UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

EXPRESSÃO DE GENES MITOCONDRIAIS E DE CRESCIMENTO ASSOCIADOS À EFICIÊNCIA ALIMENTAR DE CODORNAS SUBMETIDAS A DIFERENTES AMBIENTES TÉRMICOS

Autora: Débora Marques Voltolini
Orientadora: Profª Drª Eliane Gasparino

MARINGÁ
Estado do Paraná
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Tese apresentada, como parte das exigências para obtenção do título de DOUTORA EM ZOOTECNIA, no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá - Área de Concentração Produção Animal

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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

APROVADA em 26 de fevereiro de 2014.

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(Orientadora)

"É muito melhor arriscar coisas grandiosas, alcançar triunfos e glórias, mesmo se expondo ao fracasso, do que formar fila com os pobres de espírito, que nem gozam muito, nem sofrem muito, porque vivem nessa penumbra cinzenta que não conhece vitória nem derrota"

Franklin Delano Roosevelt

Dedico esse trabalho aos meus pais, Paulo Ademir Sant'ana Marques e Cleusa Sommer Marques, pelo apoio, encorajamento, amor e pelos ensinamentos que formaram o alicerce de minha história.

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BIOGRAFIA

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Em março de 2011, ingressou no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá, em nível de doutorado, área de concentração Produção Animal - Melhoramento Genético Animal, sob a orientação da Professora Drª. Eliane Gasparino.

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RESUMO

O trabalho foi estruturado como um único experimento envolvendo um arranjo fatorial, no qual foram analisados a expressão gênica e o desempenho de codornas. Os fatores, linhagens (postura e corte), eficiência alimentar (alta e baixa eficiência alimentar) e exposição ao estresse térmico (frio e calor) foram considerados. Desta forma, o experimento foi analisado considerando as linhagens separadamente, primeiro postura e depois corte. As análises de expressão gênica em animais domésticos vem aumentando dada a necessidade de se compreender melhor os mecanismos moleculares e bioquímicos envolvidos com a nutrição e ambiência. As análises com a expressão dos genes mitocondriais e genes relacionados ao crescimento possibilitaram a confirmação de alguns resultados já esperados, em função da ação desses genes; entretanto, também nos trouxeram desafios na explicação de resultados não esperados, onde hipóteses foram propostas. Quando analisamos a expressão desses genes em animais de postura, podemos observar que a expressão de IGF-I foi maior no fígado de codornas de alta eficiência alimentar (EA) do que em codornas de baixa EA em condições de estresse por calor ou frio. Animais de alta EA também mostraram maior expressão de mRNA GHR, independente da temperatura. A expressão de UCP no fígado foi menor em aves de alta EA, e maior em condições de estresse por frio. A expressão de ANT foi menor no fígado de codornas expostas ao calor. No músculo, maior expressão de ANT foi observada para aves de alta EA e para animais do estresse por frio. A maior expressão de COX III no músculo foi observada para aves de alta EA e que foram submetidas ao estresse por frio. No fígado, valores de expressão muito mais altos, também foram observados em codornas do estresse por frio. Os resultados das análises de expressão no músculo e fígado das codornas de corte são semelhantes aos encontrados para postura. A expressão de IGF-I foi maior no fígado de aves de alta EA do que em aves de baixa

EA no conforto e sob estresse por calor. No músculo, independente da temperatura, aves de alta EA mostraram maior expressão de IGF-I. Codornas de alta EA também apresentaram maior expressão de GHR em condições de conforto térmico. Com relação ao ambiente, maior expressão foi observada em aves no conforto, e menor expressão de GHR, observada em aves em estresse por calor. A expressão de UCP no fígado foi menor em aves de alta EA e menor em estresse térmico por calor. Aves de alta e baixa EA mostraram maior expressão de ANT em condições de estresse por frio. Comparações entre aves de alta e baixa EA revelaram maiores níveis de transcrição deste gene em aves de alta EA em condições de conforto e de estresse por calor. Maior expressão de COX III foi observada no fígado e músculo de codornas em condições de conforto térmico. Neste trabalho, além de machos, também foi avaliada a expressão dos mesmos genes em codornas de corte fêmeas, no qual foi observado maior expressão de mRNA IGF-I no fígado e no músculo de aves de alta EA, e maior expressão de GHR no músculo. A maior expressão de ANT foi observada em aves de alta EA e que permaneceram em conforto térmico. No fígado, a expressão de UCP foi estatisticamente similar entre as codornas, independente do ambiente (Tº) ou da eficiência alimentar. Entretanto, comparações entre aves de alta e baixa EA no músculo revelaram maior expressão de UCP em aves de baixa EA. Maior expressão de COX III foi observada em codornas que permaneceram no conforto; e aves de alta EA mostraram maior expressão deste gene que aves de baixa EA. Nossos resultados sugerem que a temperatura ambiental afeta a expressão de genes relacionados ao crescimento e à produção de energia pelas mitocôndrias, e que codornas que apresentam diferentes eficiências alimentares podem responder diferentemente aos estímulos do ambiente.

Palavras-chave: Expressão gênica, *Coturnix coturnix*, hormônio do crescimento, genes mitocondriais.

ABSTRACT

This work was structured as a single experiment involving a factorial arrangement where the gene expression and performance of quails were analyzed. The factors lineage (layer and meat), feed efficiency (high and low-efficiency) and thermal stress exposition (cold and heat) were considered. Thus, the experiment results were analyzed considering the lineages separately, first layer then meat. The gene expression analysis of domestic animals is increasing, due to the need to better understand the molecular and biochemical mechanisms involved on nutrition and ambiance. The analysis of mitochondrial and growth related genes, enabled the confirmation of some expected results, considering the action of such genes; although this data also brought other challenges such as explaining unexpected results, where hypothesis might be drawn. When we analyzed the expression of these genes on layer quails, we could observe that IGF-I mRNA expression was higher in the livers of high feed efficiency (FE) quail than in the livers of low-FE quail under both heat and cold stress conditions. High-FE birds also showed higher GHR mRNA expression independent of temperature. UCP mRNA expression in the liver was lower in high-FE birds and higher under cold stress compared with the other conditions. IGF-I mRNA expression was higher in the muscle of high-FE quail under the three conditions tested, and UCP mRNA expression was higher under cold stress. The ANT mRNA expression was lower in the liver of heat stress animals. In the muscle greater ANT mRNA expression was observed for high FE and for cold stress animals. The greatest COX III mRNA expression in the muscle was observed for high FE animals and that were subjected to cold stress. In the liver there was a much higher expression of COX III mRNA in cold stress animals. The analysis results of gene expression on muscle and liver of meat quails are similar to the ones of layer quails. IGF-I mRNA expression was higher in the livers of high-FE quail than in

