	XM_004945362	AAGTGCTGAAGCAACCATTGC	Forward	61
		AAGGCCACACTCTCCCTTCTG	Reverse	
avUCP	NM_204107	TGGCAGCGAAGCGTCAT	Forward	59
		TGGGATGCTGCGTCCTATG	Reverse	
avANT	AB088686	GCAGCTGATGTCGGCAAA	Forward	56
		CAGTCCCCGAGACCAGAGAA	Reverse	
NRF1	NM_001030646	GGCCAACGTCCGAAGTGAT	Forward	55
		CCATGACACCCGCTGCTT	Reverse	
Ski	M28517	GGCCCTGCTGCTTTCTCA	Forward	75
		AGGTTCCGCTGGGTCTTTG	Reverse	
FoxO1	NM_204328	GCCTCCTTTTCGAGGGTGTT	Forward	64
		GCGGTATGTACATGCCAATCTC	Reverse	
PPARα	AF163809	CAAACCAACCATCCTGACGAT	Forward	64
		GGAGGTCAGCCATTTTTTGGA	Reverse	
PGC-1β	XM_414479	TTGCCGGCATTGGTTTCT	Forward	66
		CACGGGAAGCCACAGGAA	Reverse	
PPARγ	NM_001001460	CACTGCAGGAACAGAACAAAGAA	Forward	67
		TCCACAGAGCGAAACTGACATC	Reverse	
Cox5a	XM_001233020	GCGTGCAGACGGTTAAATGA	Forward	59
		TCCTTCACCACCTCCAGAATG	Reverse	
CoxIV	NM_001030577	CGGTCGGCAGGATGTTG	Forward	55
		AGGGCTCTTCTCCCGATGA	Reverse	
mtDNA	X52392	ACACCTGCGTTGCGTCCTA	Forward	58
		ACGCAAACCGTCTCATCGA	Reverse	
mtSSBP1	NM_001278007	CACAGACAGGTGATATCAGTCAGAAG	Forward	65
		GAGGCCTGGTCTGAAGACAGA	Reverse	
TFAM	NM_204100	GAACAGCGGCCAAAAAAGAA	Forward	60
		ACGCAGCGAAGCTTTGGA	Reverse	
PGC-1α	NM_001006457	GAGGATGGATTGCCTTCATTTG	Forward	62
		GCGTCATGTTCATTGGTCACA	Reverse	
MFN1	NM_001012931	CGGTGGTTTTGAGCCCATT	Forward	57
		GAAGCCTGGCACCCAAATC	Reverse	
MFNF2	XM_004947503	ATGTGCCTGTGACACGTTCAC	Forward	63
		TCGAGTGTCAGGCAGCTTCTT	Reverse	
DNM1	XM_001233249	GAACTTTCGCCCCGATGA	Forward	57
		TGGACCATCTGAAGCAGAGCTT	Reverse	
OPA1	NM_001039309	CCCAAGCAGGATCCAACAA	Forward	73
		AACAACTGCAAAGTAACCCAAAGC	Reverse	
OMA1	XM_422503	TCACTATGATTTGGGCCATCTG	Forward	59
		GATCCGCTGGCCAACAAC	Reverse	
MTFP1	XM_004934441	CCGCCATCCCTGTCATCA	Forward	64
		ATGCTGGAATCCATCAGGAAA	Reverse	
MTFR1	NM_204543	CAGATCAGAAGTGTGGTCGAAAAC	Forward	65
		AATGTCCAGGCTGATGTAGTTCAC	Reverse	

Table 3.1 (Cont'd).

Gene	Accession Number[a]	Primer Sequence (5' --> 3')	Orientation	Product Size, bp
MFF1	XM_001233529	TGGAGTACACCGAAGGAATCAGT	Forward	64
		TGAGGAGCCACTTTGAGTTTCTC	Reverse	
18S	AF173612	TCCCCTCCCGTTACTTGGAT	Forward	60
		GCGCTCGTCGGCATGTA	Reverse	

[a]Accession numbers refer to Genbank (National Center for Biotechnology Information).

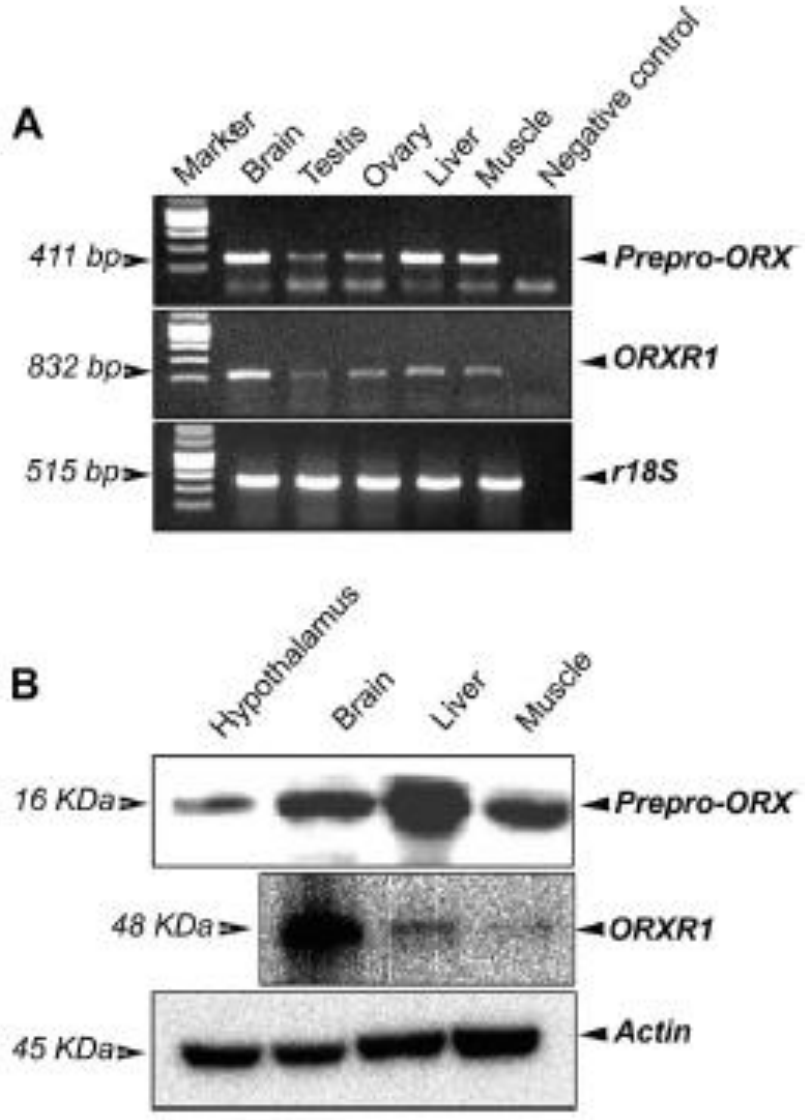


Figure 3.1 Prepro-orexin and its related receptor are expressed in broiler chicken muscle. A: total RNA (1 µg) was reverse transcribed and subjected to RT-PCR, as described in MATERIALS AND METHODS. Brain, testis, and ovary were used as positive controls. B: 70 µg total protein extracted from each tissue were electrophoresed and blotted onto polyvinylidene difluoride membrane. Preproorexin (ORX) and orexin receptor 1 (ORXR1) expression was detected by immunoblot using rabbit anti-mouse ORX and rabbit anti-rat ORXR1 antibodies. Hypothalamus and brain were used as positive controls. The figure is a representative picture from one animal.

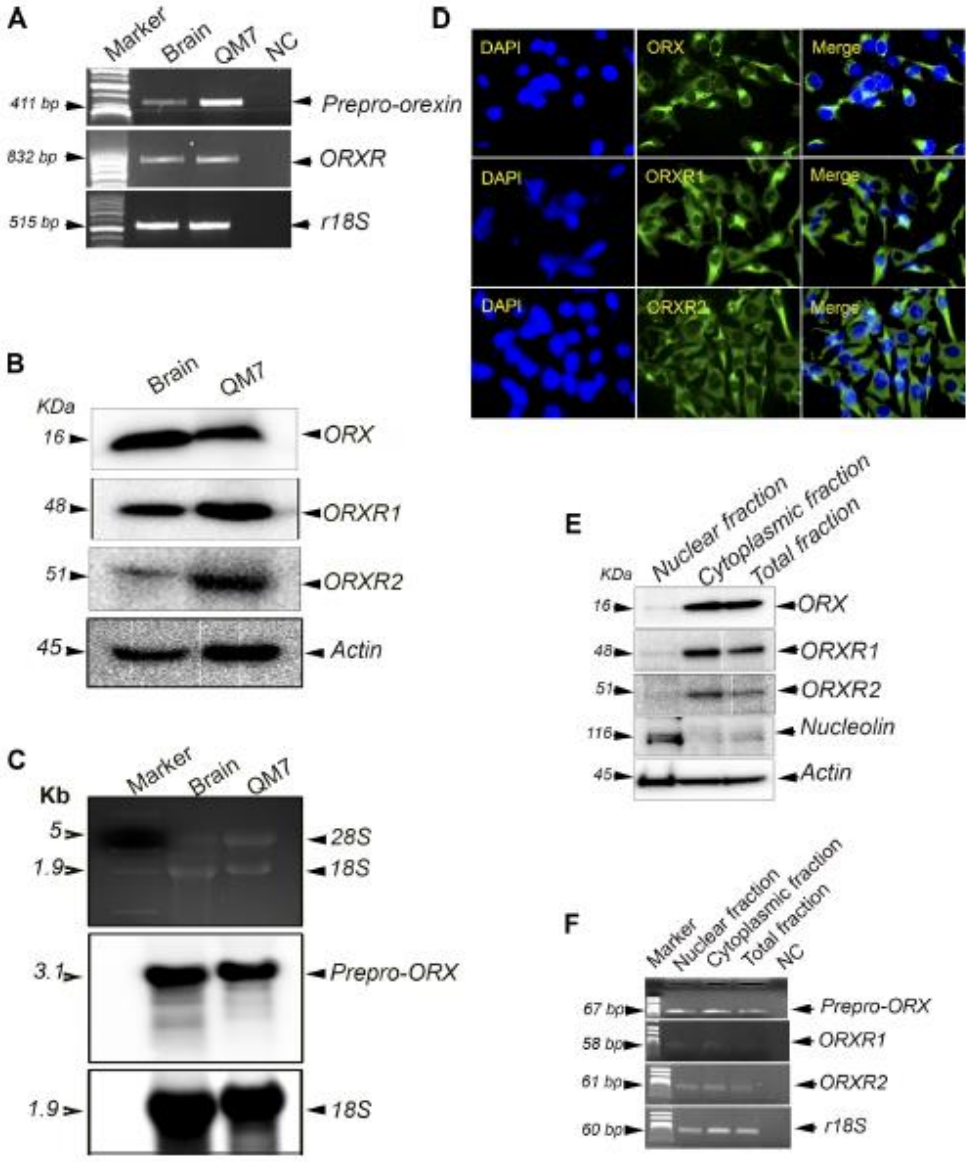


Figure 3.2 Characterization of ORX and its receptors (ORXR1/2) in QM7 cell line. A: RT-PCR. Total RNA (1 µg) was isolated from chicken brain and QM7 cells and subjected to RT-PCR, as described in MATERIALS AND METHODS. B and E: Western blot. Total, nuclear and cytoplasmic protein were electrophoresed and blotted onto PVDF membrane. ORX and ORXR1/2 were detected using rabbit anti-mouse ORX and rabbit anti-rat ORXR1/2 antibodies. Nucleolin was used as a positive control for the nuclear fraction. C: Northern blot. Total RNA (10 µg) was separated by agarose gel electrophoresis and transferred to a nylon membrane and hybridized with specific biotin-labeled DNA probe toward chicken ORX, and 18S. Hybridization signals were detected by FluorChem M MultiFluor system. D: immunofluorescence staining. Intracellular ORX system distribution visualized by fluorescent microscope in the presence of a secondary antibody conjugated with Alexa Fluor 488 (green) and DAPI (blue). F: real-time quantitative PCR (qPCR). RNA was isolated from different cellular fraction (total, cytoplasmic, and nuclear) and subjected to RT-qPCR in the presence of primers specific for chicken ORX and ORXR1/2, as described in MATERIALS AND METHODS.

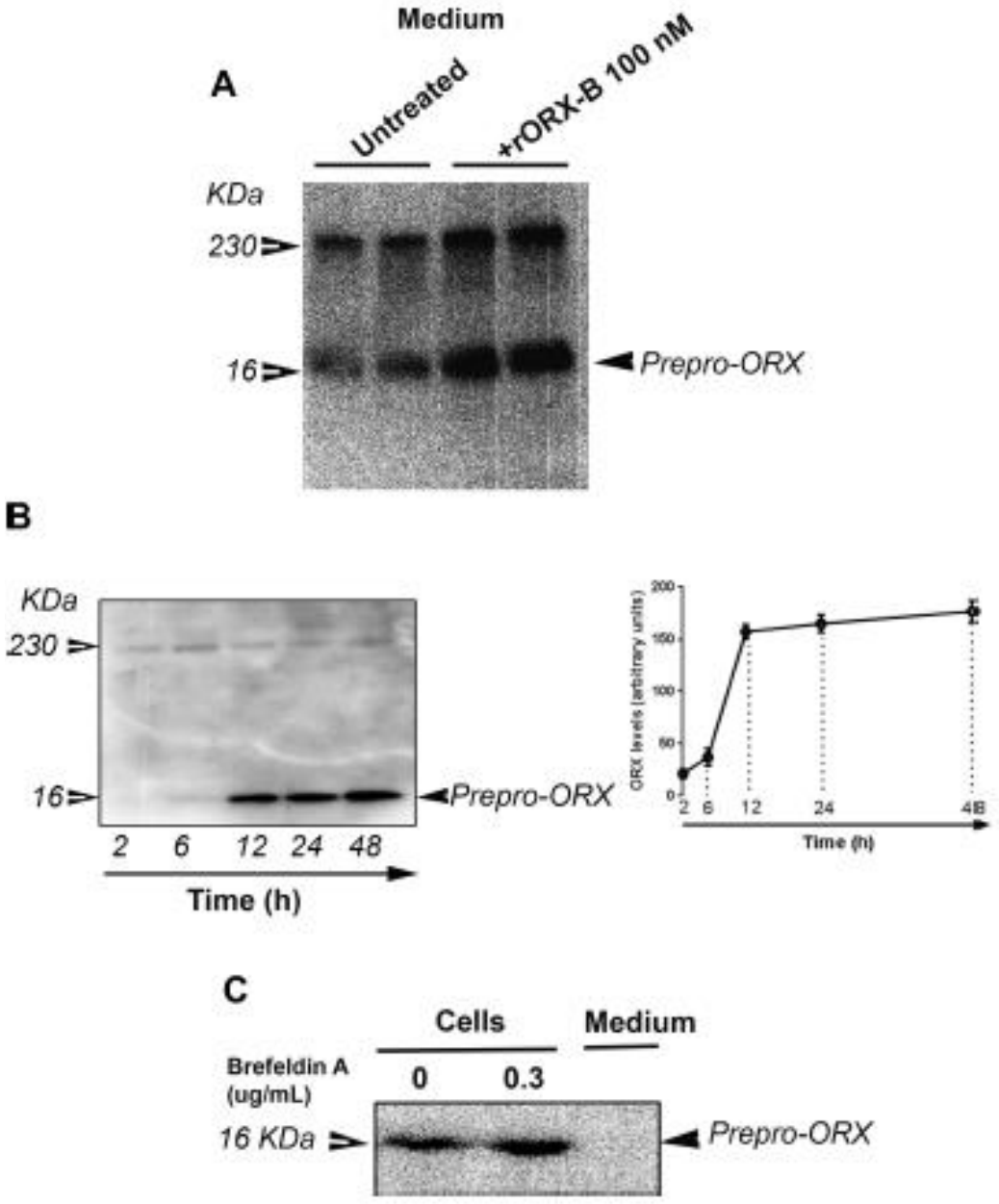


Figure 3.3 Secretion of orexin by QM7 cells. A: cell monolayers were incubated in serum-free medium with or without rORX-B (100 nM) for 24 h. B: QM7 cells were incubated in serum-free medium for different time periods. C: cells were incubated in serum-free medium with or without brefeldin A (0.3 µg/ml) for 12 h. Medium and/or cell lysates were subjected to immunoblot analysis using anti-orexin antibody, as described in MATERIALS AND METHODS.

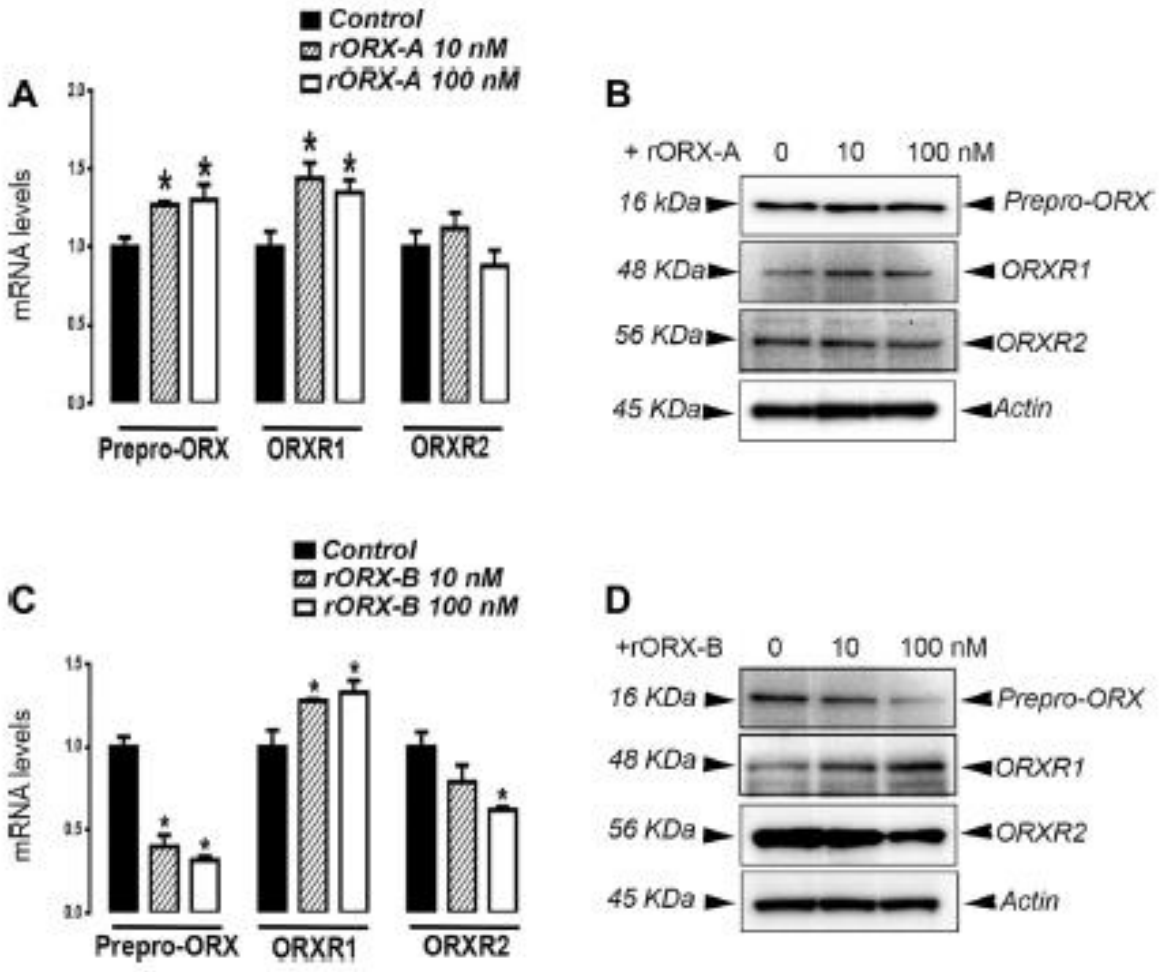


Figure 3.4 Effect of orexin treatment on orexin system expression in QM7 cells. Cells were treated with 0 (control), 10, or 100 nM of recombinant orexin A and B for 24 h. Total RNA and protein were isolated as described in MATERIALS AND METHODS. Relative expression of ORX and ORXR1/2 was determined by QPCR using $2^{-\Delta\Delta Ct}$ method (A, C). Protein levels were measured by Western blot analysis (B, D). Data are expressed as means ± SE (n = 6). *Significant difference between orexin-treated and control cells ($P < 0.05$).