the livers of low-FE quail under both comfortable and cold stress conditions. In the muscle, regardless of the environment, high-FE birds showed higher IGF-I mRNA expression. High-FE birds also showed higher GHR mRNA expression under comfortable conditions. Regarding the environment, higher expression was observed in birds at comfortable conditions, and lower expression in birds under heat stress. UCP mRNA expression in the liver was lower in high-FE birds and lower under heat stress compared with the other conditions. Low and high-FE birds showed greater ANT mRNA expression under cold stress. Comparisons of the low and high-FE birds revealed higher transcription levels of this gene in high-FE birds when housed in comfortable conditions and when the quails were submitted to heat stress. Greater mRNA COX III expressions were observed in the liver and muscle of quails under comfortable conditions. In this work, besides male quails, it was evaluated the expression the same genes on female meat quails, in which it was observed greater IGF-I mRNA expression in the liver and muscle of high-FE birds, and greater GHR mRNA expression in the muscle. The greatest ANT mRNA expression was observed for high FE-birds and that remained under comfortable conditions. In the liver, UCP mRNA expression was statistically similar among the quails, independent of the environment (T^{o}) and feed efficiency. However, comparisons of the low- and high-FE birds, in the muscle, revealed higher levels of UCP mRNA in low-FE birds. The COX III mRNA expression in the liver suffered environmental temperature and feed efficiency effects. Higher expression was observed in animals that remained under comfortable conditions; and high FE-birds showed higher expression than low FE- birds. Our results suggest that air temperature affects the expression of genes related to growth and mitochondrial energy production, and quail with different feed efficiencies respond differently to environmental stimuli.

Keywords: Gene expression, *Coturnix coturnix*, growth hormone, mitochondrial gene.

I. INTRODUÇÃO

O desenvolvimento de pesquisas científicas com avanços nas áreas de nutrição e genética na avicultura melhorou os índices alimentares e reduziram os custos de produção, possibilitando aumento na produtividade. Os gastos com a alimentação representam cerca de 70% do total dos custos de produção na pecuária; dentro disto, a eficiência alimentar se torna cada vez mais um aspecto importante e de grande interesse nas pesquisas recentes (Lassiter et al., 2006), entre estas, estão as pesquisas em nutrigênomica.

A taxa de crescimento está atrelada à eficiência alimentar, bem como a deposição de massa muscular. A eficiência de um animal em converter alimentos em peso corporal está relacionada à eficiência na produção de energia. Estudos mostram que aves com menor produção de ATP, em função de menor eficiência mitocondrial de produzir ATP a partir de substratos, apresentam pior eficiência alimentar ou conversão alimentar (Bottje & Carstens, 2009).

O desempenho das aves também é afetado pelas condições ambientais a que estes animais são expostos. A produção pode ser prejudicada em função de altas temperaturas, que levam os animais ao estresse térmico (Oliveira et al., 2006). O estresse térmico está associado a alterações metabólicas que envolvem o estresse oxidativo. De acordo com Yang et al. (2010), aves submetidas ao estresse por altas temperaturas podem apresentar redução na atividade da cadeia respiratória mitocondrial, seguida por maior produção de espécies reativas de oxigênio (ROS).

A busca por animais cada vez mais eficientes deixa clara a necessidade de conhecer melhor como todos esses fatores estão envolvidos no desempenho das aves, em função das modificações ocorridas por mudanças fisiológicas em nível celular / molecular.

Coturnicultura

A codorna pertence à ordem das Galináceas, família das Faisánidas, subfamilia dos Perdicinae e do gênero Coturnix, existindo grandes quantidades de espécies, a mais conhecida e difundida é a *Coturnix coturnix*, conhecida como codorna europeia ou selvagem. Com a introdução desta ave no Japão e, através de cruzamentos, surgiu então a subespécie *Coturnix coturnix japonica*, conhecida como codorna japonesa ou doméstica.

O que diferencia a codorna europeia da codorna japonesa é o peso na fase adulta, sendo a codorna europeia é mais indicada para corte pelo seu maior peso (170 g, aproximadamente), enquanto que a codorna japonesa é mais indicada para a postura, por atingir pesos menores na fase adulta (150 g) e alta postura de ovos. Ainda há a codorna americana, conhecida por Bob White, esta juntamente com a europeia são as codornas mais indicadas para a produção de carne.

As codornas possuem altos índices de produtividade, rápido crescimento, pequeno porte, facilmente manipuláveis, ciclo reprodutivo curto, com postura regular. Todas essas vantagens fazem da codorna um animal muito utilizado, não apenas para a produção de carne e ovos, mas também como modelo experimental para as aves domésticas em pesquisas laboratoriais (Oliveira, 2002).

Genética

- Genes mitocondriais:

O melhoramento genético proporcionou grande melhora no desempenho das aves nas ultimas décadas (Harvenstein et al., 2003). Estudos recentes têm mostrado que animais mais eficientes em converter alimentos em peso corporal podem apresentar alterações na expressão de genes para proteínas da cadeia transportadora de elétrons, o que pode influenciar a utilização de nutrientes e alterar o gasto energético corporal. Estas pesquisas sugerem que animais com maior consumo de ração residual possuem falha no transporte de elétrons/prótons, reduzindo a eficiência de produção de ATP pelas mitocôndrias, o que influencia negativamente a conversão alimentar (Bottje & Carstens, 2009; Krueger et al., 2008).

Todas as células necessitam de energia para realizar suas atividades. As mitocôndrias são as organelas responsáveis por transformar a energia química dos metabólitos em energia facilmente acessível à célula (Schauss et al., 2010) e estão presentes em quase todas as células eucarióticas (Alberts et al., 2011). Essa energia é acumulada em compostos lábeis, dos quais o principal é o ATP. De acordo com Bottje et al. (2006), as mitocôndrias são responsáveis pela produção de 90% de toda a energia requerida pelas células.

As mitocôndrias contém seu próprio DNA, bem como um sistema completo de transcrição e tradução, o que permite sintetizar algumas de suas próprias proteínas. O número de mitocôndrias presentes em diferentes tipos celulares varia muito e pode mudar com a necessidade de energia da célula (Alberts et al., 2011).

Cada mitocôndria é envolta por duas membranas altamente especializadas: as membranas externa e interna criam dois compartimentos mitocondriais, um espaço interno denominado de matriz e um espaço intermembranas muito mais estreito. A membrana externa contém muitas moléculas de uma proteína canal denominada porina, deixando a membrana externa altamente permeável a muitas moléculas, incluindo pequenas proteínas. Em contrapartida, a membrana interna é impermeável a passagens de íons e a maioria das pequenas moléculas. A membrana mitocondrial interna é o sítio de transporte de elétrons e bombeamento de prótons e contém a ATP-sintase. A maioria das proteínas embebidas na membrana interna é componente da cadeia transportadora de elétrons, necessários para a fosforilação oxidativa (Alberts et al., 2011).

Quase toda energia disponível da queima de carboidratos, gorduras e outros alimentos é, inicialmente, armazenada na forma de molécula carreadora ativada, gerada durante a glicólise e o ciclo do acido cítrico – NADH e $FADH_2$. Essas moléclas carreadoras doam seus elétrons de alta energia para a cadeia transportadora de elétrons. Os elétrons são rapidamente passados ao longo da cadeia até o oxigênio molecular para formar água. A energia liberada durante a passagem dos elétrons é utilizada para bombear prótons através da membrana mitocondrial interna. O gradiente de prótons resultante promove a síntese de ATP (Figura 1). Esse mecanismo quimiosmótco de síntese de ATP é denominado de fosforilação oxidativa (Alberts et al., 2011).

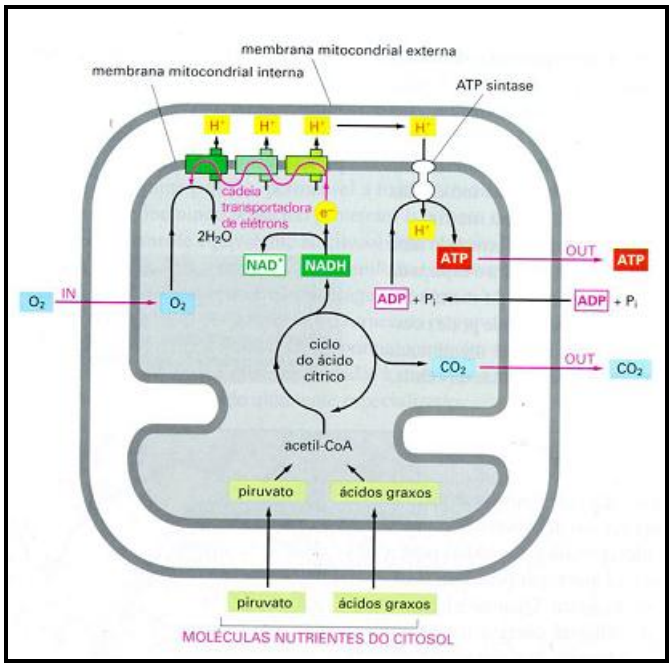

Figura 1: Cadeia transportadora de elétrons e fosforilação oxidativa.

A cadeia transportadora de elétrons possui mais de 40 proteínas, sendo que a maioria delas está envolvida na cadeia transportadora e estão agrupadas em três grandes complexos: o complexo NADH-desidrogenase, o complexo do citocromo b-C_1, o compexo citocromo-oxidase. Os complexos são sítios de bombeamento de prótons, e cada um pode ser visto como uma máquina proteica que bombeia prótons através da membrana à medida que elétrons são tranferidos por meio dela. O gradiente eletroquímico de prótons, é utilizado para promover a síntese de ATP pela ATP-sintase. A ATP-sintase cria uma via eletrolítica através da membrana mitocondrial interna que permite aos prótons fluírem de volta a matriz mitocondrial, a favor de seu gradiente eletroquímico. À medida que os prótons fazem sua passagem pela enzima, eles são utilizados para dirigir a reação desfavorável entre ADP e P_i para produzir ATP (Alberts, et al., 2011).

Durante a síntese de ATP, cerca de 2 a 4% do total de O_2 utilizado como aceptor de elétrons pelas mitocôndrias, não é totalmente reduzido a água (Bottje et al., 2006), no entanto os mesmos formam compostos químicos que ainda são oxidantes potentes, conhecidos como espécies reativas de oxigênio (ROS).

As proteínas mitocondriais, UCPs (proteínas desacopladoras), ANT (transportador de nucleotídeos de adenina) e COXIII (citocromo c oxidase subunidade III) tem se

tornado tema de pesquisas recentes, por suas importâncias na produção de ATP (Bottje et al., 2009; Ojano-Dirain et al., 2007).

As proteínas desacopladoras (UCPs) são transportadores presentes na membrana interna da mitocôndria capazes de desviar a energia de síntese de ATP, para a produção de calor catalisado por um vazamento de prótons através do interior da membrana interna (Ledesma et al., 2002). De acordo com Vidal-Puig (2000), o que a UCP faz é fornecer uma rota nova para os prótons, que não através da ATP sintase. A UCP aviária tem sido descrita como um agente que possibilita a redução da produção de ROS, por causar um leve desacoplamento na produção de ATP (Abe et al., 2006). Embora a UCP tenha o efeito benéfico de evitar danos ao DNA e as proteínas celulares, uma vez que reduzem a produção de radicais livres, observa-se que a maior expressão do gene UCP pode piorar a conversão alimentar, já que pode reduzir a produção de ATP (Ojano-Diran, et al., 2007). Com a diminuição da síntese de ATP pode ocorrer aumento do catabolismo dos nutrientes como forma de manter a produção de energia.

A expressão de UCP também é influenciada pelo estresse térmico, aves expostas a altas temperaturas apresentaram menor expressão do gene UCP (Mujahid et al., 2006), e pelo estado nutricional, a expressão de UCP é aumentada no músculo esquelético de aves em jejum (Evock-Clover et al., 2002). Baixas temperaturas têm sido constantemente associadas a maior expressão de UCP em diversos tecidos e espécies (Dridi etal., 2008; Toyomizu et al., 2002). Os autores associam essa maior expressão a uma tentativa do animal em manter a temperatura corporal adequada.

Outra proteína envolvida no processo de síntese energética é o ANT (transportador de nucleotídeos de adenina), responsável pelo deslocamento do ADP do citosol para a mitocôndria e pelo deslocamento do ATP através da membrana interna mitocondrial (Ojano-Dirain et al., 2007). Portanto, o ANT possui a função de aumentar a quantidade de ADP para ser transformado em ATP por ação da ATP sintase. A função mitocondrial pode ser prejudicada pela incapacidade de troca do ADP/ATP entre citosol e membrana, assim pode ser que haja alguma ligação entre a expressão do ANT com a expressão do fenótipo da eficiência alimentar (Bottje et al., 2006).

Ojano-Dirain et al. (2007) relataram que aves com menor expressão do gene ANT tiveram pior conversão alimentar em função da menor eficiência em produzir ATP. Nicoletti et al. (2005) verificaram redução na expressão do ANT com o aumento da idade. Segundo esses autores, alterações na expressão de subunidades da cadeia respiratória podem representar uma resposta adaptativa celular ao acúmulo de danos às

proteínas e / ou DNA mitocondrial que ocorre pelo aumento na quantidade de ROS. A expressão do gene ANT sofre influência da temperatura, havendo maior expressão em aves expostas a clima frio (Toyomizu et al., 2002), e do estado nutricional, no qual observa-se maior expressão em aves submetidas a jejum (Toyomizu et al., 2006).