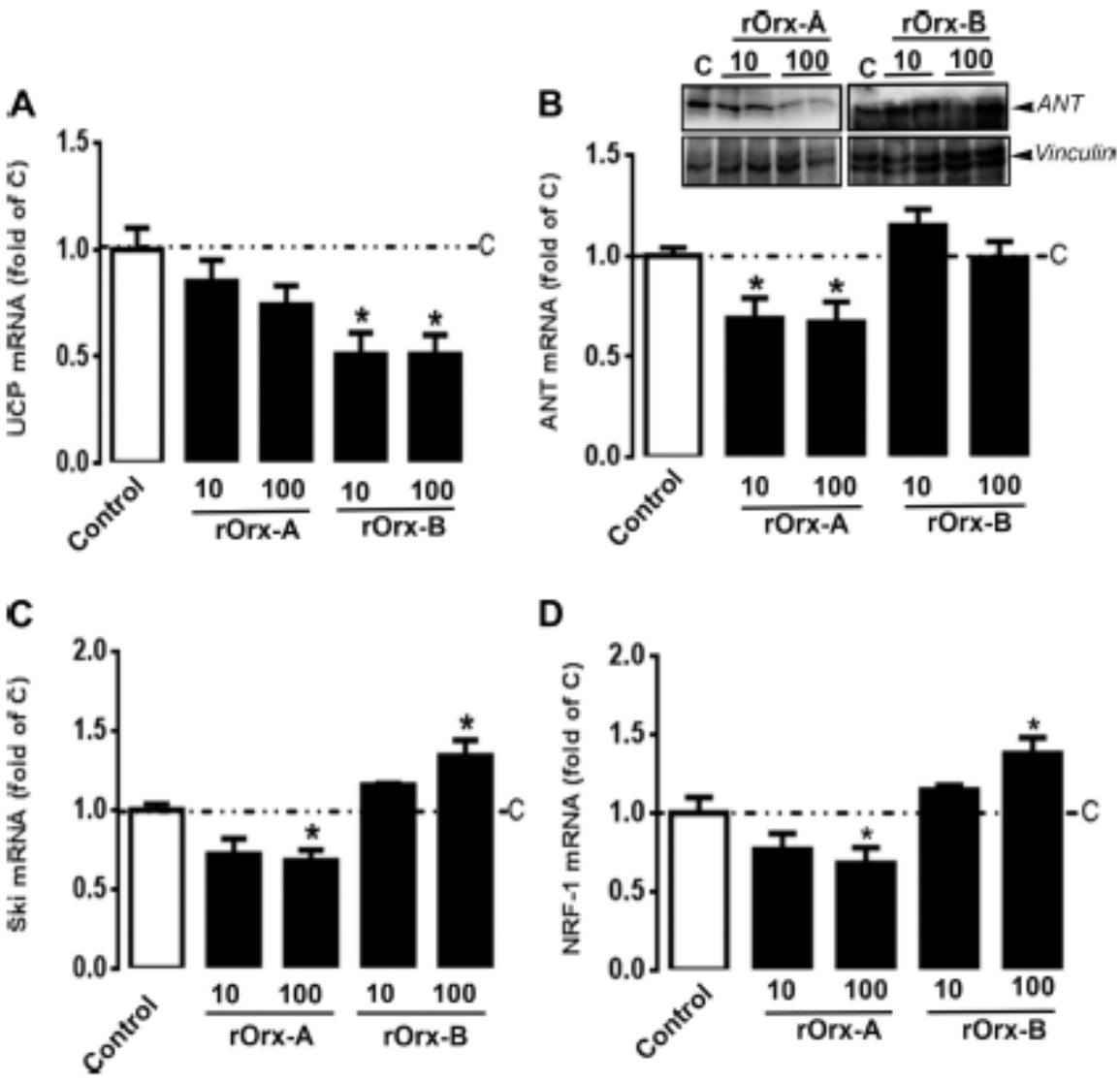


Figure 3.5 Effect of orexin treatment on mitochondrial-related genes in QM7 cells. Cells were treated with recombinant orexin A or B (10 and 100 nM) for 24 h and the relative abundance of avian (av)-UCP (A), av-adenosine nucleotide translocator (ANT; B), Ski (C), and nuclear respiratory factor 1 (NRF-1; D) were determined by QPCR. Untreated cells were used as control. Protein levels of av-ANT were measured by Western blot analysis. Data are expressed as means ± SE (n = 6). *Significant difference between orexin-treated and control cells ($P < 0.05$).

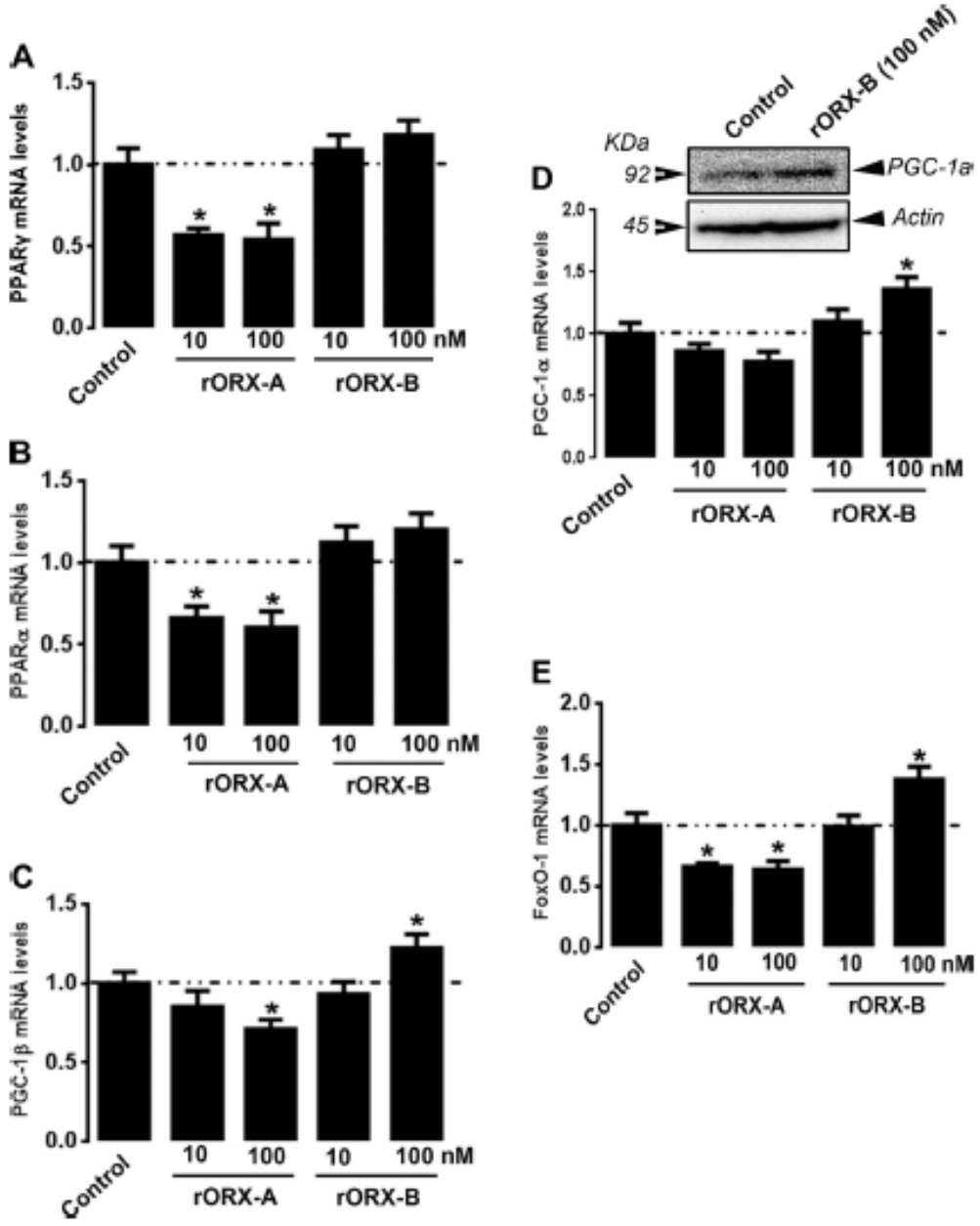


Figure 3.6 Effect of orexin treatment on mitochondrial-transcriptional regulators in QM7 cells. The relative expression of peroxisome proliferator-activated receptor γ (PPARγ (A), PPARα (B), PGC-1β (C), PGC-1α (D), and FoxO-1 (E) was determined by real-time PCR. Protein levels of PGC-1α were determined by Western blot analysis. Data are means ± SE; n = 6. *Significant difference between orexin-treated and control cells ($P < 0.05$).

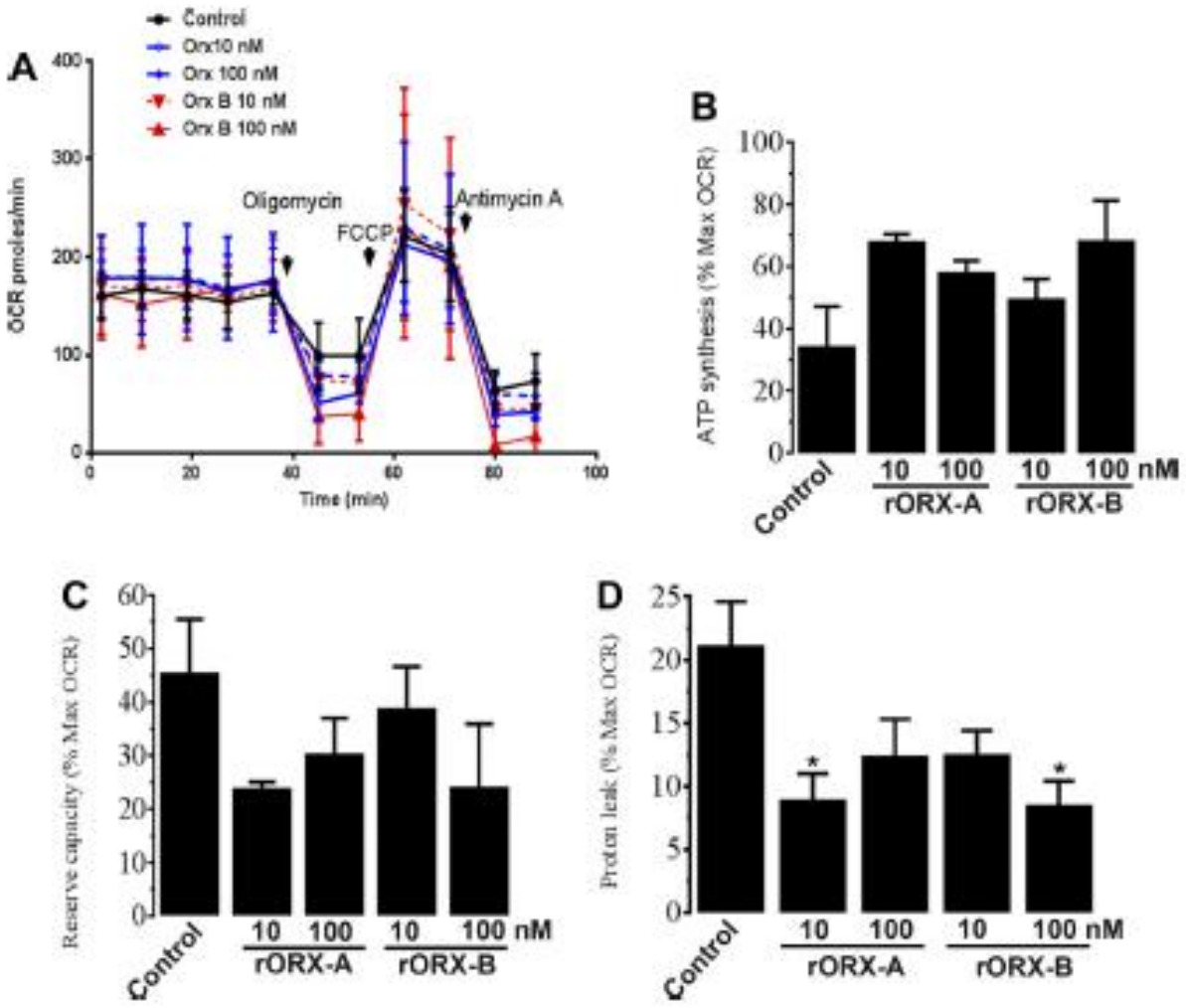


Figure 3.7 Effect of orexin treatment on mitochondrial bioenergetics in QM7 cells. A: oxygen consumption rate (OCR) was determined using XF24 Flux Analyzer. Bioenergetic parameters, including OCR due to ATP synthesis (B), mitochondrial oxygen reserve capacity (C), and proton leak (D) were determined as described in MATERIALS AND METHODS. The values represent the means ± SE (n = 6). *Significant difference between orexin-treated and control cells ($P < 0.05$).

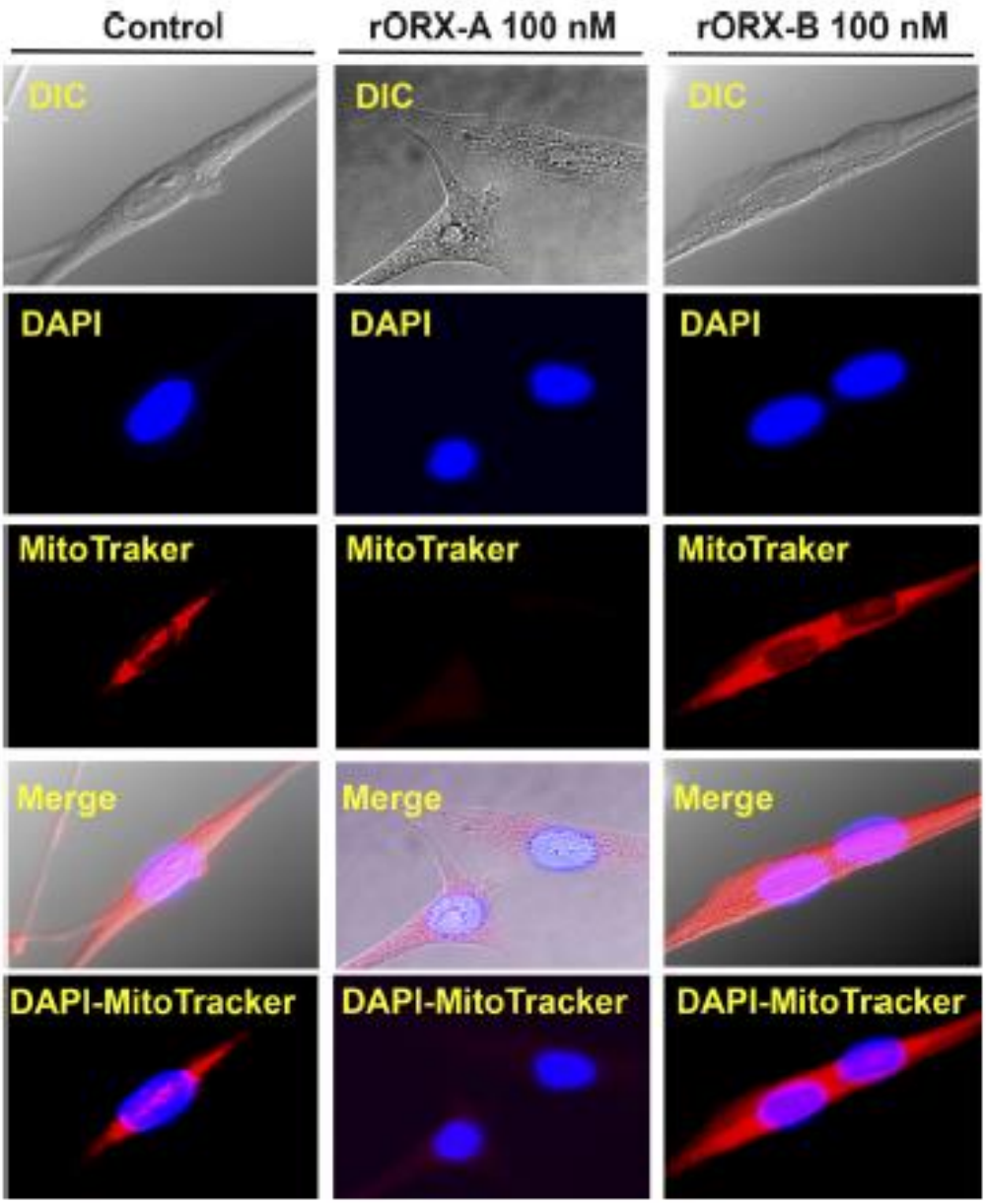


Figure 3.8 Effect of orexin treatment on mitochondrial distribution in QM7 cells. Cells were cultured in chamber slides and treated with 100 nM of rORX-A or rORX-B for 24 h. Mitochondria were visualized with MitoTracker Red CMX Ros (75 nM) under a fluorescent microscope. Representative images acquired and deconvoluted are shown. DIC, differential interference contrast. DAPI, 4',6-diamidino-2-phenylindole.

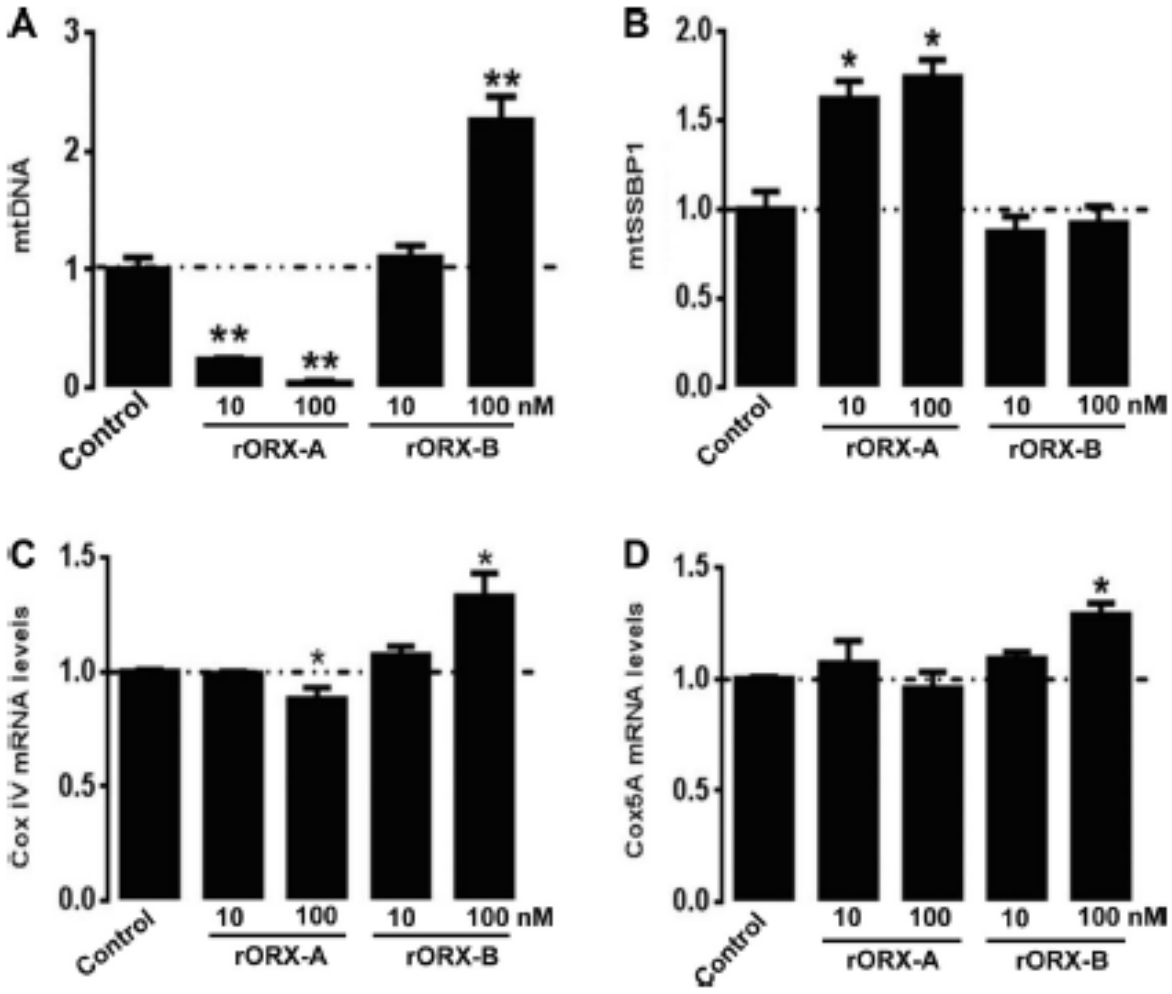


Figure 3.9 Effect of orexin treatment on mitochondrial DNA and mass in QM7 cells. QM7 cells were treated with orexins (10 and 100 nM) for 24 h, as described in MATERIALS AND METHODS. The levels of mtDNA (A) and the relative expression of mtSSBP1 (B), mitochondrial markers CoxIV (C), and Cox5a (D) were determined by real-time PCR. The values represent the means ± SE (n = 6). *Significant difference between orexin-treated and control cells ($P < 0.05$).

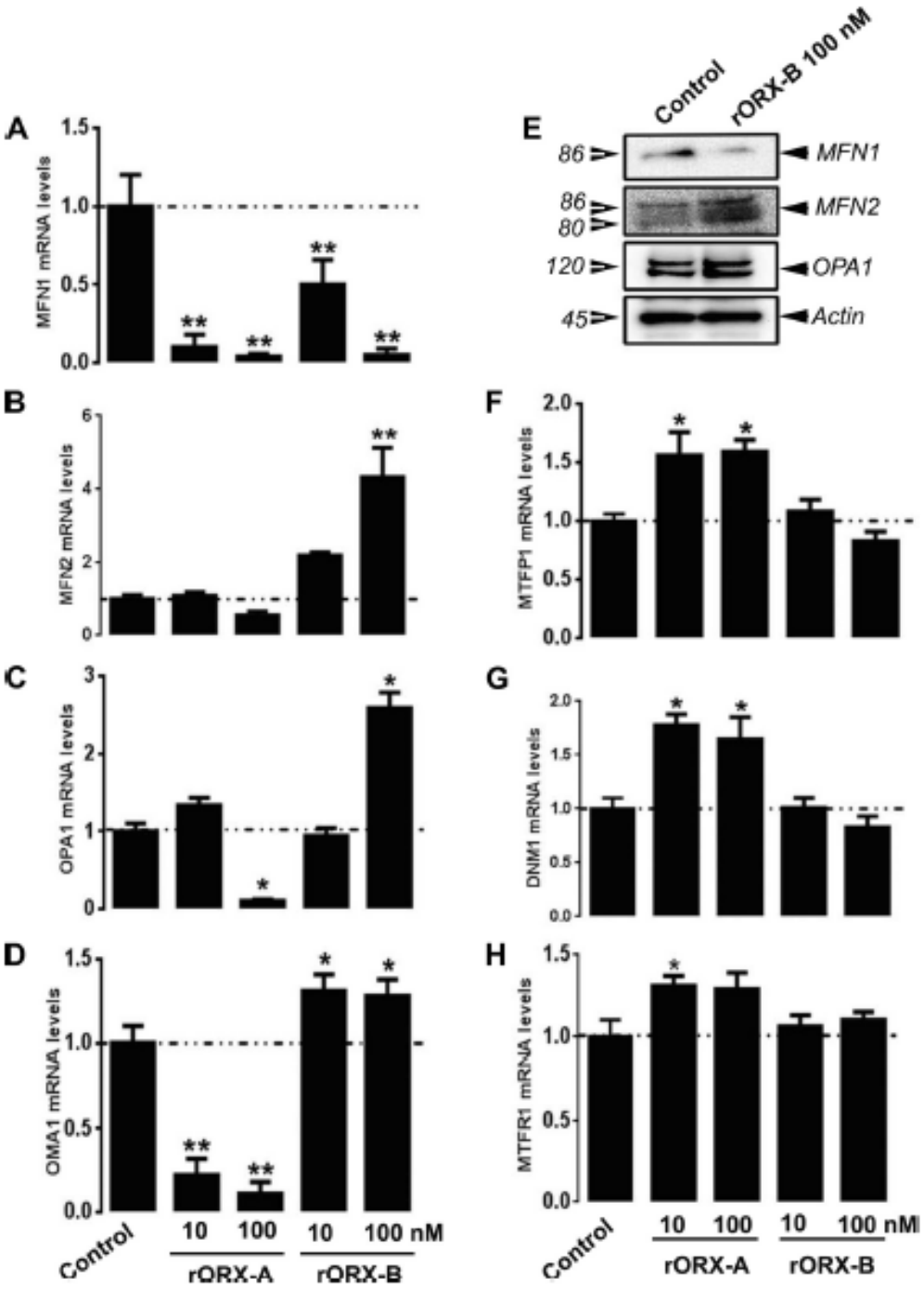


Figure 3.10 Effect of orexin treatment on mitochondrial dynamics-related genes in QM7 cells. QM7 cells were treated with orexins (10 and 100 nM) for 24 h, as described in MATERIALS AND METHODS. The relative expression of four genes involved in mitochondrial fusion, MFN1 (A), MFN2 (B), OPA1 (C), OMA1 (D) and three genes involved in mitochondrial fission, MTFP1 (F), DNM1 (G), and MTFR1 (H) was determined by real-time PCR. The protein levels of MFN1, MFN2, and OPA1 were determined by Western blot analysis (E). The values represent the means ± SE (n = 6). *Significant difference between orexin-treated and control cells ($P < 0.05$).

CHAPTER 4

Muscle development and insulin signaling-related gene expression in breast muscle associated with feed efficiency in pedigree male broilers

Kentu Lassiter, Byungwhi Caleb Kong, Alissa Piekarski-Welsher, Sami Dridi, and Walter G. Bottje

Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, Arkansas, United States of America

4.1 ABSTRACT

Background: The objective of this study was to determine mRNA expression of genes involved in muscle development and hypertrophy, and the insulin receptor signaling pathway in breast muscle associated with the phenotypic expression of feed efficiency (FE). Breast muscle samples were obtained from Pedigree Male (PedM) broilers (~8 wk old) that had been individually phenotyped for FE between 6 and 7 wk of age. The high FE group gained more weight but consumed the same amount of feed compared to the low FE group. Total RNA was extracted from breast muscle (n = 5 per group) and mRNA expression of target genes was determined by real-time quantitative PCR.

Results: The results indicate that several genes known to enhance muscle formation and growth [AMP-activated protein kinase alpha 1 and 2 (AMPKα1, AMPKα2), heat shock 70 kDa protein (HSP70), myogenin, creatine kinase, neutrophil cytosolic factor 2 (NCF2), mitogen-activated protein kinase kinase 6 (MAP2K6), mitogen-activated protein kinase kinase kinase 7 (MAP3K7)] were upregulated ($P < 0.05$) in high FE, whereas genes known to inhibit muscle hypertrophy, differentiation, and fiber size [myostatin, caveolin-3, activin receptor II B, Smad 2, Smad 3, IGF-binding protein 3 (IGFBP-3)] were downregulated in the muscle of high FE PedM broilers compared to the Low FE phenotype. Differentially expressed genes in the insulin signaling pathway included insulin receptor substrate 1 (IRS-1) and regulatory-associated protein of mechanistic target of rapamycin (RAPTOR) that were upregulated ($P < 0.05$) whereas SHC-transforming protein 1 (SHC-1) and glucose transporter 8 (GLUT-8) that were downregulated ($P < 0.05$) in high compared to Low FE breast muscle. Increased abundance of eukaryotic translation and initiation genes (determined by binomial distribution analysis) from RNAseq data was observed in the high FE compared to the low FE breast muscle.

Conclusion: These findings suggest that the expression of genes involved in muscle development-differentiation and protein synthesis gene expression was enhanced in breast muscle of PedM broilers exhibiting a high FE phenotype compared to those exhibiting a low FE phenotype.

4.2 INTRODUCTION

Feed efficiency (FE) is one of the most important genetic traits in animal production since feed costs account for roughly 70% of the total required to bring an animal to market weight [1]. In a Pedigree Male (PedM) broiler FE model, animals with Low FE exhibited higher mitochondrial reactive oxygen species (ROS) production and higher oxidative stress compared to those with high FE (see review [2]). Although ROS causes oxidation at high levels, low levels of mitochondrial ROS are important in signal transduction and control various physiological processes affecting cell growth and proliferation (reviewed by [3, 4]). Therefore, it was hypothesized that inherent gene expression in the low FE phenotype is modulated by mitochondrial ROS production [5, 6].

To develop a comprehensive understanding of the cellular basis of FE, global gene and protein expression studies were conducted on breast muscle obtained from PedM broilers exhibiting high and low FE phenotypes [6-8]. These studies have provided insight into fundamental mechanisms of FE in muscle but have also provided paradoxical findings. For example, despite clear enrichment of cytoskeletal or muscle fiber gene expression occurring in the low FE phenotype [6, 8], myostatin (MSTN**)**, which is well known to inhibit muscle development ([9, 10] reviewed by [11]), was up-regulated in breast muscle of low FE PedM broilers [12]. The expression of adenosine monophosphate-activated protein kinase 1 (AMPK), which is a sensor of energetic status in the cell, was elevated in a cDNA microarray in muscle of the high FE PedM phenotype ([13]. AMPK activates energy production pathways (e.g. glycolysis, lipolysis, oxidative phosphorylation) and inhibits energy consuming pathways (e.g. lipogenesis, protein synthesis) (reviewed by [14]). Thus, the increased expression of AMPK in the high FE phenotype could conceivably be inhibiting protein synthesis that appears to run counter to the fact that these animals gained more body weight compared to the low FE PedM

phenotype [5, 6]. Therefore, the objective of this study was to conduct targeted analysis of genes involved in muscle development, protein synthesis, and energy metabolism; particularly genes in part of the myostatin and insulin signaling pathways that are differentially expressed in breast muscle obtained from PedM broilers exhibiting a high or low FE phenotype.

4.3 MATERIALS AND METHODS

4.3.1 TISSUES AND ANIMALS

Breast muscle analyzed in this study were from the same experiment in which transcriptomics [6, 8] and proteomics [7] investigations have been conducted. The Pedigree Male (PedM) broilers were individually phenotyped for FE as previously described [5]. Briefly, FE (amount of body weight gain/amount of feed consumed) was determined between 6 and 7 wk of age on a group of 100 PedM broilers housed in individual cages. Birds were provided access to feed and water ad libitum. All birds received the same corn-soybean based diet (20.5% protein, 3,280 kcal/kg) ad libitum during the feed efficiency trial. From this group of 100, birds with the highest and lowest FE were selected. Feed efficiencies for the low- and high-FE groups (n = 6 per group) in the present study were 0.46 $\pm$ 0.01 and 0.65 $\pm$ 0.01, respectively. In the high FE group, greater efficiency was obtained by greater weight gain without a difference in feed intake during the week of phenotyping. The difference in mean FE (0.19) between the groups is identical to that reported in our initial investigation [5]. Birds were humanely euthanized, breast muscle tissue (pectoralis major) was quickly excised and flash frozen in liquid nitrogen, and stored at -80 ºC. All procedures for animal care complied with the University of Arkansas Institutional Animal Care and Use Committee (IACUC Protocol #14012).

4.3.2 RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted by TRIzol reagent (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer's recommendations, DNase-treated, and reverse-transcribed (Quanta Biosciences, Gaithersburg, MD). The concentration and purity of RNA were determined for each sample using a Take 3 microvolume plate and a Synergy HT multimode microplate reader (BioTek, Winooski, VT). The reverse-transcription (**RT**) products (cDNAs) were amplified by real-time quantitative PCR (7500 real-time PCR system, Applied Biosystems, Thermo Fisher Scientific, Foster City, CA) with Power SYBR Green Master Mix (Applied Biosystems). Oligonucleotide primers used in this study and genes that were examined are provided in Table 1.

4.3.3 STATISTICAL ANALYSIS

Comparison of mean expression values for qRT-PCR between the high and low FE groups were made using student t-test. Differences were considered significant at $P < 0.05$ with some qualifications. Binomial distribution analysis was used as previously described to assess differences in the number of genes associated with eukaryotic initiation and translation factors involved in protein synthesis of unreported data (see Table 2) contained in a transcriptomic dataset [8, 15]. Briefly, the numbers of molecules in which average values were numerically higher (H) or lower (L) in breast muscle of the high FE compared to the low FE PedM phenotype were determined and used in the exact binomial distribution analysis test offered in the 2010 version of Microsoft Excel™. There was no cutoff based on significant or fold difference in expression for a given transcript involved in the bionomial distribution analysis that was conducted.

4.4 RESULTS AND DISCUSSION

The major goal of this study was to conduct targeted gene expression analysis using qRT-PCR to confirm results and extend the understanding of fundamental mechanisms of FE presented in transcriptomic and proteomic studies conducted in breast muscle of PedM broilers exhibiting high and low FE phenotypes [6-8, 16]. The discussion below will be directed to results associated with a) muscle development and myostatin signaling, and b) nutrient sensing, protein synthesis and insulin signaling followed by a summary that will incorporate information from a transcriptomic dataset obtained previously [8] but not reported.

4.4.1 MUSCLE DEVELOPMENT AND MYOSTATIN SIGNALING

The mRNA expression of MYOG, MAP2K6, MAP3K7, HSP70, and NCF2, genes that promote muscle development and differentiation, were higher in high FE compared to low FE broilers (Figure 1). Myogenin (MYOG), a key regulatory transcription factor involved muscle development during myogenesis [17-19], was upregulated in the high FE phenotype (Figure 1A). Previous studies in broilers reported a positive relationship between increased breast muscle weight and increased mRNA levels of MYOG [20, 21]. Myozenin (Myoz-2), however, was not differentially expressed (Figure 1B). Myozenin (Myoz-2) is a protein associated with actin and myosin found on the z-line in skeletal muscle and part of the contractile apparatus [22]. In a recent transcriptomic study, Myoz-2 expression was down-regulated in breast muscle of the high FE PedM broiler phenotype [8]. We do not have an explanation for this discrepancy in expression between the present study and Bottje et al. [8] at this time.

Expression of two mitogen activated kinase enzymes, MAP2K6 and MAP3K7 involved in muscle development and response to environmental stress (see review [23]), were elevated in the high FE phenotype (Figure 1C and 1D). Specifically, MAP2K6 activates p38 MAP kinase and

plays an important part in its signal transduction pathway [24] ; whereas MAP3K7 is involved in activation of nuclear factor kappa β (NFκB) in addition to other MAP kinases [25]. There were no differences in expression of MAP2K1, MAP2K2, and MAP2K4 however (data not shown).

Expression of CAV-3 mRNA was downregulated in the high FE phenotype in this study (Figure 1E). CAV-3 is a member of the caveolin family, and serves as the muscle-specific isoform of the protein [26]. Mutations and expression differences of CAV-3 can result in certain muscle myopathies [27]. Caveolae are sub-cellular structures that function in cell signaling by aiding internalization of hormonal signals after the hormone binds to the target receptor on the cell surface. Zhu et al. [28] indicated that CAV-3 expression was upregulated during muscle hyperplasia when compared to expression levels during muscle hypertrophy in pigs. Zhu et al. [28] hypothesized that CAV-3 might be used as a genetic marker for meat production in swine. Altered CAV-3 expression, however, may be detrimental to muscle development. Smythe and Rando [29] reported that both increases or decreases in CAV-3 expression in mouse muscle cells made them more susceptible to oxidative stress and decreased survival through PI(3)K/Akt signaling. Thus, the up regulation of CAV-3 in the low FE phenotype compared to the low FE phenotype would potentially enhance muscle development as indicated by Zhu et al. [28] but could also contribute to higher oxidative observed in the low FE PedM broiler [2] as indicated by Smythe and Rando (2006) [29]. Since the expression of CAV-1 protein, which is involved in insulin signaling (see below), was elevated in the high FE breast muscle [7] it is not clear what role the CAV1 and CAV3 genes are contributing to the PedM broiler FE model in the present study.

Heat shock protein 70 (HSP70) mRNA expression was elevated in the high FE breast muscle (Figure 1F). This increase might be beneficial for muscle function as HSP70 has been reported to maintain muscle fiber integrity and enhance muscle regeneration and recovery from

damaging stress stimuli [30]. HSP70 is responsible for correct folding and assembly of nuclear-encoded proteins targeted for import into the mitochondria where they assemble with mitochondrial DNA encoded proteins [31]. HSP70 is also an important chaperone for proteins encoded by mitochondrial DNA [32]. Previously, we reported that HSP90 gene expression was elevated in muscle of the low FE phenotype and hypothesized this elevation was a response to higher oxidative stress in these animals [13], whereas HSPB2 (heat shock p27 kDa protein 2) protein expression was elevated in the high FE breast muscle [7]. Thus, there appears to be distinct differences in expression of different members of the family of heat shock proteins in the high and low FE phenotypes. The elevation of HSP70 gene expression observed in the present study could be beneficial for muscle integrity and mitochondrial function.

Neutrophil cytosolic factor 2 (NCF2) encodes a subunit of NADPH/NADH oxidase and is a critical component of NADPH oxidase 2 (NOX2) [33]. NCF2 was elevated in high FE breast muscle in the present study (Figure 1G) which concurs with findings in commercial broilers [34]. Similarly, NOX2 generates superoxide in the sarcoplasmic reticulum and is a major source of oxidative stress in muscle [33, 35]. NOX2 is a downstream target of nuclear factor erythroid 2-like 2 (NFE2L2) which coordinates antioxidant response to oxidative stress by activating expression of genes that contain an antioxidant response element in their promoter regions [36, 37]. From downstream target expression analysis, NFE2L2 was predicted to be activated in animals with high FE [7, 34].

Expression levels of the inhibitory gene MSTN (a member of the TGF-β family), one of its receptors (ActRIIB), and two transcription factors of MSTN signaling, SMAD2 and SMAD3, were elevated in the low FE compared to high FE breast muscle (Figure 2). In the cDNA microarray study [6, 13], data analyses using Ingenuity Pathway Analysis (IPA) software predicted

that several target molecules and mechanisms may contribute to differences in muscle growth, development, and differentiation between the high and low FE phenotype. Myostatin was downregulated in a microarray dataset in the high FE phenotype (i.e. upregulated in low FE phenotype; [12]). Myostatin is a member of the TGF-β family of molecules and is a strong negative regulator of skeletal muscle growth [9, 10] and differentiation and proliferation of turkey satellite cells [38]. The expression of the MSTN antagonist, FSTN, was not different between the two phenotypes (Figure 2B). Studies investigating the relationship between MSTN and growth performance in broilers show that MSTN is a polymorphic gene in which different alleles of the gene can affect performance [39-41]. Thus, differences in FE in the PedM broiler line in this study could be attributed in part to different haplotypes of the MSTN gene. Figure 3, adapted from Lee and Glass [42], summarizes the initial steps in MSTN signaling expression in the present study that would potentially exert a negative effect on muscle hypertrophy and differentiation in the low FE phenotype.

4.4.2 ENERGY SENSING

In the present study, both isoforms of the catalytic subunit of 5'AMP activated protein kinase (AMPKα1 and AMPKα2) were upregulated in the high FE phenotype (Figure 4A and 4B) which verifies the previous results [13]. AMPKα was also predicted to be activated in the high FE PedM phenotype in a proteomics study conducted on the same set of tissues [7]. AMPK expression increases in response to low energy levels (sensed by an increase in the AMP:ATP ratio) and stimulates ATP production by increasing energy-producing reactions (e.g. oxidative phosphorylation and glycolysis) and inhibiting pathways that consume ATP (e.g. fatty acid synthesis and gluconeogenesis) [43, 44]. Upregulation of AMPK stimulates mitochondrial biogenesis and mitochondrial electron transport chain activity [43-46].

Interestingly, creatine kinase (muscle isoform, CKM) gene expression was elevated in the high FE phenotype (Figure 4C) which is opposite to a decrease in CKM protein expression in the high FE phenotype [7]. The reason for this discrepancy is not apparent but gene and protein expression often do not go hand in hand. Increased expression of several proteins suggests that the muscle of the high FE phenotype PedM broiler may have enhanced capabilities for mitochondrial oxidative phosphorylation as well as the ability to shuttle creatine and phosphorylated creatine in and out of mitochondria [7, 47].

4.4.3 PROTEIN SYNTHESIS

When active, the mechanistic target of rapamycin complex 1 (mTORC1) complex has many functions that include promoting cell growth by modulating gene transcription and increasing protein synthesis [48]. The mTOR pathway was hypothesized to play a role in the phenotypic expression of feed efficiency due in part to upregulation of the mTORC1 complex in breast muscle of the high FE phenotype [12] and mTOR expression was reported to be higher in the high FE compared to the low FE phenotype [49]. Two major components of mTORC1 complex are mTOR and regulatory associated protein of mTOR (RAPTOR) [50]. In the present study, RAPTOR mRNA expression was upregulated ($P < 0.05$) but mTOR was moderately downregulated ($P < 0.08$) in the high FE phenotype (Figure 4D and 4E). It is possible that the increase in RAPTOR gene expression could have a positive effect on mTOR protein expression [50]. We do not have an explanation for the discrepancy of mTOR expression in this study and that of Piekarski [49] at this time.

Key downstream targets of mTORC1 that enhance protein synthesis are p70S6k, and eukaryotic translation initiation factor 4E (EIF4E). The expression of p70S6k, which is activated by insulin and refeeding [51], was higher in the low FE birds ($p < 0.08$) (Figure 4F). In humans,

mTOR expression is dysregulated during disease states such as cancer, diabetes, and heart disease [52] and can affect the expression of downstream genes involved in insulin signaling. Therefore, it is possible that elevated mitochondrial ROS production reported in the low FE phenotype [5], may partially explain the moderate upregulation of mTOR and p70S6k seen in those birds. This observation may also hold true for PRKAR1A and GLUT-8, two other members of the insulin signaling pathway that were upregulated in the low FE phenotype (see below). A definitive characterization of the mTOR pathway in this PedM broiler model of feed efficiency will require protein expression analysis that includes the measurement of phosphorylated proteins which is beyond the scope of the present study. It should be pointed out that although mTOR was detected in western blots of broiler breast muscle, the phosphorylated mTOR was not detected [49].

Although there was no difference in mRNA expression of EIF4E in the present study (data not shown), binomial distribution analysis of gene expression of a recent RNAseq dataset [8] revealed a significant skew (Binomial distribution P value = 0.00001) favoring eukaryotic initiation and elongation factors in the high FE phenotype (Table 2). Similarly, a genetic architecture favoring ribosome assembly and protein translation also was present in the high FE phenotype breast muscle [15]. These components of protein translation in the context of our feed efficiency model will be described in greater detail below (see Figure 6, and Summary).