O COX III também está presente na cadeia transportadora de elétrons e está relacionado com a eficiência da fosforilação oxidativa. A citocromo c oxidase, subunidade III (COX III), é uma subunidade do complexo proteico IV da mitocôndria, responsável pelo bombeamento de prótons e pelo transporte de elétrons. O COX III possui grande relevância na eficiência energética mitocondrial (Scheffler, 1999) e menor expressão deste gene pode ocorrer pela menor eficiência celular ou maior dano oxidativo pela produção de substâncias reativas ao oxigênio (Kemp et al., 2003).

Ojano-Dirain et al. (2007) sugerem que maior produção de ROS e maior oxidação proteica são observadas consistentemente em aves com baixa eficiência alimentar, sugerindo que este fator pode alterar a expressão de genes mitocondriais. Estes autores também sugerem que os níveis de expressão dos genes mitocondriais, como o COXIII, podem variar de acordo com os níveis de danos causados pelo estresse oxidativo. Esta hipótese é corroborada pelos resultados de Barazzoni et al. (2000) que verificaram redução na expressão do gene COX III relacionada com alteração na capacidade oxidativa da mitocôndria em animais mais velhos, e por Li et al. (2002) que verificaram que não só a expressão desse gene, mas também sua atividade é afetada pela quantidade de ROS presente no tecido.

A coordenação entre os complexos da cadeia respiratória é necessária para que a produção de energia seja eficiente, e assim, para que os animais tenham maior eficiência alimentar. Qualquer desequilíbrio pode provocar vazamento de elétrons e geração de ROS.

- Genes do crescimento:

O crescimento das aves é dado pela ação do eixo somatotrófico, com ação principal do hormônio do crescimento (GH). A ação do GH sobre o crescimento pode ocorrer de forma direta, entretanto, seus efeitos são dados principalmente pela ação do IGF-I (fator de crescimento semelhante à insulina I). A presença de GH no organismo induz à síntese e à liberação deste hormônio (Becker et al., 2001).

O GH é produzido pelas células somatotrópicas da hipófise, sua síntese é estimulada pela ligação entre o hormônio liberador de GH (GHRH), sintetizado pelo hipotálamo, e seu receptor (GHRHR) (Martinelli Júnior et al., 2002). A ação do GH sobre o IGF-I é mediada pelo receptor de GH (GHR), já que deve haver a ligação GH-GHR para estimular a síntese e liberação do IGF-I. O GHR é uma proteína transmembrana de 620 aminoácidos, seu domínio extracelular é responsável pela ligação do GH, e o domínio intracelular está envolvido na sinalização do GH. Esta proteína é codificada por um gene localizado no braço curto do cromossomo 5, composto por dez éxons e nove íntrons, sendo o fígado o local de maior expressão do gene GHR (Leung et al., 1987).

Para estimular a síntese de IGF-I, o GH provoca a dimerização do seu receptor, ligando-se a ele (Brown et al., 2005). Essa mudança conformacional é responsável pela ligação da proteína JAK2 (Janus Kinase 2) ao complexo, formado anteriormente pelo GH e GHR, e assim, pelo início da sinalização intracelular, que modula as ações do GH. Com a ligação da JAK2, diversas rotas biológicas podem ser induzidas (Burfoot et al., 1997). Uma das rotas ativadas com a formação do complexo GH-GHR-JAK2 é a rota que envolve os fatores de transcrição denominados de STATs (Transdutores do sinal e ativadores da transcrição). Neste processo, as STATs são fosforiladas, o que fazem com que estas formem complexos com outras proteínas e se movam para o núcleo, agindo na indução da síntese de mRNA IGF-I (Kofoed et al., 2003). O complexo pode ativar também as proteínas quinases MAPK (Proteína cinase ativadora de mitose), por intermédio do recrutamento da molécula adaptadora GrB2 (Proteína 2 ligada ao receptor de fator de crescimento). As MAPKs fosforilam moléculas responsáveis pela transcrição no núcleo, agindo na síntese proteica (Guyoti, 2009). A última rota bioquímica que pode ser ativada envolve as proteínas IRS-I (receptor de insulina substrato I), as quais quando fosforiladas servem como locais de ligação para uma variedade de moléculas sinalizantes, como PI3K (Fosfaditilinositol-3 cinase), envolvidas no metabolismo e crescimento (Carter-Su et al., 1996).

Kuhn et al. (2002) afirmam que níveis sanguíneos de GH estão correlacionados inversamente com a expressão de GHR. Kim (2010) sugere que o GH inibe a expressão de GHR diretamente ou por intermédio da liberação de IGF-I, e estimula a formação do complexo GH-GHR pelas células.

O IGF-I é uma proteína de estrutura semelhante à insulina. É sintetizado principalmente no fígado, entretanto, sua produção ocorre também em outros tecidos,

sendo fundamental para o crescimento adequado do animal. Como não há um local de armazenamento deste hormônio, a maioria do IGF-I está na circulação ligada a um complexo formado por uma proteína ligadora de IGF-I (IGFBP-3 ou IGFBP-5) e por uma subunidade proteica ácido-lábil (ALS). A ligação desta proteína ao complexo estende a meia vida do IGF-I por cerca de 15 h (Kim, 2010).

Segundo Scanes (2009), o IGF-I possui importante papel na taxa de crescimento corporal de aves, e quanto menor o nível de IGF-I encontrado, pior será o crescimento corporal. Tem-se observado maior síntese e menor degradação proteica em aves com maior nível de IGF-I plasmático, o que resulta em maior deposição de músculo esquelético (Colon & Kita, 2002).

Expressão gênica - qRT-PCR

Na produção animal, características de interesse, como eficiência alimentar e reprodutiva, são expressas em função da genética do animal, do ambiente a que este é exposto, e também em função da interação entre estes dois fatores.

Os animais devem adaptar seus metabolismos a condições fisiológicas e ambientais que estão em constante mudanças, e requerem respostas metabólicas coordenadas pela expressão de genes específicos na presença ou ausência de nutrientes apropriados (Averous et al., 2003). O estudo da influência de determinados nutrientes na dieta de aves sobre a expressão de genes pode auxiliar a explicar diferenças obtidas no desempenho, em função das modificações ocorridas por mudanças fisiológicas.

A técnica de transcrição reversa, seguida pela PCR em tempo real (qRT-PCR), é um dos métodos que pode ser utilizado para análise da expressão gênica. Esta técnica, considerada altamente sensível, permite que até mesmo pequenas diferenças na expressão de determinado gene seja observada (Pfaffl, 2001). Outras características positivas da qRT-PCR são resultados rápidos de quantificação e alta acurácia (Ferraz, 2009).