4.4.4 INSULIN SIGNALING

Targeted expression of genes associated with insulin signaling are presented in Figure 5. Insulin signaling and its pleiotropic effects on gene expression and muscle development in chickens is not as thoroughly understood and differs in certain ways when compared to mammals [53]. Unlike mammals, in chicken muscle only SHC-1 (not IRS-1) is activated by changes in nutritional status, suggesting that chickens have a tissue-specific regulation of insulin

signaling that is yet to be fully understood [54, 55]. Although there was no difference insulin receptor gene expression (data not shown), insulin-like substrate 1 (IRS-1) was upregulated in the high FE birds (Figure 5A), which concurs with the predicted activation of IRS-1 reported by Kong et al. [7] (2016). Additionally, insulin signaling requires insulin receptor endocytosis and is particularly dependent on CAV-1 [56]. CAV-1 protein expression was 9 fold higher in the high FE compared to low FE breast muscle [7] and could be instrumental in facilitating insulin signaling in the high FE PedM broiler.

It has also been suggested that p70S6k is involved in a negative feedback that inhibits IRS-1 activation by phosphorylating its serine residues [57]. Since p70S6k was marginally upregulated ($P < 0.08$) in the Low FE phenotype in the current study, it may be inhibiting IRS-1 activity, and thus increased SHC-1 expression (Figure 5B) may help to maintain the insulin signaling pathway in low FE. Both IRS-1 and SHC-1 are activated by tyrosine phosphorylation activity mediated by phophoinositide-3 kinase (PI3K) when insulin binds to the insulin receptor. There were no differences in PI3K expression in the present study, however (data not shown). While IDE plays a role in insulin signaling and insulin activity [58, 59], a mitochondrial form of IDE is capable of cleaving mitochondrial leader signals of nuclear DNA-encoded mitochondrial proteins [60], thus making the enzyme important in mitochondrial function. Although we observed higher IDE protein expression in the high FE phenotype [7], IDE gene expression was not differentially expressed in the present study (data not shown). The upregulation of anti-proliferative IGFBP-3 in the low FE phenotype (Figure 5C) concurs with Zhou et al. [34] (2015). It has been reported that IGFBP3 may modulate the interaction of IGFs in the extracellular matrix [61]. Spangenburg et al. [62] (2003) reported that IGFBP3 (both gene and protein) was detected in rat soleus muscle

(Type 1 muscle fiber) but not in Type I and II muscle fibers in gastrocnemius muscle. IGFBP3 was also demonstrated to play a role in differentiation in human myoblasts [63].

4.5 SUMMARY

Figure 6 provides a summary of results by combining the mRNA expression data presented above (Figure 1-5) with findings obtained from previous global expression studies conducted on the same group of animals used in the present study [7, 8]. Genes shown with a solid color (pink, green, or gray) are ones that were up-regulated, down-regulated or not different, respectively, in the high compared to the low FE phenotype. Genes or processes shown with hatched or dotted fill depict findings from previous studies as described below.

The down-regulation of Smad 2,3 in the high FE phenotype could result in less inhibition of muscle development relative to the low FE muscle phenotype. Rictor was predicted to be inhibited in the high FE based on expression of downstream target molecules in a proteomics dataset obtained from the same groups of high and low FE PedM broilers [7]. The combined effects of down-regulation of Smad 2,3 and predicted inhibition of Akt could be hypothesized to lower the inhibition of Rheb through the intermediary components TSC1 and TSC2. We did not measure TSC1 and 2 or Rheb in the present study; TSC1 and 2 were not differentially expressed in transcriptomic data ([8], unreported observation). The upregulation of Raptor (a key step in initiating protein synthesis) in the high FE phenotype could hypothetically enhance protein synthesis. It should be noted that in a previous transcriptomic study [12], mTORC1 was upregulated in the high FE phenotype. Increased abundance of genes associated with eurkaryotic translation and initiation (see Table 2) combined with increased abundance of genes and proteins associated with ribosome biosynthesis and mRNA translation that we reported previously [8] could foster the infrastructure needed to support protein synthesis in the high FE phenotype.

There also appears to be a number of components that would favor mitochondrial energy production and an energetic infrastructure in the high FE phenotype. PGC1a was predicted to be activated in the proteomics study reported previously [7]. Components of the creatine phosphate shuttling between mitochondria and the cytosol including VDAC, ANT and mitochondrial creatine kinase (mt-CK) were upregulated in the high FE phenotype [47]. Gene expression of the muscle isoform of CK (CKM) was elevated in the high FE phenotype in the present study as was the expression of the brain isoform of CK (CKB) protein [47]. However, the CKM protein was downregulated in the high FE phenotype [47]. Enzyme kinetic studies conducted on the mitochondrial and cytosolic fractions from high and low FE phenotypes may be needed in order to fully characterize this system. Nonetheless, better coupling of high FE compared to low FE mitochondria that includes lower mitochondrial ROS production and oxidative stress [2, 5] indicates that mitochondrial energetic capabilities are enhanced in the high FE phenotype. Enhanced production of mitochondrial oxidative phosphorylation in the high FE phenotype would help support the energetic demand associated with protein synthesis that in turn would support muscle development.

The increased expression of NCF2, Myog, Map3K7 and Map2K6 combined with reduced expression of CAV3 and Smad 2,3 would all exert positive effects on muscle development in the high FE phenotype. Although Map4K4 was not differentially expressed in the present study, Map2K4 was predicted to be inhibited in the high FE phenotype based on downstream expression of proteins [7]. The down regulation of IGFBP3 in the high FE phenotype would exert a negative effect on muscle development, however.

4.6 REFERENCES

1. Willems OW, Miller SP, Wood BJ: Aspects of selection for feed efficiency in meat producing poultry. World's Poult Sci 2013, 69:77-87.

2. Bottje W, Carstens GE: Association of mitochondrial function and feed efficiency in poultry and livestock species. J Anim Sci 2009, 87:E48-E63.

3. Finkel T: Signal transduction by reactive oxygen species. J Cell Biol 2011, 194:7-15.

4. Bleier L, Wittig I, Heide H, Steger M, Brandt U, Dröse S: Generator specific targets of mitochondrial ROS. Free Rad Biol Med 2015, 78:1-10.

5. Bottje WG, Iqbal M, Tang Z, Cawthon DC, Okimoto R, Wing T, Cooper M: Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult Sci 2002, 81:546-555.

6. Kong B, Song J, Lee JY, Hargis BM, Wing T, Lassiter K, Bottje WG: Gene expression in breast muscle associated feed efficiency in a single male broiler line using a chicken 44k microarray. I. Top differentially expressed genes. Poult Sci 2011, 90:2535-2547.

7. Kong B, Lassiter K, Piekarski-Welsher A, Dridi S, Reverter-Gomez A, Hudson NJ, Bottje WG: Proteomics of Breast Muscle Tissue Associated with the Phenotypic Expression of Feed Efficiency within a Pedigree Male Broiler Line: I. Highlight on Mitochondria. PLoS ONE 2016, 11(5):e0155679.

8. Bottje WG, Kong B, Reverter A, Waardenberg AJ, Lassiter K, Hudson NJ: Progesterone signalling in broiler skeletal muscle is associated with divergent feed efficiency. BMC Systems Biol 2017, 11:1-16 (DOI 10.1186/s 12918-017-0396-2).

9. McPherron AC, Lawler AM, Lee SJ: Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 1997, 387:83-90.

10. McPherron AC, Lee S: Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci (USA) 1997, 94:12457-12461.

11. Kollias HD, McDermott JC: Transforming growth factor-β and myostatin signaling in skeletal muscle. J Appl Physiol 2008, 104:579-587.

12. Bottje WG, Kong B, Lee JY, Washington T, Baum JI, Dridi S, Wing T, Hardiman J: Potential roles of mTOR and protein degradation pathways in the phenotypic expression of feed efficiency in broilers. J Biochem Physiol 2014, 3:1-8.

13. Bottje WG, Kong BW, Song JJ, Lee JY, Hargis BM, Lassiter K, Wing T, Hardiman J: Gene expression in breast muscle associated feed efficiency in a single male broiler line using a chicken 44k microarray II. Differentially expressed focus genes.. Poult Sci 2012, 91:2576-25887.

14. Jeon SM: Regulation and function of AMPK in physiology and diseases. Exp Mol Med 2016, 48(7):e245–. http://doi.org/10.1038/emm.2016.81.

15. Bottje WG, Lassiter K, Piekarski-Welsher A, Dridi S, Reverter A, Hudson NJ, Kong B: Proteogenomics Reveals Enriched Ribosome Assembly and Protein Translation in Pectoralis major of High Feed Efficiency Pedigree Broiler Males. Frontiers Physiol Avian 2017, 8 article 306:1-11.

16. Bottje W, Kong BW: Feed efficiency: Mitochondrial function to global gene expression. J Anim Sci 2013, 91:1582-1593.

17. Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S: The myoD gene family: nodal point during specification of the muscle cell lineage. Science 1991, 251(4995):761-766.

18. Ohkawa Y, Yoshiura S, Higashi C, Marfella CGA, Dacwag CS, Tachibana T, Imbalzano AN: Myogenin and the SWI/SNF ATPase Brg1 maintain myogenic gene expression at different stages of skeletal myogenesis. J Biol Chem 2007, 282:6564-6570.

19. Faralli H, Dilworth EJ: Turning on myogenin in muscle: A paradigm for understanding mechanisms of tissue-specific gene expression. Comp Func Genomics 2012, Article ID836374 (doi:10.1155/2012/836374).

20. Wen C, Chen X, Chen GY, Wu P, Chen YP, Zhou YM, Wang T: Methionine improves breast muscle growth and alters myogenic gene expression in broilers. J Anim Sci 2014, 92:1068-1073.

21. Xiao Y, Wu. C, Li K, Gui G, Zhang G, Yang H: Association of growth rate with hormone levels and myogenic gene expression profile in broilers. J Anim Sci Biotechnol 2017, 8:43-doi:10.1186/s40104-017-0170-8.

22. Takada F, Vander Woude DL, Tong H, Thompson TG, Watkins SC, Kunkel LM, Beggs AH: Myozenin: An a-actinin and g-filamin-binding protein of skeletal muscle z-lines. Proc Nati Acad Sci USA 2001, 98:1595-1600.

23. Davis RJ: Signal transduction by the JNK group of MAP kinases . Cell 2000, 103:239-252.

24. Raingeaud J, Whitmarsh AJ, Barrett T, Dérijard B, Davis RJ: MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol Cell Biol 1996, 16(3):1247-1255.
25. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K: The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 1999, 398 (6724):252-256.

26. Biederer C, Ries S, Drobnik W, Schmitz G: Molecular cloning of human caveolin 3. Biochim Biophys Acta 1998, 1406:5-9.

27. Woodman WE, Sotgia F, Galbiati F, Minetti C, Lisanti MP: Caveolinopathies: Mutations in caveolin-3 cause four distinct autosomal dominant muscle diseases. Neurol 2004, 62:538-543.

28. Zhu Z, Li Y, Mo. D, Li K, Zhao S: Molecular characterization and expression analysis of the porcine caveolin-3 gene. Biochem Biophys Res Comm 2006, 346:7-13.

29. Smythe GM, Rando TA: Altered caveolin-3 expression disrupts PI(3) kinase signaling leading to death of cultured muscle cells. Exp Cell Res 2006, 312:2816-2825.

30. Senf SM: Skeletal muscle heat shock protein 70: diverse functions and therapeutic potential for wasting disorders. Frontiers Physiol 2013, 4:1-6.

31. Truscott K, Brandner NK, Planner N: Mechanisms of protein import into mitochondria. Curr Biol 13, 13:R326-337.

32. Hermann JM, Stuart RA, Craig EA, Neupert W: Mitochondrial heat shock protein 70, a molecular chaperone for proteins encoded by mitochondrial DNA. J Cell Biol 1994, 127:893-902.

33. Ferreira LF, Laitano O: Regulation of NADPH oxidases in skeletal muscle. Free Rad Biol Med 2016, 98:18-28.

34. Zhou N, Lee WR, Abasht B: Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens' feed efficiency. BMC Genomics 2015, 16:195-215.

35. Dikalov S: Crosstalk between mitochondria and NADPH oxidases . Free Rad Biol Med 2011, 51:1289-1301.

36. Kobayashi A, Kang M, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi TI, Yamamoto M: Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteosomal degradation of Nrf2. Mol Cell Biol 2004, 24:7130-7139.

37. Kobayashi A, Kang M, Watai Y, Tong KI, Shibata T, Koji U, Yamamoto M: Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mo Cell Biol 2006, 26:221-229.

38. McFarland DC, Velleman SG, Pesall JE, Liu C: Effect of myostatin on turkey myogenic satellite cells and embryonic myoblasts. Comp Biochem Physiol 2006, 144:501-508.

39. Gu Z, Zhu D, Li N, Li H, Deng X, Wu C: The single nucleotide polymorphisms of the chicken myostatin gene are associated with skeletal muscle and adipose growth. Science in China Ser C Life Sciences 2004, 47:26-31.

40. Ye X, Brown SR, Nones K, Coutinho LL, Dekkers JCM, Lamont SJ: Associations of myostatin gene polymorphisms with performance and mortality traits in broiler chickens. Genet Sel Evol 2007, (73):79.

41. Bhattacharya TK, Chatterjee RN: Polymorphism of the myostatin gene and its association with growth traits in chicken. Poult Sci 2013, 92(4):910-915 doi:10.3382/ps2012-02736.

42. Lee S, Glass DJ: Treating cancer cachexia to treat cancer. Skel Musc 2011, 1:1-5 http://doi.org/10.1186/2044-5040-1-2.

43. Hardie DG, Scott JW, Pan DA, Hudson ER: Management of cellular energy by the AMP-activated protein kinase system. FEBS Letters 2003, 546:113-120.

44. Carling D: The AMP-activated protein kinase cascade - A unifying system for energy control. Trends Biochem Sci 2004, 29:18-24.

45. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001, 108:1167-1174.

46. Hardie DG: AMP- activatred/ SNF1 protein kinases: conserved guardians of cellular energy. Nature Rev Mol Cell Biol 2007, 8:774-785.

47. Bottje WG, Lassiter K, Dridi S, Hudson N, Kong B: Enhanced expression of proteins involved in energy production and transfer in breast muscle of pedigree male broilers exhibiting high feed efficiency. . Poult Sci 2017, 96:2454-2458.

48. Laplante M, Sabatini DM: Regulation of mTORC1 and its impact on gene expression at a glance. J Cell Sci 2013, 126:1713-1719.

49. Piekarski A: Autophagy and its potential role in stress and feed efficiency in avian tissues. 2015. *University of Arkansas, Fayetteville* 2015.

50. Kim DK, Sarbassov DD, Siraj MA, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM: mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 2002, 110:163-175.

51. Bigot K, Taouis M, Tesseraud S: Refeeding and insulin regulate S6K1 activity in chicken skeletal muscles . J Nutr 2003, 133(2):369-373.
52. Dowling RJ, Topisirovic I, Fonseca BD, Sonenberg N: Dissecting the role of mTOR: lessons from mTOR inhibitors. Biochim Biophys Acta 2010, 1804(3):433-439.

53. Dupont J, Rideau N, Simon J: Endocrine Pancreas. In *Sturkie's Avian Physiology (6 edition).* Edited by Scanes CG. New York: Elsevier; 2015:613-631.

54. Dupont J, Derouet M, Simon J, Taouis M: Nutritional state regulates insulin receptor and IRS-1 phosphorylation and expression in chicken. Am J Physiol 1998, 274:E309-E316.

55. Dupont J, Derouet M, Simon J, Taouis M: Effect of nutritional state on the formation of a complex involving insulin receptor IRS-1, the 52 kDa Src homology/collagen protein (Shc) isoform and phosphatidylinositol 3'-kinase activity . Biochem J 1998, 335:293-300.

56. Cohen AW, Combs TP, Scherer PE, Lisanti MP: Role of caveolin and caveolae in insulin signaling and diabetes. Am J Physiol Endocrinol Metab 2003, 285:E1151-E1160.

57. Duchene S, Metayer S, Audouin E, Bigot K, Dupont J, Tesseraud S: Refeeding and insulin activate the AKT/p70S6 kinase pathway without affecting IRS1 tyrosine phosphorylation in chicken muscle . Dom Anim Endo 2008, 34:1-13.

58. Fawcett J: Biologically active insulin-derived peptides. Prot Peptide Lett 2014, 21:584-588.

59. Leissring MA, Malito E, Hedouin S, Reinstatler L, Sahara T, Abdul-Hay SO, Choudhry S, Maharvi GM, Fauq AH, Huzarska M, May PS, Choi S, Logan TP, Turk BE, Cantley LC, Manolopoulou M, Tang W, Stein RL, Cuny GD, Selkoe DJ: Designed inhibitors of insulin-degrading enzyme regulate the catabolism and activity of insulin. PLoS One 2010, 5(5):e10504.

60. Leissring MA, Farris W, Wu X, Christodoulou DC, Haigis MC, Guarente L, Selkoe DJ: Alternative translation initiation generates a novel isoform of insulin-degrading enzyme targeted to mitochondria. Biochem J 2004, 383:439-446.

61. Stewart CEH, Rotwein P: Growth, differentiation and survival: Multiple physiological interactions for insulin-like growth factors. Physiol Rev 1996, 76:1005-1026.

62. Spangenburg EE, Abraha T, Childs TE, Pattison JS, Booth FW: Skeletal muscle IGF-binding protein-3 and -5 expression are age, muscle, and load dependent. Am J Physiol Endocrinol Metab 2003, 284:E340-350.

63. Foulstone EJ, Savage PB, Crown AL, Jeff MPH, Stewart CEH: Role of insulin-like growth factor binding protein-3 (IGFBP-3) in the differentiation of primary human adult skeletal myoblasts. J Cell Physiol 2003, 195:70-79.

4.7 TABLES AND FIGURES

Table 4.1 Oligonucleotide for quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers.

Gene Symbol	Gene Name	Accession Number[a]	Primer Sequence (5' ➔ 3')	Orientation	Product Size (bp)
ActRIIA	Activin Receptor IIA	NM_205367.1	GCCATCTCACACAGGGACAT TACCTTCGTGTGCCAACCTG	Forward Reverse	146
ActRIIB	Activin Receptor IIB	NM_204317.1	CGTGACCATCGAAGAGTGCT CACGATGGAGACAAGGCAGT	Forward Reverse	130
ALK4	Activin-like Kinase 4	XM_001231300.3	CCGCTACACGGTGACCATAG TCCCAGGCTTTCCCTGAGTA	Forward Reverse	107
ALK5	Actvin-like Kinase 5	NM_204246.1	GGCAGAGCTGTGAGGCATTA CTAGCAGCTCCGTTGGCATA	Forward Reverse	73
AMPKα1	AMP activated Kinase α 1	NM_001039603.1	CCACCCCTGTACCGGAAATA GGAAGCGAGTGCCAGAGTTC	Forward Reverse	68
AMPKα2	AMP activated Kinase α 2	NM_001039605.1	TGTAAGCATGGACGTGTTGAAGA GCGGAGAGAATCTGCTGGAA	Forward Reverse	62
CAV-3	Caveolin-3	NM_204370.2	CGTTGTAAAGGTGGATTTCGAGG ACCAGTACTTGCTGACGGTG	Forward Reverse	110
CK(m)	Creatine Kinase (muscle isoform)	NM_205507.1	TGGGTTACATCCTGACGTGC CTCCTCGAATTTGGGGTGCT	Forward Reverse	101
FSTN	Follistatin	NM_205200.1	CCCGGGCATGCTCGTA TGCGCTGTGTGATCTTCCAT	Forward Reverse	60
GLUT-8	Glucose Transporter-8	NM_204375.1	GGCATCGTGGTTTGGGTCTA ATCCACAAGGTAGCCTCCCA	Forward Reverse	73
HSP-70	Heat Shock Protein 70	JO2579	GGGAGAGGGTTGGGCTAGAG TTGCCTCCTGCCCAATCA	Forward Reverse	55
IDE	Insulin Degrading Enzyme	XM_421686.5	GCCCATTTGCTTACGTGGAT GTTGAGGGAGTCTTTGAGTAGTTCAA	Forward Reverse	77
IGF-1	Insulin-like Growth Factor 1	NM_001004384.2	GCTGCCGGCCCAGAA ACGAACTGAAGAGCATCAACCA	Forward Reverse	56
IGFBP-3	IGF-1 Binding Protein	NM_001101034.1	ATCAGGCCATCCCAAGCTT GATGTGCTGTGGAGGCAAATT	Forward Reverse	59

Table 4.1 (Cont'd).

Gene Symbol	Gene Name	Accession Number[a]	Primer Sequence (5' ➔ 3')	Orientation	Product Size (bp)
IRS-1	Insulin Receptor Substrate 1	NM_005544.2	GCGCAAGGTGGGCTACCT CGCGCGCAGTACGAAGA	Forward Reverse	64
MAP2K6	Mitogen activated Kinase kinase 6	XM_003642348.2	TGTCTCAGTCGAGAGGCAAA TGGAGTCTAGATCCCTGGGT	Forward Reverse	105
MAP3K7	Mitogen activated Protein kinase 7	XM_004940375.1	CCTGATGATGCAGGTAAGACCA TCTTTGGAGTTCGGGCATGG	Forward Reverse	107
MSTN	Myostatin	NM_001001461.1	ATGCAGATCGCGGTTGATC GCGTTCTCTGTGGGCTGACT	Forward Reverse	59
mTOR	Mechanistic target of rapamycin	XM_417614.5	CATGTCAGGCACTGTGTCTATTCTC CTTTCGCCCTTGTTTCTTCACT	Forward Reverse	77
MYOG	Myogenin	NM_204184.1	GGAGAAGCGGAGGCTGAAG GCAGAGTGCTGCGTTTCAGA	Forward Reverse	62
MYOZ2	Myozenin	NM_001277827.1	CAACACTCAGCAACAGAGGC GTATGGGCTCTCCACGATTTCT	Forward Reverse	120
NCF2	Neutrophil Cytosolic Factor 2	XM_004943279.1	TCTTTGCTTGCGAGGTGGT TTTCTGGTGTCTTGGGCCTG	Forward Reverse	111
P70S6K	70 kDa ribosomal Protein S6 kinase	NM_001109771.2	GTCAGACATCACTTGGGTAGAGAAAG ACGCCCTCGCCCTTGT	Forward Reverse	60
PRKAR1A	cAMP dependent Kinase Iα	NM_001007845.1	GTGGGAGCGCCTTACTGTAG CAGCTGTGCCCTCCAAGATA	Forward Reverse	119
RAPTOR	Regulatory protein of mTOR	XM_004946275.1	GGCTACGAGCTCTGGATCTG TGACATGACAAGCTAACTGCC	Forward Reverse	70
SHC-1	SHC-transforming Protein 1	NM_001293280.1	CTGCTCAAGCAGGAAGAGAGAAA GCGTGTCTTGTCCACGTTCT	Forward Reverse	110
SMAD2	Mothers against Decapentaplegic homolog 2	NM_204561.1	TGAGTATAGGCGGCAGACCG AAGGGGAGCCCATCTGAGTC	Forward Reverse	107
SMAD3	Mothers against Decaplegic homolog 3	NM_204475.1	CCCACCGTTGGACGATTACA GGAGGAGGTGTCTCTGGGAT	Forward Reverse	99
18S		AF173612	TCCCCTCCCGTTACTTGGAT GCGCTCGTCGGCATGTA	Forward Reverse	60

Table 4.2 Expression of eukaryotic translation elongation and initiation factors list obtained from an RNAseq dataset of breast muscle tissue (from Bottje et al., 2017) showing log2 high feed efficiency – log2 low feed efficiency (M), the gene symbol and the gene name. A negative or positive M value indicates expression was numerically lower or higher in the high feed efficiency phenotype compared to the low feed efficiency phenotype. Of the 56 sequences that were detected, 43 were higher (positive M value) in the high feed efficiency phenotype and 13 were lower (negative M value) in the high FE compared to the low FE phenotype. The skew in the high FE phenotype was significant (binomial P value = 0.0002).

M	Symbol	Entrez Gene Name
-0.67	EEF1A1	eukaryotic translation elongation factor 1 alpha 1
-0.18	EIF6	eukaryotic translation initiation factor 6
-0.17	EIF4A2	eukaryotic translation initiation factor 4A2
-0.15	EIF2AK2	eukaryotic translation initiation factor 2 alpha kinase 2
-0.11	EIF2AK3	eukaryotic translation initiation factor 2 alpha kinase 3
-0.10	EIF3H	eukaryotic translation initiation factor 3 subunit H
-0.07	EIF3E	eukaryotic translation initiation factor 3 subunit E
-0.07	EIF3M	eukaryotic translation initiation factor 3 subunit M
-0.05	EIF2AK1	eukaryotic translation initiation factor 2 alpha kinase 1
-0.05	EIF4H	eukaryotic translation initiation factor 4H
-0.04	EIF3L	eukaryotic translation initiation factor 3 subunit L
-0.03	EIF4A3	eukaryotic translation initiation factor 4A3
-0.01	EIF4ENIF1	eukaryotic translation initiation factor 4E nuclear import factor 1
0.00	EIF2S3	eukaryotic translation initiation factor 2 subunit gamma
0.00	EIF3A	eukaryotic translation initiation factor 3 subunit A
0.02	EEF1AKMT1	eukaryotic translation elongation factor 1 alpha lysine methyltransferase 1
0.04	EEF2	eukaryotic translation elongation factor 2
0.05	EEF1A1	eukaryotic translation elongation factor 1 alpha 1
0.05	EIF2B5	eukaryotic translation initiation factor 2B subunit epsilon
0.05	EIF4E	eukaryotic translation initiation factor4E
0.06	EIF3I	eukaryotic translation initiation factor 3 subunit I
0.06	EIF4G2	eukaryotic translation initiation factor 4 gamma 2
0.06	EIF5A2	eukaryotic translation initiation factor 5A2
0.07	EIF2B1	eukaryotic translation initiation factor 2B subunit alpha
0.08	EIF4E3	eukaryotic translation initiation factor 4E family member 3
0.10	EIF1B	eukaryotic translation initiation factor 1B
0.10	EIF3B	eukaryotic translation initiation factor 3 subunit B
0.11	EIF2D	eukaryotic translation initiation factor 2D
0.11	EIF3F	eukaryotic translation initiation factor 3 subunit F
0.12	CTIF	cap binding complex dependent translation initiation factor
0.12	EIF4E2	eukaryotic translation initiation factor 4E family member 2

Table 4.2 (Cont'd).

M	Symbol	Entrez Gene Name
0.14	EIF2AK4	eukaryotic translation initiation factor 2 alpha kinase 4
0.14	HBS1L	HBS1 like translational GTPase
0.17	EIF4G3	eukaryotic translation initiation factor 4 gamma 3
0.17	TPT1	tumor protein, translationally-controlled 1
0.18	EIF1AY	eukaryotic translation initiation factor 1A, Y-linked
0.19	EIF1	eukaryotic translation initiation factor 1
0.19	EIF3D	eukaryotic translation initiation factor 3 subunit D
0.21	EIF2B2	eukaryotic translation initiation factor 2B subunit beta
0.21	EIF4G1	eukaryotic translation initiation factor 4 gamma 1
0.22	EEF1D	eukaryotic translation elongation factor 1 delta
0.22	EIF2B3	eukaryotic translation initiation factor 2B subunit gamma
0.23	MTO1	mitochondrial tRNA translation optimization 1
0.24	EIF2B1	eukaryotic translation initiation factor 2B subunit alpha
0.24	EIF5	eukaryotic translation initiation factor 5
0.25	EIF2B4	eukaryotic translation initiation factor 2B subunit delta
0.26	TMA16	translation machinery associated 16 homolog
0.28	EIF2A	eukaryotic translation initiation factor 2A
0.28	EIF2S1	eukaryotic translation initiation factor 2 subunit alpha
0.28	EIF5B	eukaryotic translation initiation factor 5B
0.29	EEF1B2	eukaryotic translation elongation factor 1 beta 2
0.32	EIF3J	eukaryotic translation initiation factor 3 subunit J
0.33	MTIF2	mitochondrial translational initiation factor 2
0.41	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1
0.43	MTIF3	mitochondrial translational initiation factor 3
0.45	MTRF1	mitochondrial translational release factor 1
0.61	MSS51	MSS51 mitochondrial translational activator
0.66	MTRF1L	mitochondrial translational release factor 1 like

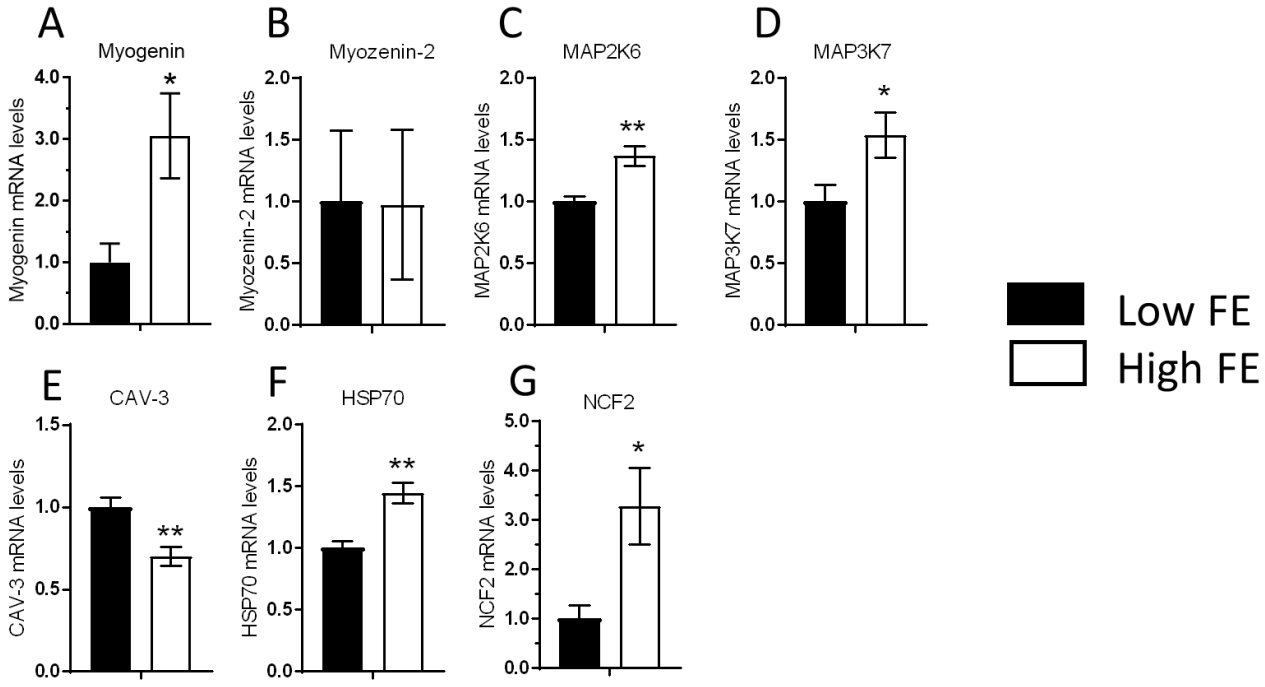


Figure 4.1 Differentially expressed genes that are involved in muscle development and differentiation in breast muscle from Pedigree Male (PedM) broilers exhibiting high or low feed efficiency (FE) phenotype. Relative expression of mRNA is shown for myogenin (A), myozenin-2 (B), mitogen activated protein kinase kinase 6 (MAP2K6) (C), MAP kinase kinase kinase 7 (MAP3K7) (D), caveolin 3 (CAV-3) (E), heat shock protein 70 (HSP70) (F), and neutrophil cytosolic factor 2 (NCF2) (G). Bars represent the mean ± SE (n=5). Mean values were different at $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

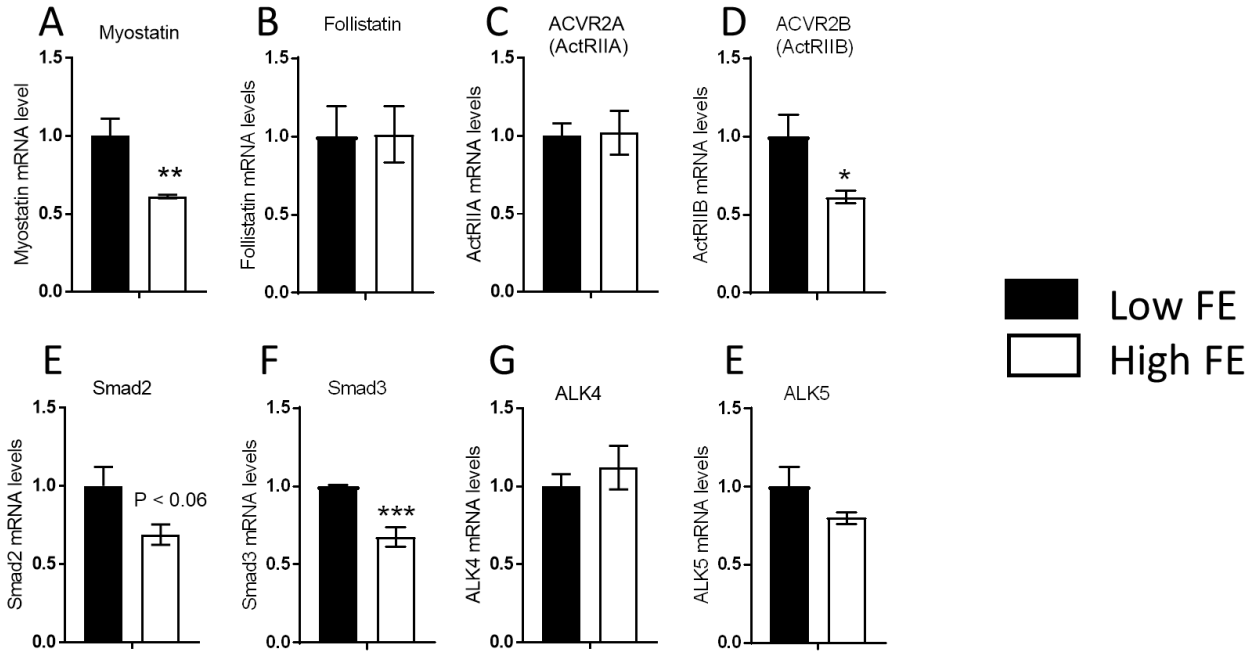


Figure 4.2 Differentially expressed genes that are involved in myostatin signaling in breast muscle from Pedigree Male (PedM) broilers exhibiting high or low feed efficiency (FE) phenotype. Relative expression of mRNA is shown for myostatin (A), follistatin (B), activin receptor type IIA and IIB (ActIIA and Act IIB) (C,D), Mothers against decapentaplegic homolog 2 and 3 (SMAD2 and SMAD3) (E, F), and activin receptor-like kinase 4 and 5 (ALK 4 and ALK 5) (G, H). Bars represent the mean ± SE (n=5). Mean values were different at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P < 0.001$.

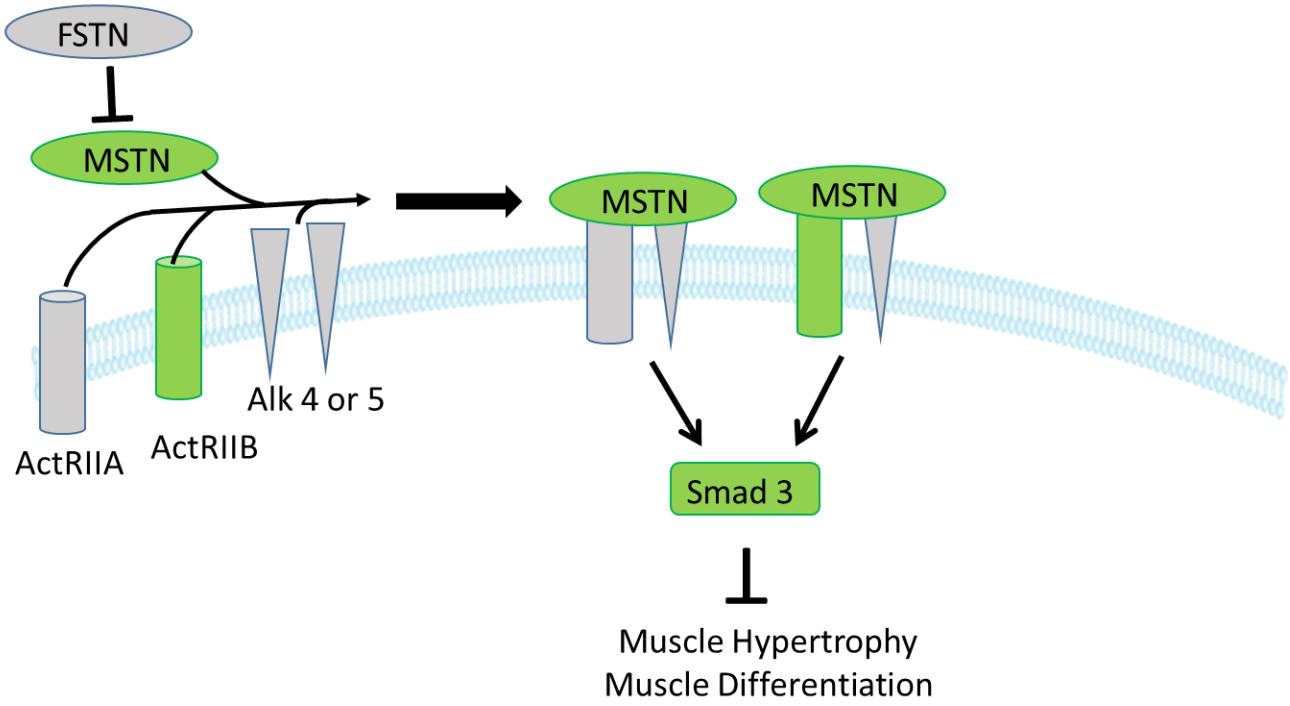


Figure 4.3 Myostatin signaling in muscle cells. Myostatin initially binds to one of the two activin type II receptors, and then binds to one or both of the activin-like kinase type I receptors 4 and 5 (ALK 4,5). Binding with myostatin activates the ALK4 and ALK 5 type I receptors resulting in phosphorylation of the transcription factors SMAD 2,3 that leads to inhibition of muscle growth and differentiation. Gene expression of the molecules shown in green were downregulated in the high FE phenotype (upregulated in the low FE phenotype) whereas molecules in gray were not differentially expressed between the groups. The figure is adapted from Lee and Glass (2011).

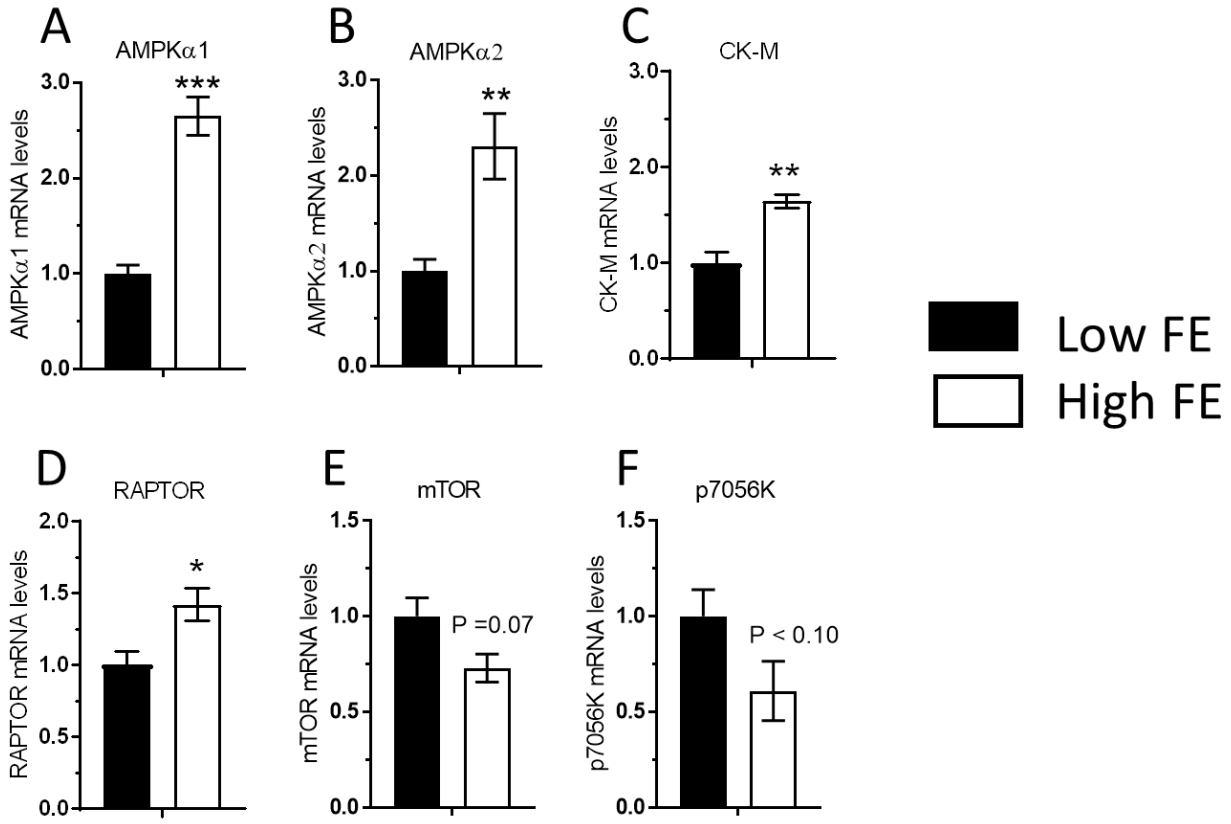


Figure 4.4 Differentially expressed genes that are involved in A) muscle metabolism (energy sensing/storage), and B) protein synthesis via nutrient sensing in breast muscle from Pedigree Male (PedM) broilers exhibiting high or low feed efficiency (FE) phenotype. Relative expression of mRNA is shown for AMP-activated protein kinase alpha subunits 1 and 2 (AMPKα1 and AMPKα2), creatine kinase muscle isoform (CKM), regulatory-associated protein of mTOR (RAPTOR), mechanistic target of rapamycin (mTOR), and 70 kDa ribosomal protein S6 kinase (P70S6K). Bars represent the mean ± SE (n=5). Mean values were different at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P < 0.001$ (***).