A quantificação do material genético pela PCR em tempo real utiliza reagentes fluorescentes que possibilitam a detecção da formação do produto da PCR. Esses reagentes podem ser de dois tipos: uma sonda específica que reconhece sequências do genoma; ou um corante fluorescente que apresenta alta afinidade ao DNA dupla-fita, ligando-se a qualquer sequência amplificada. Essa ligação reagente-*amplicon* faz com

que aumente a fluorescência e permite detectar o produto da PCR conforme este se acumula durante os ciclos da reação (Hunt, 2012).

Os resultados da quantificação da qRT-PCR podem ser dados na forma relativa ou absoluta. A quantificação relativa é baseada na expressão de um gene alvo em relação a um gene referência. Este é utilizado para minimizar o erro da PCR em tempo real. A normalização destes genes, denominados de genes *housekeeping*, controlam a entrada das quantidades de RNA na transcrição reversa, já que são genes constitutivos e não variam em condições experimentais. Os mais comumente utilizados incluem a β-actina, gliceraldeído-3-fosfato desidrogenase (GAPDH) e 18S do RNA ribossomal (Huggett, 2005). Já o método de quantificação absoluta é baseado em uma curva padrão, através da qual é determinado o número de cópias do transcrito de interesse (Livak & Schmittgen, 2001).

Um dos motivos pelo qual a qRT-PCR é considerada uma técnica eficiente na quantificação dos ácidos nucleicos, é que os resultados obtidos são gerados durante a fase exponencial da reação de PCR. É denominado de Ciclo *Threshold* (Ct) o ponto em que a reação atinge o limiar da fase exponencial (Novais & Pires-Alves, 2004). O sinal dos compostos fluorescentes aumenta juntamente com a quantidade dos *amplicons*, assim, a amostra que apresentar maior quantidade de cópias de transcritos alcançará primeiramente o limiar de detecção (Bustin et al., 2005).

A fluorescência emitida pelo corante SYBR Green I indica a amplificação de DNA complementar total (cDNA); para determinar se está ocorrendo a amplificação da região de interesse é utilizada a curva de *melting*. A temperatura de *melting* é a temperatura em que metade das fitas de DNA está na forma de fita simples, e a outra metade está na forma de dupla hélice. Neste ponto, a fluorescência está diminuída e o software plota a taxa de mudança da fluorescência relativa com o tempo no eixo Y e a temperatura no eixo X. Esta curva atingirá o pico na temperatura de *melting* (Tm). Todos os produtos da PCR para um determinado *primer* devem apresentar a mesma Tm, se isso não estiver ocorrendo pode ser sinal de formação de dímeros de *primers*, de amplificação de região não específica, ou algum outro problema. A temperatura de *melting* de uma determinada dupla hélice depende da sua composição de bases e também de seu tamanho (Hunt, 2012).

Estresse térmico

As aves são animais endotérmicos, capazes de produzir calor internamente, e dependem de conforto térmico para expressar o máximo desempenho permitido por seu potencial genético (Miragliotta, 2005). Na zona de conforto térmico, denominada de zona termoneutra, toda a energia produzida pelo organismo é direcionada para fins produtivos, ou seja, não há gasto de energia para termorregulação (Macari et al., 2004).

Para manter o equilibrio térmico, as aves primeiramente dispõe de mecanismos ambientais e posturais, entretanto, quando estes não são eficientes, entram em ação respostas fisiológicas, coordenadas pelo hipotálamo. O sistema termorregulatório baseia-se em quatro unidades funcionais: os receptores, neurônios sensíveis ao frio e calor; o centro controlador, hipotálamo; os efetores, neurônios responsivos ao frio e calor; e o sistema passivo, responsável por executar a resposta determinada pelo hipotálamo (Furlan & Macari, 2002).

Esses mecanismos de termorregulação, entretanto, são eficientes somente quando a temperatura ambiente está dentro de certos limites, denominados de zona de tolerância. O ponto no qual a temperatura ambiental está abaixo desta zona é denominado temperatura crítica inferior, e o ponto no qual a temperatura ambiental está acima, denominado de temperatura crítica supeiror (Abreu & Abreu, 2012).

A característica mais utilizada pelos pesquisadores na determinação da faixa de termoneutralidade das aves é a idade das mesmas (Moura, 2001). A ave recém-nascida, por exemplo, possui uma grande relação entre área e volume corporal, o que acarreta em dificuldades para reter calor corporal. Além disso, sua capacidade termorregulatória ainda não se encontra bem desenvolvida até os 10 a 15 dias após o nascimento. A zona de conforto térmico para codornas situa-se entre 38ºC e 65% de unidade relativa (UR) na primeira semana de idade, 32ºC e 60% de UR na segunda semana de idade, 27ºC e 60% de UR na terceira semana de idade e 21ºC a 25ºC e 60% de UR a partir da quarta semana de idade (Reis, 1980 e Singh & Narayam, 2002).

Quando as aves estão sofrendo por estresse térmico por calor, ocorre a tentativa de diminuir a produção de calor metabólico, e aumentar a dissipação de calor. De acordo com Macari et al. (2004), os principais métodos de dissipação de calor são a vasodilatação e o aumento da evaporação através do aumento na frequência respiratória. Frequência respiratória aumentada pode ocasionar alcalose repiratória, e assim,

desequilíbrio eletrolítico relacionado à redução na ingestão de alimentos e, portanto pior desempenho (Silva, 2004).

Oliveira et al. (2006) observaram que altas temperaturas (35ºC), durante a produção de frangos de corte, influenciaram negativamente o consumo de ração e o ganho de peso, e também o rendimento de cortes nobres (coxa, sobrecoxa e peito). Esses autores observaram, ainda, que esses efeitos negativos são acentuados pelo aumento da umidade relativa do ar.

Como o relatado pelos referidos autores, o estresse calórico depende não somente da temperatura, mas também da umidade relativa do ar, da idade, do tamanho e da fase produtiva das aves; as respostas fisiológicas ao estresse também dependerão destes fatores (Abreu & Abreu, 2012).

Assim, como altas temperaturas, estresse térmico por frio também causa alterações no metabolismo animal. O aumento da exigência de energia que ocorre em animais submetidos às baixas temperaturas implica em mudanças no sistema cardiovascular, essas mudanças ocorrem para que a nova demanda energética seja suprida (Blahová et al., 2007). A concentração circulante do hormônio T3 parece estar envolvida na regulação da taxa de crescimento pela baixa temperatura ambiental. A concentração de T3 tem sido correlacionada negativamente com a temperatura e positivamente com a ingestão de alimentos em frangos (Yahav, 2000).

Outra forma que baixas temperaturas podem afetar o desempenho das aves é através da modulação das proteínas desacopladoras (UCPs). Baixas temperaturas têm sido constantemente associadas a maior expressão de mRNA UCP em diversos tecidos e espécies (Dridi etal., 2008; Toyomizu et al., 2002). Os autores associam essa maior expressão a uma tentativa do animal em manter a temperatura corporal adequada.

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II. OBJETIVOS

Objetivos Gerais:

Entender a influência do estresse térmico sobre a expressão de alguns genes envolvidos com o desempenho do animal e correlacionar com a eficiência alimentar deles.

Objetivos Específicos:

avaliar o desempenho de codornas de corte e postura em ambiente termoneutro, e dividi-las em grupos de alta e baixa eficiência alimentar;

avaliar a expressão dos genes de crescimento: receptor do hormônio do crescimento (GHR) e fator de crescimento semelhante à insulina I (IGF-I);

avaliar a expressão dos genes mitocondriais: UCP (proteína desacopladora), ANT (transportador de nucleotídeos de adenina) e COXIII (citocromo c oxidase subunidade III);

avaliar a expressão dos genes em tecidos diferentes: no fígado e músculo do peito de codornas de corte e postura de alta e baixa eficiência alimentar;

avaliar a influência do estresse térmico agudo sobre a expressão gênica de aves submetidas a três ambientes: conforto, estresse por calor (38ºC) de 12 h e estresse por frio (10ºC) de 12 h.

III. IGF-I, GHR AND UCP MRNA EXPRESSION IN THE LIVER AND MUSCLE OF HIGH- AND LOW-FEED-EFFICIENCY LAYING JAPANESE QUAIL AT DIFFERENT ENVIRONMENTAL TEMPERATURES

(Journal: Livestock Science)

Abstract: In this study, we analyzed insulin-like growth factor I (IGF-I), growth hormone receptor (GHR) and uncoupling protein (UCP) mRNA expression in the muscle and liver of high- (0.23 g/g) and low- (0.17 g/g) feed-efficiency (FE) Japanese quail at three different air temperatures: comfortable (25ºC), heat stress (38ºC) for 12 hours or cold stress (10ºC) for 12 hours. Total RNA was extracted from the liver and breast muscle of each quail, and cDNA was amplified using specific primers for the target genes. Expression was analyzed using quantitative real-time PCR (qRT-PCR). IGF-I mRNA expression was higher in the livers of high-FE quail than in the livers of low-FE quail under both heat and cold stress conditions. High-FE birds also showed higher GHR mRNA expression independent of temperature. UCP mRNA expression in the liver was lower in high-FE birds and higher under heat stress compared with the other conditions. IGF-I mRNA expression was higher in the muscle of high-FE quail under the three conditions tested, and UCP mRNA expression was higher under cold stress. Our results suggest that air temperature affects the expression of genes related to growth and mitochondrial energy production, and quail with different feed efficiencies respond differently to environmental stimuli.

Keywords: cold stress, feed efficiency, GHR, heat stress, IGF-I, UCP

Introduction

Characteristics governing animal production, such as feed and reproductive efficiency, are expressed as a function of the animal's genetics, the environment to which the animal is exposed and the interaction between these two factors.

Birds are endothermic animals and thus require comfortable temperatures in order to channel all the energy they produce toward animal production (Macari et al., 2004). Changes in air temperature, above or below the comfortable range, can negatively affect animal performance. Animals exposed to low temperatures undergo cardiovascular system changes in order to meet their increased energy requirements (Blahová et al., 2007). The hormone T3 appears to be involved in regulating the growth rate at low air temperatures. The circulating level of T3 has been negatively correlated with temperature and positively correlated with feed ingestion in chicken (Yahav, 2000).

Low temperatures can also affect bird performance via uncoupling protein (UCP). UCP is a protein located in the internal membrane of mitochondria that is responsible for redirecting energy from ATP production to heat production (Vidal-Puig, 2000). Because it causes uncoupling during energy production, studies have shown that chickens with high UCP mRNA expression also have lower feed efficiency (Ojano-Dirain et al., 2007). Low temperatures have been linked to higher UCP mRNA expression in several tissues and species (Dridi et al., 2008; Toyomizu et al., 2002). This higher level of expression has been attributed to the animal's attempts to maintain adequate body temperature.

Similar to low temperatures, heat stress also causes metabolic changes. In broilers, higher temperatures are correlated with decreases in feed intake , nutrient utilization efficiency, weight gain, egg production and feed efficiency (Menten et al., 2006; Akşit et al, 2006; Oliveira, 2006). Such decreased performance is mainly caused by reductions in T3 and T4 levels, changes in water and ionic balance, changes in the kinetics of important enzymes that control the concentrations of anabolic and catabolic products, depression of immune system function and changes in growth hormone concentration (Barbour et al., 2010).

In addition to air temperature (Gabillard et al., 2006; Gabillard et al., 2003), the expression of hormones, such as insulin-like growth factor I (IGF-I) and growth hormone receptor (GHR), can also be affected by many factors, including diet (Gasparino et al., 2012; Katsumata et al., 2002), tissue type and developmental stage (Berishvili et al., 2006). IGF-I mRNA expression also differs between chickens selected

for high or low growth rates. Higher IGF-I mRNA expression was found in animals with high growth rates (Beccavin et al., 2001).

Confirming the hypothesis that IGF-I has a positive affect on bird growth, studies have shown increased protein deposition in the presence of higher levels of circulating IGF-I (Carew et al., 2003; Stubbs et al., 2002), possibly due to the effect of this hormone on metabolic cycles responsible for protein synthesis (Tesseraud et al., 2007) and degradation (Sacheck et al., 2004).

Therefore, based on the hypothesis that air temperature can affect hormones and proteins important for bird performance, and that animals with different feed efficiencies respond differently to environmental stimuli, this study sought to analyze IGF-I, GHR and UCP expression in the muscle and liver of high- and low-feed-efficiency (FE) laying quail kept in three environments: comfortable, heat stress (38ºC) for 12 hours and cold stress (10ºC) for 12 hours.

Materials and Methods

The experiment was conducted at the Iguatemi Experimental Farm at the State University of Maringá and the experimental procedure was approved by the Brazilian Animal Ethics Committee. First, 400 male laying quails (*Coturnix coturnix japonica*), born from the same incubation, were conventionally raised for 28 days under the same experimental conditions. At this time, the birds were transferred to individual cages and underwent an adaptive period for seven days. Feed efficiency was calculated as the increase in body weight relative to feed intake from 35 to 42 days of age. Feed consumption and weight gain during the test period were measured individually. During this time, the birds were kept at a comfortable temperature (25 ± 0.9°C with 60 ± 1.2% relative humidity (RH)). The animals had free access to food and water throughout the experiment. The feed was formulated for the two phases of the birds' lives according to Rostagno et al. (2011). Starter feed was provided during the first 14 days of life, and growth feed was provided from 15 days of age. At 42 days of age, the animals were separated into two groups: the 36 birds with the highest FE (high-FE) and the 36 birds with the lowest FE values (low-FE) (Table 2). These groups were then divided into three environmental conditions: comfortable (25ºC, according to Pinto et al., 2003), heat stress (38ºC) for 12 hours and cold stress (10ºC) for 12 hours, with 12 animals in each group.