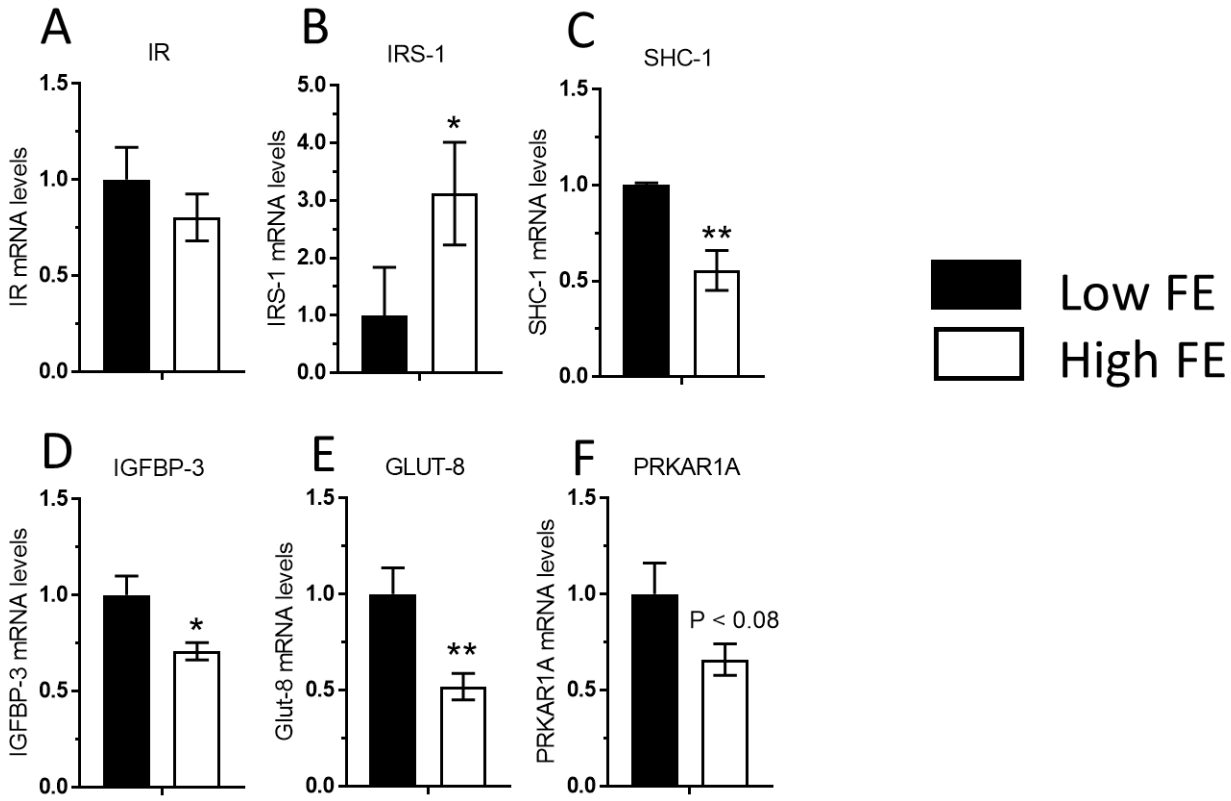


Figure 4.5 Gene expression associated with the insulin signaling pathway in breast muscle from Pedigree Male (PedM) broilers exhibiting high or low feed efficiency (FE) phenotype. Relative expression of mRNA is shown for; A) insulin receptor (IR), B) insulin receptor substrate 1 (IRS-1), C) SHC-transforming protein 1 (SHC-1), D) insulin-like growth factor-binding protein 3 (IGFBP-3), E) glucose transporter 8 (GLUT-8), and F) cAMP-dependent protein kinase type I-alpha regulatory subunit (PRKAR1A). Bars represent the mean ± SE (n=5). Mean values were different at $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

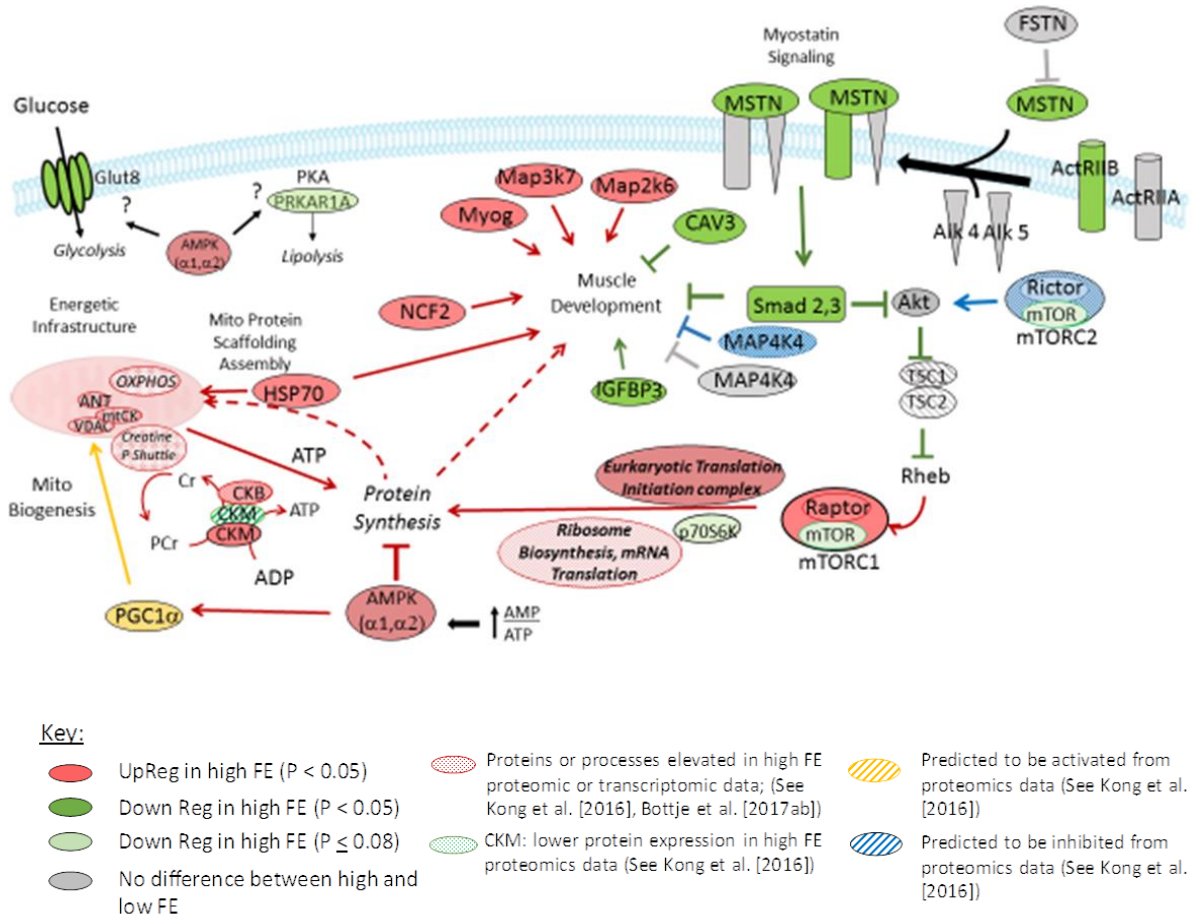


Figure 4.6 Diagrammatic representation of targeted gene expression analysis conducted in the present study combined with information obtained from results from global protein and gene expression studies [7, 8]. Genes expression that would enhance muscle development in the high FE phenotype include the upregulation of Myog (Myogenin), Map3K7, Map2K6, and NCF2 and down regulation of CAV3 and Smad 2,3. Map4k4 was not differentially expressed in the present study but was predicted to be inhibited in a proteomic study [7] (Kong et al., 2016). Components that would potentially enhance protein synthesis include Raptor and eukaryotic translation and initiation complex expression (see Table 2) and components supporting ribosome assembly and mRNA translation (see Bottje et al., [8]). Although upregulation of AMPK in high FE would presumably inhibit protein synthesis, stimulation of mitochondrial biogenesis through peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (see Kong et al. [7]) and enhanced energetic infrastructure and creatine kinase shuttle expression (Bottje et al., [47]) could provide ATP needed to support protein synthesis in high FE breast muscle. The downregulation of Glut 8 and PRKAR1A that are involved in glycolysis and lipolysis, respectively, appears to not be compatible with the elevation of AMPK in the high FE PedM phenotype.

CHAPTER 5

Identification of mitochondrial hormone receptors in avian muscle cells

Kentu Lassiter, Sami Dridi, Elizabeth Greene, Byungwhi Caleb Kong, and Walter G. Bottje

Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, Arkansas, United States of America

Published:

Lassiter, K., S. Dridi, E. Greene, B. C. Kong, and W. Bottje. 2018. Identification of mitochondrial hormone receptors in avian muscle cells. Poult. Sci. 97: 2926-2933.

5.1 ABSTRACT

The major objective of this study was to assess the expression of mitochondrial hormone receptors for progesterone (PR), estrogen (ER), glucocorticoid (GR), thyroid (TR), and insulin (IR) in avian muscle cells (quail muscle 7, QM7) and in breast muscle of quail and broilers. Visualization of receptor location in QM7 cells was accomplished by immunofluorescence. QM7 cells were stained with Mito Tracker Deep Red CMX, fixed in methanol, immune stained with anti-PR, -GR, -TR, -IR, and -ER primary antibodies overnight at 4°C, and visualized with Alexa Fluor 488-conjugated secondary antibody. After staining the nucleus with 4',6-diamidino-2-phenylindole (DAPI), images were obtained by immunofluorescence microscopy. Merged images revealed the presence of all five hormone receptors on mitochondria in QM7 cells. Western blot analysis identified; a) the β-isoform of the PR, b) the α-isoform of GR, c) the α–receptor of TR, d) the β–subunit of IR, and e) the α-isoform of the ER on mitochondria isolated from broiler breast muscle. Similar results were obtained in quail breast muscle mitochondria with the exception that the α-isoform of the GR was not detected. To our knowledge, this is the first report of hormone receptors (PR, TR, GR, IR, and ER) on mitochondria in avian cells. We hypothesize that these receptors could play important roles in regulating mitochondrial function in avian muscle cells.

Key Words: mitochondrial hormone receptors, muscle cells, steroid hormones, thyroid hormone, insulin.

5.2 INTRODUCTION

Steroid hormones, thyroid hormone, and insulin are well known to influence fundamental cellular and organismal processes such as metabolism, growth, and development. There is accumulating evidence in mammals that these hormones may act on mitochondria by two general mechanisms. The first is an indirect mechanism in which the hormone is conveyed to the nucleus where it binds to hormone response elements followed by cell signaling that, among other cell processes, enhance mitochondrial protein transcription, mitochondrial biogenesis and subsequently, mitochondrial function (see reviews by Wrutniak-Cabello et al. 2001; Chen et al., 2005; Psarra et al. 2006). In the second mechanism, the hormone binds directly to mitochondrial receptors to initiate effects on mitochondria gene expression and function (e.g. Tsiriyotis et al., 1997; Psarra, et al. 2008; Du et al. 2009; Dai et al. 2013; Feng et al., 2014).

From global gene and global protein expression studies conducted in muscle tissue, we have evidence that several hormones and/or hormone receptors including progesterone, estrogen, thyroid hormone, corticosterone, and components of insulin signaling are associated with the phenotypic expression of feed efficiency in a Pedigree Male broiler line (Bottje et al., 2012; 2016; Kong et al., 2011; 2016). Progesterone receptor and two other transcription factors involved in progesterone signaling were identified as having a major impact on phenotypic expression of feed efficiency (Bottje et al., 2017). This latter finding is somewhat curious as these studies were conducted in immature male broiler breeders in which endogenous progesterone levels would not typically be considered as having major effects on cell function.

Studies investigating progesterone receptors in mammalian mitochondria have shown that the hormone plays a role in controlling oxidative phosphorylation during periods of high metabolic activity or in metabolically active tissues (Dai et al., 2013; Price and Dai, 2015). The

binding of hormones to mitochondrial receptors stimulates the activation of transcription factors and genes encoding components of the oxidative phosphorylation system, which increases mitochondrial biogenesis and the capacity for cellular respiration (Psarra et al., 2006). Progesterone and estrogen reduced mitochondrial reactive oxygen species production and oxidative damage in brain cells (Irwin et al. 2008). Pretreatment of animals with progesterone reduced the effects of traumatic brain injury by preserving mitochondrial function in nerve tissue (Robertson and Sarawati, 2015). Progesterone was also effective in maintaining mitochondrial function by initiating transcription when the hormone-receptor complex binds to hormone-response elements located on both nuclear and mitochondrial DNA (Psarra et al., 2006).

Although mitochondrial hormone receptors have been identified in mammalian tissues, to our knowledge, there are no reports of avian mitochondrial hormone receptors. The presence of hormone receptors in avian mitochondria would give additional insight into how mitochondrial function is controlled and if this might related to genetic traits such as feed efficiency. Therefore, the purpose of this study is to determine if mitochondrial hormone receptors for progesterone, estrogen, glucocorticoid, thyroid, and insulin are present in both an avian muscle cell line and intact avian muscle tissues.

5.3 MATERIALS AND METHODS

5.3.1 ANIMALS AND BREAST MUSCLE TISSUE COLLECTION

Breast muscle tissue used in this study was obtained from male Cobb-500 broilers and male Japanese quail. The animals used in this study were raised under standard conditions, with a standard diet and provided access to feed and water *ad libitum*. Birds were humanely euthanized, breast muscle tissue (*Pectoralis major*) was quickly excised and flash frozen in

liquid nitrogen, and stored at -80 C º. All procedures for animal care complied with the University of Arkansas Institutional Animal Care and Use Committee (IACUC Protocols #13039 and #14012).

5.3.2 CELL CULTURE

Quail muscle 7 (**QM7**) cells were grown in cell culture medium M199 (ThermoFisher, Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Life Technologies) at 37°C under a humidified atmosphere of 95% air and 5% CO_2 as previously described (Lassiter et al., 2015).

5.3.3 SEPARATION OF NUCLEAR, CYTOPLASMIC, AND MITOCHONDRIAL COMPONENTS

Nuclear and cytoplasmic proteins were extracted from QM7 cells and breast muscle using the Membrane, Nuclear, & Cytoplasmic Protein Extraction Kit (BSP002) (Bio Basic Inc., Ontario, Canada), according to the manufacturer's recommendations. Mitochondria were isolated from QM7 cells and broiler/quail breast muscle using Thermo Scientific Mitochondria Isolation Kits 89874 and 89801(Pierce Biotechnology, Rockford, IL), respectively, according to the manufacturer's recommendations.

5.3.4 ANTIBODIES

Immunofluorescence assessment of mitochondrial hormone receptors in QM7 cells utilized affinity purified rabbit polyclonal antibodies for anti-progesterone receptor (**PR**), anti-estrogen receptor (**ER**), anti-glucocorticoid receptor (**GR**), anti-thyroid hormone receptor (**TR**), and anti-insulin receptor (**IR**) used in this study were obtained from Santa Cruz Biotechnology (Dallas,

TX). The anti-PR was raised against a peptide mapping at the C-terminus of PR of human origin, and the anti-GR was raised against a peptide mapping at the C-terminus of GRα of human origin. The anti-TR was raised against amino acids 1 to 408 that represents the full length TRα1 of chicken origin, yet this antibody is able to detect both α and β isoforms. The anti-IR was raised against amino acids 128 to 205 that represents the α-isoform of human origin.

With the exception of the TR polyclonal antibody, initial studies revealed extensive non-specific binding of polyclonal antibodies for the remaining hormone receptors in breast muscle tissue. Thus, monoclonal antibodies were used to detect the PR, IR, and GR hormone receptors in broiler and quail breast muscle by Western blot analysis. Protein G purified mouse monoclonal anti-estrogen receptor (**ER**) (Thermo Fisher Scientific, Rockford, IL) was raised against the C-terminus (aa 302-595) of human ER expressed in *E. coli*. Mouse monoclonal anti-IR and anti-GR (Santa Cruz Biotechnology, Dallas, TX) were raised against the C-terminus of IR of human origin and amino acids 121-420 of GR of human origin, respectively. The protein G purified mouse monoclonal anti-PR (Abcam Inc., Cambridge, MA) was raised against PR originating from chick oviduct cytosol.

To verify the separation of mitochondria from cytosolic and nuclear compartments, western analysis was conducted on each cell fraction using polyclonal antibodies (Cell Signaling Technology Inc., Danvers MA) for: a) the 32 kDa voltage-dependent anion channel (**VDAC**) protein found exclusively in mitochondria (Columbini et al., 1996), b) the 75 kDa glucose regulated protein 75 (**GRP-75),** a protein found in the mitochondria and cytosol, but not in the nucleus, (Mizzen et al., 1991; Mazkereth et al., 2016), c) glyceraldehyde 3 phosphate dehydrogenase (**GAPDH**) located primarily in the cytosol (Lodish et al., 2000), and d) the 110 kDa phosphoprotein nucleolin that is expressed in the nucleolus (Tutega and Tutega, 1999). The

VDAC protein was detected using protein A/peptide affinity chromatography-purified polyclonal antibody for human VDAC (Cell Signaling Technology Inc., Danvers, MA). GRP-75 was detected with a human GRP-75 monoclonal antibody (Thermo Fisher Scientific, Waltham MA). GAPDH was detected using a polyclonal anti-GAPDH raised against amino acids 1-335 that represent full length GAPDH of human origin (Santa Cruz Biotechnology, Dallas, TX). Nucleolin was detected using a polyclonal anti-nucleolin raised against amino acids 271-520 of nucleolin of human origin (Santa Cruz Biotechnology, Dallas, TX).

5.3.5 IMMUNOFLUORESCENCE

QM7 cells were grown to 50-60% confluence in chamber slides (Lab-Tek, Hatfield, PA) and stained with Mitotracker® deep red CMX dye (dihydro-X-rosamine, ThermoFisher, Molecular Probes, Life Technologies, Grand Island, NY) according to manufacturer specifications. The MitoTracker dye passively diffuses across the cell membrane and accumulate in active mitochondria. The cells were then fixed in methanol for 10 min at -20°C with protein block serum-free blocking buffer (Dako, Carpinteria, CA). The cells were then incubated with rabbit anti-PR, anti-GR, anti-TR, anti-IR, (1:200; Santa Cruz Biotechnology, Dallas, TX) or mouse anti-ER (Thermo Fisher Scientific, Rockford, IL) overnight at 4°C and visualized with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Life Technologies). The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and cover-slipped in Vectashield (Vector Laboratories, Burlingame, CA). Fluorescent images were obtained using a Zeiss Imager M2 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) with an attached CCD camera (Hamamatsu, Orca ER, Bridgewater, NJ), and Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD).

5.3.6 PROTEIN EXPRESSION

QM7 cells and breast muscle tissue were homogenized in RIPA buffer (Pierce Biotechnology) containing Halt™ Protease & Phosphatase Inhibitor Cocktail (EDTA-free, Pierce Biotechnology). Protein concentrations were determined using a Synergy HT multimode microplate reader (BioTek, Winooski, VT) and a Bradford assay kit (Bio-Rad, Hercules, CA) with BSA as a standard. Proteins (ranging from 30 to 125 µg loaded on the gel) were separated on 10% Mini-PROTEAN® TGX™ gels (Bio-Rad). The separated proteins were transferred to PVDF membranes, blocked for 1 h at room temperature, and incubated with primary antibodies (dilutions ranging from 1:100 to 1:1000) at 4°C overnight. Due to non-specific binding observed with polyclonal antibodies, monoclonal antibodies (mouse anti-ER, anti-PR, anti-IR, and anti-GR) were used for western analysis. A pre-stained molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (Bio-Rad). The PVDF membranes were incubated with the HRP-conjugated secondary antibody (1:5000) (Cell Signaling Technology) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences, Pittsburgh, PA) and captured by FluorChem M MultiFluor System (ProteinSimple, San Jose, CA). Image acquisition was performed using AlphaView software (ProteinSimple).

5.3.7 IMMUNOPRECIPITATION

Immunoprecipitation was performed prior to detection of the ER in order to reduce incidences of non-specific binding by the primary antibody observed in pilot studies. The primary antibody was first conjugated to Dynabeads™ M-280 Tosylactivated magnetic beads using a DynaMag™-2 magnet (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's instructions. The antibody-coupled beads were mixed with the protein sample

and incubated at 35°C for 30-40 min to capture the specific target protein. The bead-antibody-protein complex was resuspended in Laemmli sample buffer before proceeding directly to Western blotting.