After the stress period, the animals were euthanized by cervical dislocation, and tissue from the breast muscle (*pectoralis superficialis*) and liver were collected and stored in RNA Holder® (BioAgency Biotecnologia, Brasil) at -20ºC until RNA extraction. Animals in comfortable conditions were sacrificed immediately after the groups were separated. Only 12 (6 high-FE and 6 low-FE) of the 24 animals submitted to each experimental condition were used for gene-expression analysis.

Total RNA was extracted using Trizol® (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions (1 mL per 100 mg of tissue). All of the materials used had been previously treated with the RNase inhibitor RNase AWAY® (Invitrogen, Carlsbad, CA, USA). The tissue and Trizol mixture were triturated with a Polytron electric homogenizer until completely dissociated. Next, 200 µL of chloroform was added to the sample, and the mixture was manually homogenized for 1 minute. The samples were then centrifuged for 15 minutes at 12,000 rpm and 4ºC. The aqueous phase was collected and transferred to a clean tube containing 500 µL of isopropanol per tube and again homogenized and centrifuged for 15 minutes at 12,000 rpm and 4ºC. The supernatant was discarded, and the precipitate was washed in 1 mL of 75% ethanol. The material was once again centrifuged at 12,000 rpm for 5 minutes, and the supernatant was discarded. The pellet was dried for 15 minutes and resuspended in ultrapure RNase-free water.

The total RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. RNA integrity was analyzed using a 1% agarose gel stained with 10% ethidium bromide and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions to remove possible genomic DNA contamination.

A SuperScript^{TM} III First-Strand Synthesis Super Mix (Invitrogen Corporation, Brasil) kit was used for complementary DNA synthesis according to the manufacturer's instructions. For this reaction, 6 µL of total RNA, 1 µL of oligo dT (50 µM oligo$(dT)_{20}$) and 1 µL of annealing buffer were added to a sterile RNA-free tube. The reaction was then incubated for 5 minutes at 65ºC and placed on ice for 1 minute. Subsequently, 10 µL of 2× First-Strand Reaction Mix and 2 µL of solution containing SuperScript III reverse transcriptase enzyme and RNase inhibitor were added to the tubes. The solution was incubated for 50 minutes at 50ºC for the synthesis of cDNA. Next, the reaction was incubated for 5 minutes at 85ºC and immediately placed on ice. The samples were stored at -20ºC until use.

Real-time PCR reactions were performed using the fluorescent dye SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, USA) in a StepOnePlus v.2.2 PCR machine (Applied Biosystems, Carlsbad, CA, USA). All of the reactions were analyzed under the same conditions and normalized to the ROX Reference Dye (Invitrogen, Carlsbad, CA, USA) to correct fluctuations in the readings due to evaporation during the reaction.

The primers used in the GHR and IGF-I amplification reactions were designed based on the gene sequences deposited at www.ncbi.nlm.nih.gov (accession numbers NM001001293.1 and FJ977570.1, respectively) using the website www.idtdna.com. The UCP primers were described by Ojano-Dirain et al. (2007) (Table 1). Two endogenous controls, ß-actin and GAPDH, were tested, and ß-actin (accession number L08165) was selected because its amplification was shown to be more efficient. All of the analyses were performed in duplicate, each in a volume of 25 µL.

Table 1: qRT-PCR primers

Gene	Amplicon (bp)	Annealing Temperature (ºC)	Primer sequence (5'-3')
GHR	145	60ºC	AACACAGATACCCAACAGCC
			AGAAGTCAGTGTTTGTCAGGG
IGF-I	140	60ºC	CACCTAAATCTGCACGCT
			CTTGTGGATGGCATGATCT
UCP	41	60ºC	GCAGCGGCAGATGAGCTT
			AGAGCTGCTTCACAGAGTCGTAGA
ß-actin	136	60ºC	ACCCCAAAGCCAACAGA
			CCAGAGTCCATCACAATACC

The $2^{-\Delta CT}$ method was used for the relative quantification analysis. The results are shown as averages with standard deviations. Univariate analysis was used to test for the normality of the data. The experimental design was a completely randomized factorial set up with three environments (comfortable, heat stress and cold stress) and two feed efficiencies (high and low). Averages were compared using the Tukey test ($P<0.05$) (SAS Inst. Inc., Cary, NC).

Results

After the feed intake vs. weight gain analysis period, the birds were divided into high-FE and low-FE groups (Table 2). High-FE birds had a higher final weight, increased weight gain and a better feed conversion ratio than quail with low FE.

Table 2: Performance data for the animals separated into high- and low-FE groups

	IW (g)	FW (g)	WG (g)	FI (g)	FCR (g/g)	FE (g/g)
High-FE	119.55	145.57^{a}	24.44^{a}	107.00	4.40^{b}	0.23^{a}
Low-FE	124.00	141.44^{b}	17.44^{b}	106.74	6.15^{a}	0.17^{b}

IW: initial weight, FW: final weight, WG: weight gain, FI: feed intake, FCR: feed conversion ratio, FE: feed efficiency.
Lowercase letters indicate a significant difference by the Tukey test.

The gene expression results for IGF-I, GHR and UCP in the liver and muscle of the high- and low-FE birds in the three environments are shown in Table 3. A significant interaction between feed efficiency and environment was observed.

Table 3: IGF-I, GHR and UCP gene expression in the liver and muscles of high- and low-FE birds under three environmental conditions

	Liver					
	IGF-I		GHR		UCP	
ENV	High-FE	Low-FE	High-FE	Low-FE	High-FE	Low-FE
Comfortable	0.560^{aA}	0.111^{bA}	0.740^{aA}	0.490^{bA}	0.00022^{bB}	0.0024^{aA}
Cold	0.394^{aB}	0.117^{bA}	0.732^{aA}	0.415^{bA}	0.0017^{bA}	0.0021^{aA}
Hot	0.045^{aC}	0.044^{aB}	0.229^{aB}	0.177^{bB}	0.00015^{bB}	0.0014^{aB}
CV (%)	10.3		24.5		24.13	
P value	<.0001		<.0001		<.0001	
	Muscle					
	IGF-I		GHR		UCP	
ENV	High-FE	Low-FE	High-FE	Low-FE	High-FE	Low-FE
Comfortable	0.201^{aA}	0.181^{bA}	0.261^{aB}	0.221^{aB}	0.201^{bB}	0.516^{aB}
Cold	0.107^{aB}	0.022^{bB}	1.471^{aA}	0.593^{bA}	0.295^{bA}	0.853^{aA}
Hot	0.057^{aC}	0.028^{bB}	0.065^{aC}	0.034^{bC}	0.247^{aB}	0.289^{aC}
CV (%)	25.5		18.9		23.72	
P value	<.0001		<.0001		<.0001	

Identical lowercase letters in the same row indicate no significant difference by the Tukey test.
Identical uppercase letters in the same column indicate no significant difference by the Tukey test.