5.4 RESULTS

5.4.1 IMMUNOFLUORESCENCE IMAGING

Immunofluorescence imaging of QM7 cells is presented in Figure 1. In the third panel for each hormone, functional mitochondria were evident in the QM7 myoblast cells as indicated by their ability to import MitoTracker Red into the organelles. The yellow-orange color in the merged images in the fourth panel indicates that hormone receptors were co-localized on mitochondria of QM7 cells.

5.4.2 WESTERN BLOT ANALYSIS

To verify that the mitochondrial fraction did not contain nuclear proteins, western analysis was conducted on each of the cell fractions for GRP-75, VDAC, and GAPDH (Figure 2). Clear separation of the mitochondrial from the nuclear fraction is shown in Figure 2A by the absence of binding to GRP-75 and VDAC1. GAPDH was primarily detected in the cytoplasmic fraction with a small amount present in the nuclear fraction and a faint detection in the mitochondrial fraction (Figure 2B). There was no band present in the mitochondrial fraction at the appropriate molecular weight for nucleolin (110 kDa) but there was considerable non-specific binding in bands below 100 kDa (data not shown).

The presence of a specific isoform, subunit, or receptor for each mitochondrial hormone receptor at the target molecular weight was determined for all five hormones examined in broilers (Figure 3) and for PR, TR, IR and ER in quail (Figure 4). It is worth mentioning that the gel provided in

the product description for the GR antibody provided by the company (Santa Cruz Biotechnology) indicated the presence of two bands between approximately 90 to 110 kDa and the gel was cutoff below 80 kDa. Thus, the second band shown in Figure 3b could either be the result of non-specific binding or possibly the presence of a receptor protein fragment. No literature citation was provided for this gel, so we assume it was conducted by the company. A second band at ~ 30 to 32 kDa in the ER blot (Figure 3e) was also present in the background information on the ER antibody obtained from Thermo Fisher. In quail muscle, several bands in addition to the β isoform band of PR was apparent (Figure 4a). A very gel image was presented in the background information for the Thermo Fisher PR antibody.

5.5 DISCUSSION

The economic impact of feed efficiency has been illustrated in production animal agriculture (e.g. Emmerson 1997; Robinson and Oddy, 2004; Patience et al., 2015). Commercial broilers have been highly selected for growth and feed efficiency when compared to previous, less intensely-selected lines of birds (Zuidhof et al., 2014). Progesterone was reported to play a role in the phenotypic expression of feed efficiency in pedigree male broilers (Bottje et al., 2017). Progesterone was also predicted to be activated in the high feed efficiency pedigree male broiler phenotype based on downstream target protein expression (Kong et al., 2016). As mammalian mitochondria receptors for many hormones including progesterone (Tsiriyotis et al., 1997; Psarra, et al. 2008; Du et al. 2009; Dai et al. 2013; Feng et al., 2014), we initiated studies to determine if mitochondrial hormone receptors for progesterone, estrogen, glucocorticoid, thyroid hormone, and insulin were present in avian muscle cells *in vitro* and *in vivo*. To our knowledge, this is the first time that mitochondrial hormone receptors have been reported in any avian tissue or species.

The binding of progesterone to its receptor is typically associated with the physiological role in the reproductive function of females; i.e. development of the mammary glands and uterus, ovarian function, and sexual behavior (Connelly and Lydon, 2000). The progesterone-receptor complex functions as a transcription factor through its binding to hormone response elements in the nucleus, and the activation of Src signaling pathways in the cytoplasm that promote cell proliferation, cell differentiation and mitochondrial function (Edwards et al., 2003). Using rat brain mitochondria, Irwin et al. (2008) reported that progesterone-treated mitochondria exhibited increased cytochrome c oxidase activity, reduced oxidative damage, and enhanced antioxidant protection. Dai et al. (2013) demonstrated that treating cells with progestin increased cellular respiration and mitochondrial membrane potential. Therefore, the action of progesterone signaling appears to be beneficial to mitochondrial function, and supports findings in recent proteogenomic studies (Kong et al., 2016; Bottje et al., 2017).

The glucocorticoid receptor is a part of the superfamily of nuclear receptors (Psarra et al., 2006). The presence of glucocorticoid receptors in the mitochondria of mammalian cells has been clearly shown using immunofluorescence and immunogold electron microscopy (Scheller et al., 2000). There have been a number of reports illustrating how the presence of glucocorticoid receptors affect mitochondrial activity and the regulation of energy production. When HepG2 cells were stably transfected to overexpress mitochondrial-targeted glucocorticoid receptors, these cells showed an increase in RNA synthesis, expression of the mitochondrial protein cytochrome oxidase subunit I, and mitochondrial ATP production (Psarra and Sekeris, 2011). Treating rat skeletal muscle and C2C12 muscle cells with dexamethasone, a synthetic glucocorticoid, was shown to stimulate mitochondrial biogenesis (Weber et al., 2002). In a recent study, Morgan et al. (2016) suggested that a specific isoform of the glucocorticoid receptor (GRγ) is specialized in regulating

mitochondrial function, causing an increase in oxygen consumption, ATP production, and mitochondrial mass. Steroid receptors are translocated to the mitochondria following hormonal stimulation (Psarra et al., 2006). Translocation of the glucocorticoid receptor to the mitochondria is thought to occur by its interaction with chaperone proteins such as HSP70 (Du et al., 2009). Therefore, mitochondrial glucocorticoid receptors in avian muscle cells may play a role in regulating mitochondrial function and energy production.

Thyroid hormone plays a significant role in energy metabolism. This occurs when the hormone response element is activated by binding of the ligand-receptor complex, affecting transcription of both nuclear and mitochondrial genes that encode for subunits of the mitochondrial oxidative phosphorylation system (Scheller et al., 2003; Psarra and Sekeris, 2008). Due to this ability to interact with DNA binding sites that modulate gene and protein expression, the thyroid hormone receptor functions as a ligand-activated transcription factor (Psarra et al., 2006). Several isoforms of the thyroid hormone receptor are present in mammalian cells. The variation in number and position of amino acids in these isoforms can determine whether ligand binding promotes or inhibits transcription (Bassett et al., 2003). Two truncated thyroid receptor α isoforms (p28 and p43) that specifically targeted to the mitochondria increase mitochondrial gene expression, oxidative phosphorylation, and thermogenesis (Bassett et al., 2003). The p43 isoform is of particular interest since it is located in the mitochondrial matrix, and therefore in direct contact with the hormone response elements of mitochondrial DNA (Wrutniak et al., 1995). Interestingly, studies in which QM7 cells were transfected to overexpress p43 showed that mitochondrial activity was stimulated by this receptor isoform (Casas et al., 1999; Rochard et al., 2000). Based on the molecular weight of the thyroid receptor detected in our study which corresponds to the β isoform, it is not likely to be one of these mitochondria-specific isoforms. However, it is possible that this

β isoform is associated with non-genomic actions linked to secondary messenger signaling pathways (i.e. protein kinase and Ca^{2+} pathways) to elicit a hormone response (Losel and Wehling, 2003).

In the present study, immunofluorescence microscopy demonstrated that the insulin receptor co-localized with mitochondria in QM7 cells (Figure 1D), and that the β-subunit precursor was detected in the mitochondrial fraction of broiler (Figure 3D) and quail breast muscle (Figure 4C). However, a search of the literature does not indicate that the insulin receptor is located on the mitochondrial membrane. A relationship between mitochondrial function and insulin resistance has been reported (Montgomery and Turner, 2015). In neurons, signaling between the insulin receptor and mitochondria occurs through the generation of reactive oxygen species in the mitochondria, primarily via succinate oxidation (Pomytkin, 2012). The insulin receptor belongs to the family of receptor tyrosine kinases, where ligand binding stimulates a phosphorylation cascade that help regulate cell metabolism, proliferation, and differentiation. Recent studies have shown that certain members of the receptor tyrosine kinase family translocate to the mitochondria where the phosphorylation of mitochondrial proteins regulates metabolism and function (Ding et al., 2012; Salvi 2013). In the review by Lemmon and Schlessinger (2010), the extracellular structural domain of the human insulin receptor is very similar to that of the epidermal growth factor receptor (EGFR) which another receptor tyrosine kinase member that is known to translocate to the mitochondria (Salvi et al., 2005). The extracellular domains of both the EGFR and insulin receptor subfamilies contain large amounts of leucine and cysteine regions (Lemmon and Schlessinger, 2010).

In summary, the results of this study indicate that the avian muscle cells and breast muscle tissue express mitochondrial hormone receptors for progesterone, glucocorticoid, thyroid

hormone, and insulin. We hypothesize that the steroid hormones investigated in the current study could have direct effects on mitochondrial function in avian muscle through binding to specific mitochondrial hormone receptors and may play fundamentally important roles in avian physiology and genetic phenotypes.

5.6 REFERENCES

1. Bottje, W., Z.X. Tang, M. Iqbal, D. Cawthon, R. Okimoto, T. Wing, and M. Cooper. 2002. Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult. Sci. 81:546-555.

2. Bottje, W. G., and G. Carstens, 2009. Association of mitochondria with feed efficiency in livestock and poultry. J. Anim. Sci. 87:E48-E63.

3. Bottje, W.G., B.-W. Kong, J.J. Song, J.Y. Lee, B.M. Hargis, K. Lassiter, T. Wing, and J. Hardiman. 2012. Gene expression in breast muscle associated with feed efficiency in a single male broiler line using a chicken 44K oligo microarray. II. Differentially expressed focus genes. Poult. Sci. 91: 2576-2587.

4. Bottje, W., K. Lassiter, S. Dridi, N. Hudson, and B-W. Kong. 2016. Hormonal and mitochondrial signatures in muscle: Possible implications for mitochondrial function and feed efficiency. Poult. Sci. 95(E-Suppl. 1):75.

5. Bottje, W., B-W. Kong, A. Reverter, A. J. Waarendenberg, and N. J. Hudson. 2017. Progesterone signaling in broiler skeletal muscle is associated with divergent feed efficiency. BMC Systems Biology 11:29. DOI 10.1186/s12918-017-0396-2.

6. Casas, F., P. Rochard, A. Rodier, I. Cassar-Malek, S. Marchal-Victorion, R. J. Wiesner, G. Cabello, and C. Wrutniak. 1999. A variant form of the nuclear triiodothyronine receptor c-ErbAα1 plays a direct role in regulation of mitochondrial RNA synthesis. Mol. Cell. Biol. 19(12): 7913-7924.

7. Chen, J. Q., M. Delannoy, C. Cooke, J. D. Yager. 2005. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/patho-physiological implications. Biochim. Biophys. Acta 1746: 1-17.

8. Colombini, M., Blachly-Dyson, E. and Forte, M. 1996. VDAC, a channel in the outer mitochondrial membrane. In: "Ion Channels" Vol. 4 (Narahashi, T. ed.) pp 169-202. Plenum Publishing Corp., New York, NY.

9. Connelly, O. M., and J.P. Lydon. 2000. Progesterone receptors in reproduction: functional impact of the A and B isoforms. Steroids, 65(10): 571-577.

10. Dai, Q., A.A. Shah, R.V. Garde, B.A. Yonish, L. Zhang, N.A. Medvitz, S.E. Miller, E.L. Hansen, C.N. Dunn, and T.M. Price. 2013. A truncated progesterone receptor (PR-M) localizes to the mitochondrion and controls cellular respiration. Mol. Endo. 27:741-753. http://doi.org/10.1210/me.2012-1292.

11. Ding, Y., Z. Liu, S. Desai, Y. Zhao, H. Liu, L. K. Pannell, H. Yi, E. R. Wright, L. B. Owen, W. Dean-Colomb, O. Fodstad, J. Lu, S. P. LeDoux, G. L. Wilson, and M. Tan. 2012. Receptor

tyrosine kinase ErbB2 translocates into mitochondria and regulates cellular metabolism. Nature Comm. 3: 1271.

12. Du, J., B. McEwen, and H. K. Manji. 2009. Glucocorticoid receptors modulate mitochon¬drial function. Comm. Integrat. Biol. 2(4): 350-352.

13. Edwards, D. P., S.E. Wardell, and V. Boonyaratanakornkit. 2002. Progesterone receptor interacting coregulatory proteins and cross talk with cell signaling pathways. J. Steroid Biochem. Mol. Biol. 83(1): 173-186.

14. Emmerson, D. A., 1997. Commercial approaches to genetic selection for growth and feed conversion in domestic poultry. Poult. Sci. 76 (8): 1121-1125.

15. Feng, Q., J. R. Crochet, Q. Dai, P. C. Leppert, and T. M. Price. 2014. Expression of a mitochondria progesterone receptor (PR-M) in leiomyomata and association with increased mitochondrial membrane potential. J. Clin. Endo. Metab. 99:E390-E399.

16. Flamant, F., J. D. Baxter, D. Forrest, S. Refetoff, H. Samuels, T. S. Scanlan, B. Vennström, and J. Samarut, H. 2006. International Union of Pharmacology. LIX. The pharmacology and classification of the nuclear receptor superfamily: thyroid hormone receptors. Pharmacol. Rev. 58(4): 705-711.

17. Giangrande, P. H. and D. P. McDonnell. 1998. The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. Recent Prog. Horm. Res. 54: 291-313.

18. Irwin, R. W., J. Yao, R.T. Hamilton, E. Cadenas, R.D. Brinton, and J. Nilsen. 2008. Progesterone and estrogen regulate oxidative metabolism in brain mitochondria. Endocrinology, 149(6): 3167-3175.

19. Kong, B-W., J. J. Song, J. Y. Lee, B. M. Hargis, T. Wing, K. Lassiter, and W. Bottje. 2011 Gene expression in breast muscle associated feed efficiency in a single male broiler line using a chicken 44k microarray. I. Top differentially expressed genes. Poult. Sci. 90:2535-2547; doi:10.3382/ ps.2011-01435.

20. Kong, B., K. Lassiter, A. Piekarski-Welsher, S. Dridi, A. Reverter-Gomez, N. J. Hudson, and W. G. Bottje. 2016. Proteomics of breast muscle tissue associated with the phenotypic expression of feed efficiency within a pedigree male broiler line: I. Highlight on mitochondria. PLoS ONE 11:e0155679. doi:10.1371/journal.pone.0155679.

21. Jongsoon, L., and P. F. Pilch. 1994. The insulin receptor: structure, function, and signaling. Am. J. Physiol. 266(2): C319-C334.

22. Lee, J., A. B. Karnuah, R. Rekaya, N. A. Anthony, and S. E. Aggrey. 2015. Transcriptomic analysis to elucidate the molecular mechanisms that underlie feed efficiency in meat-type chickens. Mol. Genet. Genomics 290(5): 1673-1682.

23. Lassiter, K., E. Greene, O. B. Faulkner, B. M. Hargis, W. Bottje, and S. Dridi. 2015. Orexin system is expressed in avian muscle cells and regulates mitochondrial-related genes. Am. J. Physiol. 308(3):R173-87. doi: 10.1152/ajpregu.00394.2014.

24. Lemmon, M. A., and J. Schlessinger. 2010. Cell signaling by receptor tyrosine kinases. Cell: 141(7), 1117-1134.

25. Lodish, H., A. Berk, S. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell. 2000. Ed. W. H. Freeman. Molecular Cell Biology (4th Ed.). New York, NY.

26. Lösel, R., and M. Wehling. 2003. Nongenomic actions of steroid hormones. Nat. Rev. Mol. Cell Biol. 4(1): 46-55.

27. Mazkereth, M., F. Rocca, J-R. Schubert, C. Geisler, Y. Hillman, A. Egner, and Z. Fishelson. 2016. Complement triggers relocation of mortalin/GRP75 from mitochondria to the plasma membrane. Immunobiol. 221(12): 1395-1406.

28. Mizzen, L. A., A. N. Kabiling, and W. J. Welch. 1991. The two mammalian mitochondrial stress proteins grp 75 and hsp 58, transiently interact with newly synthesized mitochondrial proteins. Cell. Reg. 2: 165-179.

29. Montgomery, M. K., and N. Turner. 2015. Mitochondrial dysfunction and insulin resistance: an update. Endo. Connect. 4(1): R1-R15.

30. Morgan, D. J., T. M. Poolman, A. J. Williamson, Z. Wang, N. R. Clark, A. Maayan, A. D. Whetton, A. Brass, L. C. Matthews, and D. W. Ray. 2016. Glucocorticoid receptor isoforms direct distinct mitochondrial programs to regulate ATP production. Scientific Reports (6) 26419. doi:10.1038/srep26419.

31. Patience J. F., M. C. Rossoni-Serão, and N. A. Gutiérrez. 2015. A review of feed efficiency in swine: biology and application. J. Anim. Sci. Biotechnol. 6(1): 1-9. doi:10.1186/s40104-015-0031-2.

32. Pomytkin, I. A. 2012. H2O2 Signaling pathway: a possible bridge between insulin receptor and mitochondria. Curr. Neuropharm. 10(4): 311-320.

33. Price, TM and Q. Dai. 2015. The role of mitochondrial progesterone receptor (PR-M) in progesterone action. Sem. Reprod. Med. 33: 185-194.

34. Psarra, A-M. G., S. Soakidi, and C. E. Sekeris. 2006. The mitochondrion as a primary site of action of steroid and thyroid hormones: Presence and action of steroid and thyroid receptors in mitochondria of animal cells. Mol. Cell Endo. 246: 21-33.

35. Psarra, A-M.G. and C. E. Sekeris. 2008. Steroid and hormone receptors in mitochondria. IUBMB Life 60: 210-223.

36. Psarra, A. M. G., and C. E. Sekeris. 2011. Glucocorticoids induce mitochondrial gene transcription in HepG2 cells: role of the mitochondrial glucocorticoid receptor. Biochim. Biophys. Acta. 1813(10): 1814-1821.

37. Robertson, C. L., and M. Saraswati. 2015. Progesterone protects mitochondrial function in a rat model of pediatric brain injury. J. Bioeng. Biomembr. 47: 43-51. DOI 10.1007/s10863-014-9585-5.

38. Robinson, D. L., and V.H. Oddy. 2004. Genetic parameters for feed efficiency, fatness, muscle area and feeding behaviour of feedlot finished beef cattle. Livestock Prod. Sci. 90(2-3): 255-270.

39. Rochard, P., A. Rodier, F. Casas, I. Cassar-Malek, S. Marchal-Victorion, L. Daury, C. Wrutniak, and G. Cabello. 2000. Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. J. Biol. Chem. 275(4): 2733-2744.

40. Scheller, K., C. E. Sekeris, G. Krohne, R. Hock, I. A. Hansen, and U. Scheer. 2000. Localization of glucocorticoid hormone receptors in mitochondria of human cells. Eur. J. Cell Biol. 79(5): 299-307.

41. Scheller, K., P. Seibel, and C. E. Sekeris. 2003. Glucocorticoid and thyroid hormone receptors in mitochondria of animal cells. I. Rev. Cytol. 222: 1-61.

42. Salvi, M. 2013. Receptor tyrosine kinases take a direct route to mitochondria: an overview. Curr. Protein Peptide Sci. 14(7): 635-640.

43. Salvi, M., A. M. Brunati, and A. Toninello. 2005. Tyrosine phosphorylation in mitochondria: a new frontier in mitochondrial signaling. Free Rad. Biol. Med. 38(10), 1267-1277.

44. Tsiriyotis, C., D. A. Spandidos, and C. E. Sekeris. 1997. The mitochondria as a primary site of action of glucocorticoids: mitochondrial nucleotide sequences showing similarity to hormone response elements confer dexamethasone inducibility to chimaeric genes transfected in LATK-Cells. Biochem. Biophys. Res. Comm. 235: 349-354.

45. Tuteja, R., and N. Tuteja. 1999. Nucleolin: a multifunctional major nucleolar phospho¬protein. In: Crit. Rev. Biochem. Mol. Biol. 33 (6): 407–36.

46. Weber, K., P. Brück, Z. Mikes, J. H. Küpper, M. Klingenspor, and R. J. Wiesner. 2002. Glucocorticoid hormone stimulates mitochondrial biogenesis specifically in skeletal muscle. Endocrinology, 143: 177-184.

47. Wrutniak, C., I. Cassar-Malek, S. Marchal, A. Rascle, S. Heusser, J. M. Keller, J. Flechon, M. Dauca, J. Samarut, J. Ghysdael, and G. Cabello. 1995. A 43-kDa protein related to c-Erb A α1 is located in the mitochondrial matrix of rat liver. J. Biol. Chem. 270(27), 16347-16354.

48. Wyrutniak-Cabello, C., F. Casas, and G. Cabello. 2001. Thyroid hormone action in mitochondria. J. Mol. Endo. 26:67-77.

49. Zhou, N., W. R. Lee, and B. Abasht. 2015. Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens' feed efficiency. BMC Genomics 16:195-215.

50. Zuidhof, M. J., B. L. Schneider, V.L. Carney, D. R. Korver and F. E. Robinson. 2014. Growth, efficiency, and yield of commercial broilers from 1957, 1978 and 2005. Poult. Sci. 93 (12):2970-2982. : 10.3382/ps.2014-04291.

5.7 FIGURES

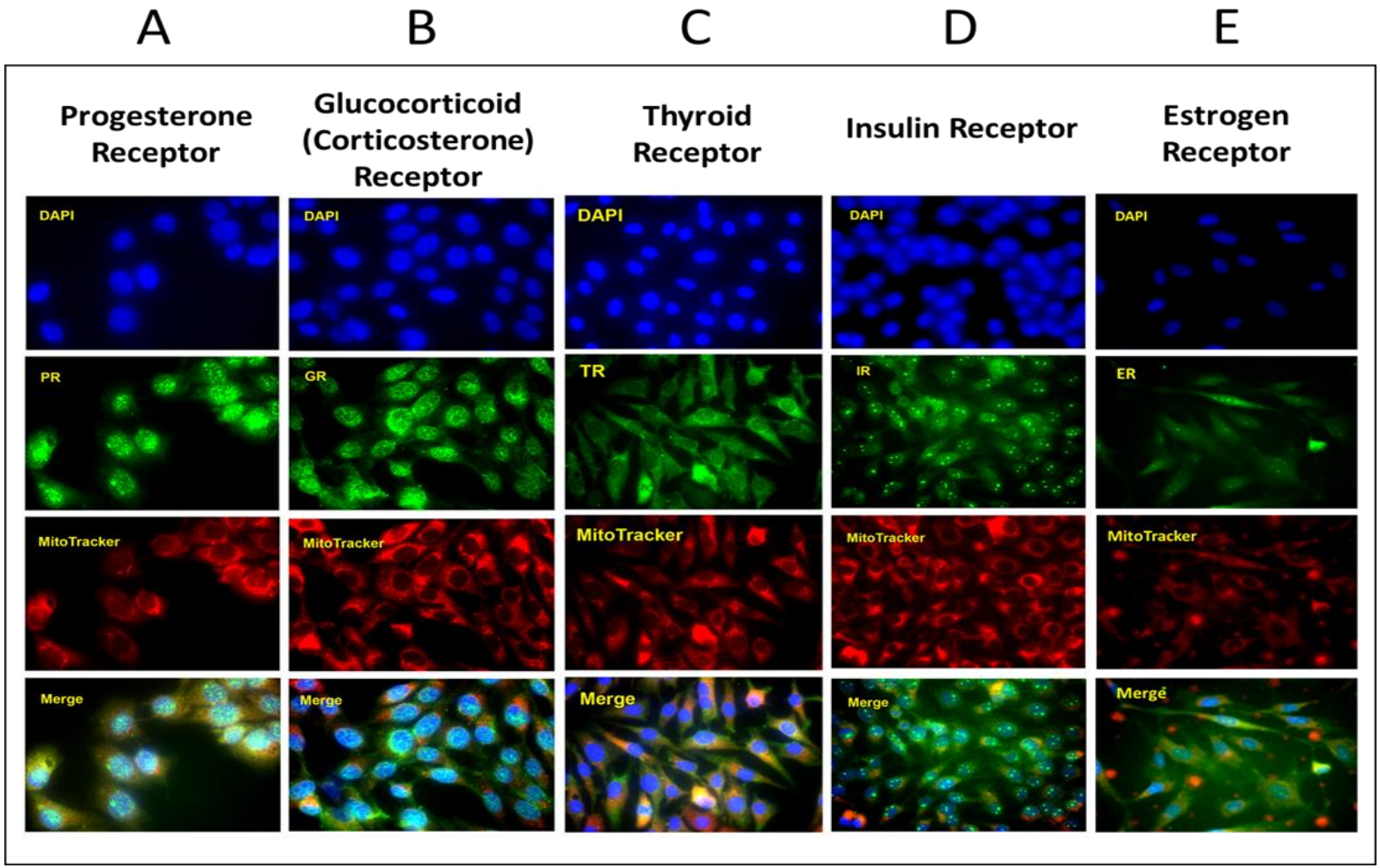


Figure 5.1 Immunofluorescent staining of QM7 cells for visualization of mitochondrial receptors for; A) Progesterone, B) Glucocorticoid, C) Thyroid, D) Insulin and E) Estrogen. The nucleus was visualized with DAPI (blue), cytoplasm with Alexafluor (green), and mitochondria with Mitotracker® deep red CMX (red). The merged images with orange to yellow coloring represents the presence of mitochondrially located hormone receptors.

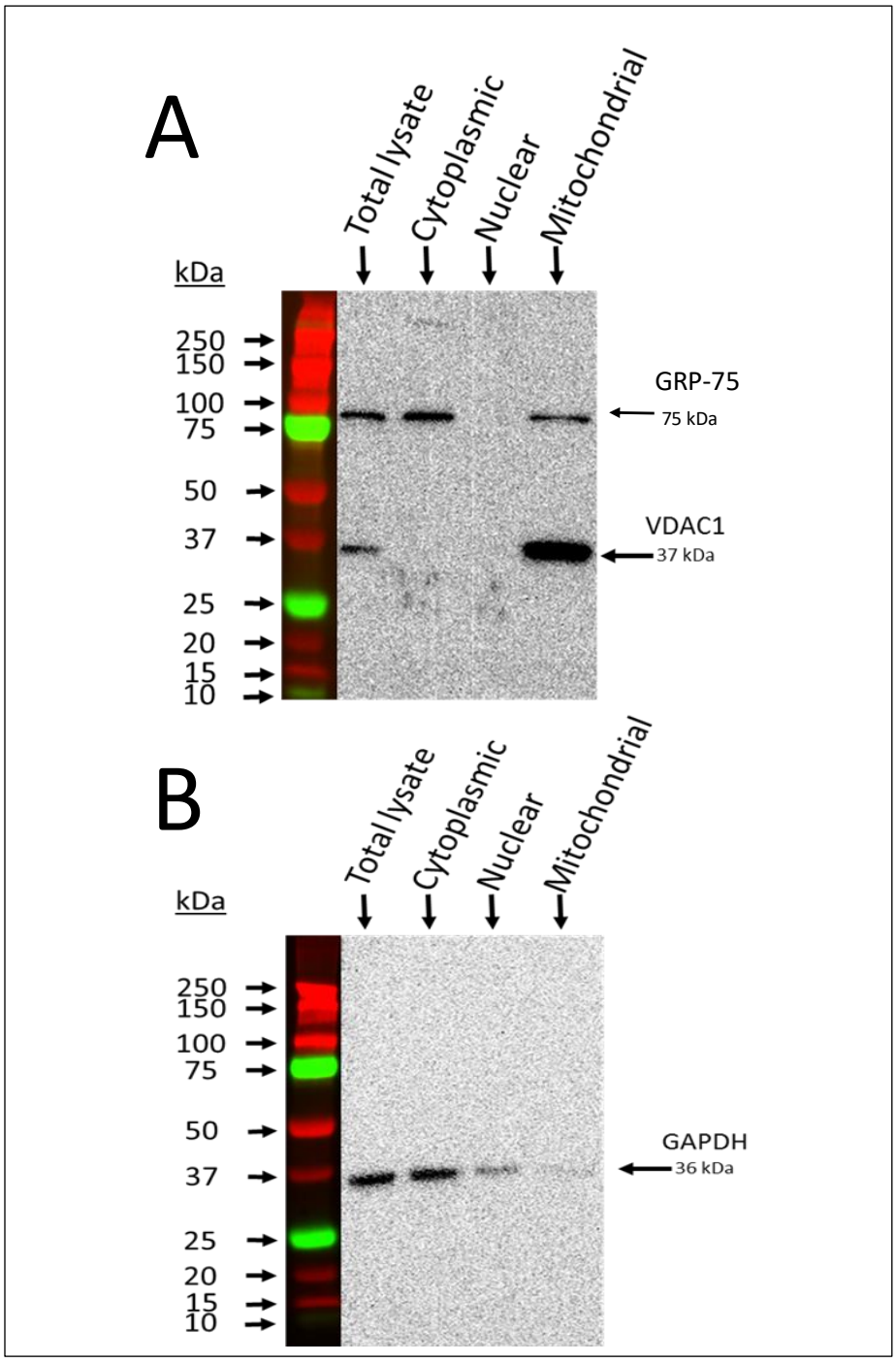


Figure 5.2 Verification of cell fractionation procedures. Gels are shown for A) Nucleolin (C23) (110 kDa) that was detected in nuclear fraction but not the mitochondrial fraction. B) Voltage dependent activation channel (VDAC) that was detected in the mitochondrial fraction only, and C) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – present primarily in the cytoplasmic fraction but not in the mitochondrial fraction.

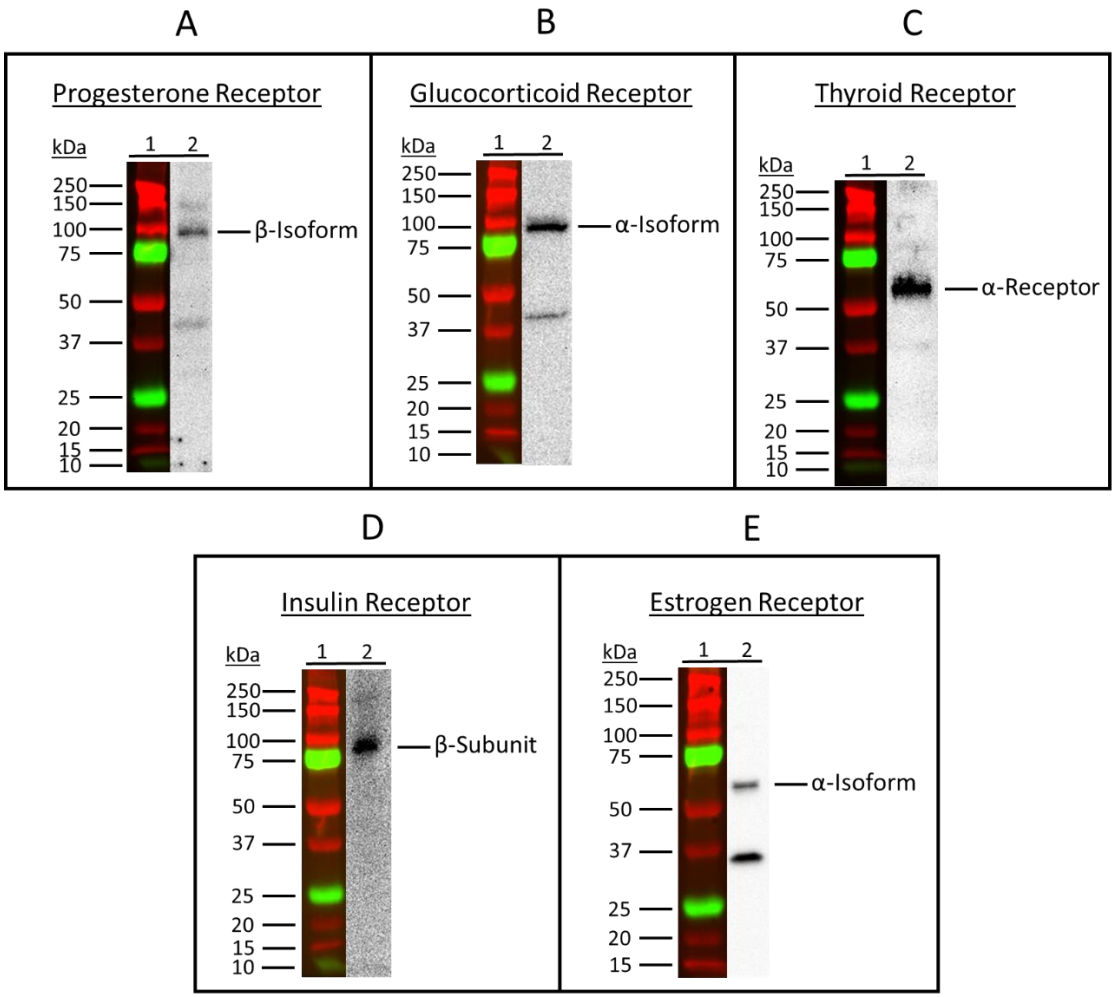
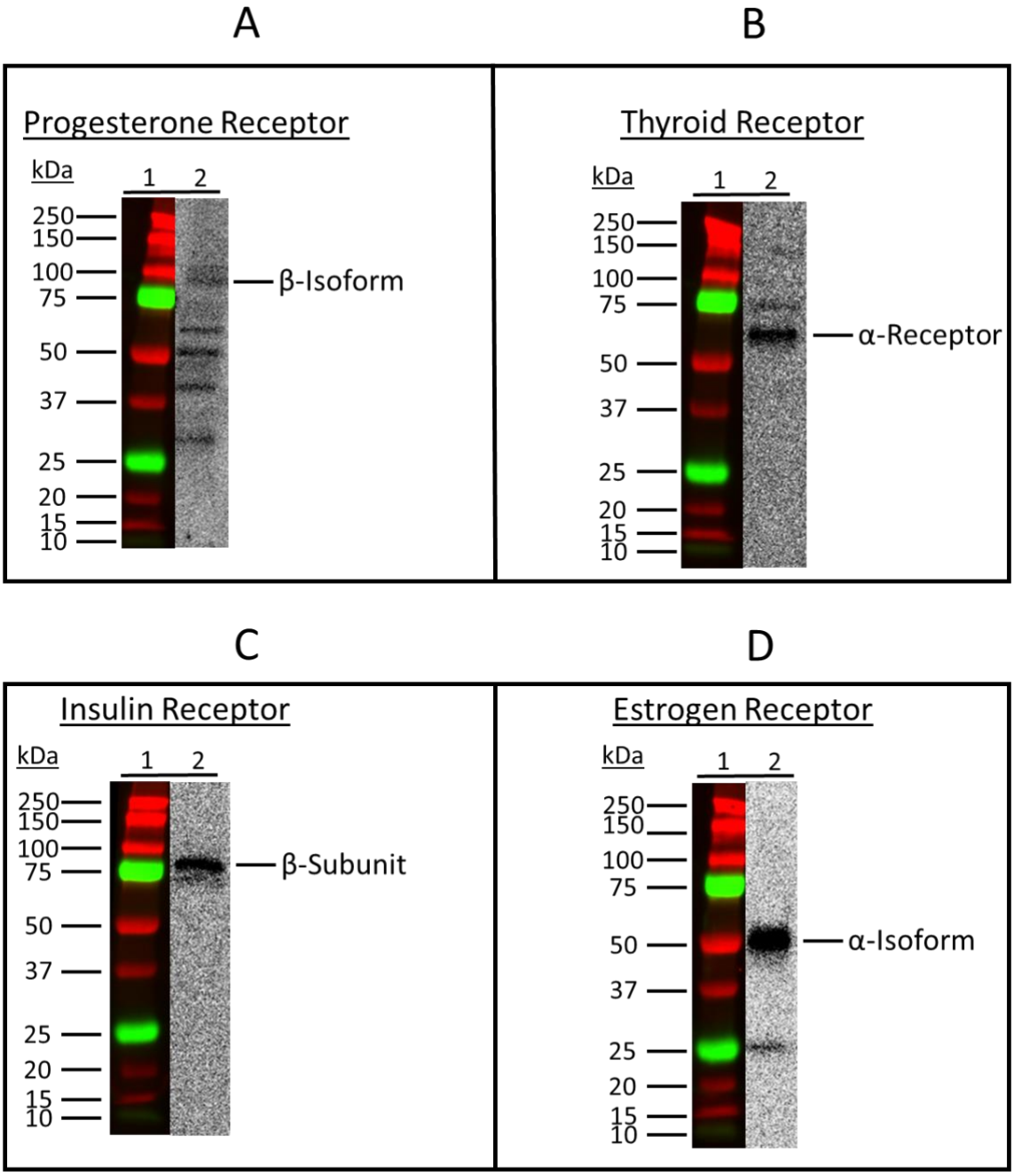


Figure 5.3 Hormone receptor expression in mitochondria isolated from breast muscle obtained from broiler for; (A) progesterone, (B) glucocorticoid, (C) thyroid hormone, (D) insulin, and (E) estrogen. The amount of protein loaded onto the gel for each lane was 75 µg.



Lane 1: Protein Standards.
Lane 2: Mitochondria isolated from quail breast muscle.

Figure 5.4 Hormone receptor expression in mitochondria isolated from breast muscle obtained from Japanese Quail for; (A) progesterone, (B) thyroid, (C) insulin, and (D) estrogen. The amount of protein loaded onto the gel for each lane was 75 µg.

6. CONCLUSIONS

In this dissertation the expression of hormones and hormone receptors as it relates to mitochondrial function and feed efficiency in broilers was investigated. Hormonal action plays an important role in maintaining cellular function and contributing to muscle development and growth. However, studies of the relationship between hormones, mitochondria, and muscle have largely been confined to mammalian species; with very little information being present concerning avian species. Therefore, the overall goal of research in this dissertation was to determine if the hormone orexin is present in avian muscle cells and affects mitochondrial physiology, investigate whether differences in the feed efficiency of broilers can be partially attributed to differences in the expression of components of hormone signaling pathways, and to determine whether hormone receptors are present within the mitochondria of avian muscle cells.

We found that the neuropeptide orexin and its related receptors indeed are expressed in both intact avian muscle cells and an immortalized avian muscle cell line. Not only is orexin present, but is also secreted by these muscle cells, indicating that avian muscle tissue may use the hormone in an autocrine or paracrine role. The application of recombinant orexin impacted the expression of a number of genes involved in mitochondrial fission/fusion and metabolism. This study provides the first evidence of the orexin system being involved in avian muscle function. Based on these findings, future studies can be developed to investigate how other hormones and their receptors, particularly steroid and thyroid hormones, participate in the regulation of growth and mitochondrial function of avian muscle.

While investigating orexin in avian muscle, concurrent analysis of the high and low feed efficient broiler phenotype was also performed. Breast muscle isolated from both groups of birds was subjected to global gene and protein expression analysis. Based on the data obtained from

those studies, genes involved in regulating muscle hypertrophy as well as the insulin signaling pathway were predicted to be determinants of the high feed efficient phenotype. Using these predictions as a guideline, real-time qPCR was performed on breast muscle samples from both groups to investigate genes involved in muscle development and hypertrophy, in addition to insulin signaling. The results indicated that a number of genes involved in enhancing muscle growth and formation (i.e. AMPK, myogenin, creatine kinase) were upregulated in the high feed efficient phenotype, whereas genes known to inhibit muscle hypertrophy, differentiation, and fiber size (i.e. myostatin, caveolin-3, IGFBP-3) were downregulated in the high feed efficient phenotype. Additionally, several genes that are members of the insulin signaling pathway were differentially expressed between the two phenotypes, though no clear pattern was established. It must be noted that the insulin signaling pathway is very complex and not fully understood in mammalian species, even less so in avian species. Therefore future studies to elucidate the intricacies of the insulin pathway in avian species is warranted. This may lead to a better understanding of how the pathway is involved in muscle growth, mitochondrial function, and the determination of the high/low feed efficient phenotype.

In the previous global protein expression analysis it was predicted that enhanced progesterone signaling within the mitochondria is a characteristic of the high feed efficient phenotype. Even though the presence of hormone receptors and hormone response elements have been identified in mammalian mitochondria, the same cannot be said for avian species. In the final study of this dissertation we were able to identify receptors for progesterone, estrogen, glucocorticoids, thyroid, and insulin in broiler and quail breast muscle tissue and an immortalized avian muscle cell line. Since the presence of hormone receptors has been shown to enhance energy production and gene/protein expression in mammalian mitochondria, in future

studies it would be interesting to compare levels of mitochondrial hormone receptor expression between the high and low feed efficient phenotypes to determine whether it can be classified as a defining trait of feed efficiency in these animals.

The increasing global demand for animal protein coupled to the increasing costs of animal production makes feed efficiency a very important topic. The more researchers are able to understand about the physiology of these animals then the more likely we are to have the ability to implement changes that have a positive impact on the poultry industry.

APPENDIX

In regard to IACUC protocols #13039 and #14012, the student (Kentu R. Lassiter) was not involved in the animal care or harvesting of tissues used for the collection of research data.

UNIVERSITY OF ARKANSAS
DIVISION OF AGRICULTURE

Center of Excellence
for Poultry Science

UNIVERSITY OF ARKANSAS
DALE BUMPERS COLLEGE
OF AGRICULTURAL, FOOD & LIFE SCIENCES

0-114 Poultry Science Building, University of Arkansas, Fayetteville, AR 72701-1201
479-575-4952 • Fax 479-575-3026 • www.poultryscience.uark.edu

To: Graduate School
From: Walter Bottje, Professor
Re: Kentu Lassiter, Dissertation – Animal Care Statement
Date: February 21, 2019

I am writing this letter to verify that the research conducted by Kentu Lassiter did not require an IACUC protocol approval. The reason is that the tissues that he analyzed for his dissertation research had been collected from a previous study that had been conducted before Kentu began work on his dissertation. I was involved in the animal handling and tissue collection which was approved by the IACUC committee under IACUC protocol numbers 13039 and 14012.

If you have any questions or need additional information, let me know. Thank you.

Walter Bottje

1. IACUC approval for orexin study and hormone receptor study.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Sami Dridi

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

DATE: May 8, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **June 30, 2016**

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

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

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

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

cnc/car

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

2. IACUC approval for gene expression study and hormone receptor study.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Walter Bottje

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

DATE: September 17, 2013

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

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#14012-** "**Molecular signatures and mechanistic modeling for improving feed efficiency in broilers**". You may begin this study immediately.

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

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **09-17-2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

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

cnc/car

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199