Our analysis of gene expression in the liver showed that high-FE birds had higher IGF-I mRNA expression than low-FE birds in both the cold-stress and comfortable

conditions. The highest IGF-I mRNA expression in high-FE animals was observed under comfortable conditions, followed by that under cold and then heat. However, IGF-I mRNA expression was similar in the low-FE animals exposed to either cold or comfortable conditions and lower in those exposed to high temperatures.

In the liver, high-FE birds had higher IGF-I mRNA expression than in the muscle in all three studied environments. In the muscle, these birds had significantly higher IGF-I mRNA expression under comfortable conditions, followed by that under cold and heat. Low-FE birds had significantly higher IGF-I mRNA expression in comfortable conditions than in the other environments.

GHR expression in the liver was higher in high-FE birds than in low-FE birds independent of environment. Lower GHR expression was observed in both high and low-FE birds exposed to heat stress, compared with the other temperatures.

GHR mRNA expression in the muscle differed between high- and low-FE animals under both the cold and hot conditions; expression of this gene was higher in high-FE animals under both conditions. High- and low-FE animals had significantly higher GHR mRNA expression under cold conditions, followed by under comfortable and hot conditions.

UCP mRNA expression in the liver was higher in low-FE birds, independent of the environment. High-FE birds had significantly higher UCP mRNA expression under cold conditions, compared with the other temperatures. No significant difference in expression of this gene between the cold and comfortable conditions was observed for low-FE birds; however, animals exposed to the high temperature had significantly lower UCP mRNA expression.

In the muscle, UCP mRNA expression was higher in high-FE birds under cold conditions, but no difference in UCP mRNA expression was observed between the hot and comfortable conditions. However, higher expression levels for this gene were observed in low-FE birds under cold conditions, followed by under comfortable and hot conditions. Comparisons of the low- and high-FE birds revealed higher levels of UCP mRNA in low-FE birds when exposed to cold stress or housed in comfortable conditions.

Discussion

All animals adjust their metabolism to adapt to constantly changing physiological and environmental conditions; this involves the control of metabolic responses by

specific genes. Therefore, we hypothesized that production characteristics, such as feed efficiency, are influenced by several biological mechanisms, and that the overall observed performance is a result of interactions between all of these factors; air temperature can affect hormones and proteins important for bird performance, and animals with different feed efficiencies respond differently to environmental stimuli.

We observed that high-FE birds gained significantly more body weight, even though they were the same breed and had similar food intake as low-FE birds. This suggests that part of the observed difference is due to differences in the expression of important genes that influence various metabolic mechanisms.

In a study of chickens selected for rapid or slow growth, Tesserau et al. (2000) observed greater weight gain, higher relative Pectoralis major muscle mass (g/Kg body weight), greater protein deposition in this muscle, increased protein synthesis and higher overall protein deposition in the rapidly growing birds. The authors suggested that the higher level of protein deposition may be mainly due to decreased protein degradation in the muscles of these birds. Rapidly growing chickens also appear to have more and larger muscle fibers (Remignon et al., 1995). This trait may be due to the fact that the satellite cells of rapidly growing birds are more responsive to IGF-I in the plasma, which may contribute to increased hypertrophy of the muscle fibers (Duclos et al., 1991).

Hormonal growth regulation involves a complex series of interactions between different hormones, with the somatotropic axis (GH, GHR and IGF-I) considered to be the most important. GH can affect growth directly, but its effects are mostly mediated through IGF-I activity. The presence of GH in an organism promotes the synthesis and release of this hormone (Becker et al., 2001). The effect of GH on IGF-I is mediated by the GH receptor (GHR) because GH-GHR binding is necessary to stimulate IGF-I synthesis and release.

Studies have shown that IGF-I plays an important role in the growth rate of birds, and the lower the IGF-I level is, the slower is the growth (Scanes et al., 2009). Increased synthesis and decreased protein degradation have been observed in chickens with high plasma IGF-I levels, resulting in increased skeletal muscle deposition (Colon and Kita, 2002). There is evidence that protein synthesis and degradation may occur via the same biochemical cycle. Murf-1 (muscle RING finger 1) and atrogin-1 are two genes involved in protein degradation in the ubiquitin-proteosome complex. Overexpression

of these atrogenes is linked to muscular atrophy, and IGF-I appears to decrease their expression (Tesseraud et al., 2007; Sacheck et al., 2004).

Supporting the idea that GH affects growth mainly through IGF-I (Vasilatos-Younken, 1999), previous studies have shown that higher GH levels in chickens may be associated with lower growth rates (Burke and Marks, 1982; Goddard et al., 1988). This suggests that GH levels do not directly explain the growth rates observed in chickens but that more information can be obtained by analyzing IGF-I (Beccavin et al., 2001). These authors observed that birds selected for higher growth rates have higher levels of IGF-I mRNA in the liver and higher circulating IGF-I levels than birds selected for low growth rates. Similar to these findings, we also observed higher IGF-I and GHR expression in the muscles and livers of high-FE birds.

Our comparisons of comfortable temperatures and heat stress showed that birds with high- or low-FE had higher IGF-I and GHR expression under comfortable conditions in both of the studied tissues. Similar results have been reported previously (Antonio, 2010). Willemsen et al. (2011) observed lower circulating IGF-I concentrations in chickens exposed to high temperatures, along with lower T3 and T4 levels, higher corticosterone levels, higher TBARS levels and higher antioxidant activity. Therefore, the authors suggested that heat stress may cause oxidative stress, contributing to the observed decrease in the birds' metabolic rate.

Decreased protein synthesis rate and capacity, growth and protein deposition have been found in chickens exposed to heat stress (Temim et al., 2000). The authors also observed lower total RNA concentrations in stressed animals. Therefore, there may be a link between these results found and the lower levels of IGF-I mRNA expression observed at high temperatures in our study; IGF-I activity is important not just for promoting protein synthesis but also for decreasing the protein degradation rate by the ubiquitin-proteosome complex (Sacheck et al., 2004).

IGF-I is mainly synthesized in the liver (Kim, 2010); however, we found that high-FE quail exposed to heat stress had higher IGF-I mRNA expression levels in the muscle. Extra-hepatic tissues expressing high IGF-I mRNA levels suggest that decreased plasma concentrations of the hormone, which may occur in animals exposed to high temperatures, can lead to higher IGF-I mRNA expression in other tissues (Katsumata et al., 2002).

Feed efficiency in birds has been frequently linked to the efficiency of energy production in the mitochondria (Ojano-Dirain et al., 2007; Bottje et al., 2006; Iqbal et

al., 2005). These studies have determined that mitochondria in low-FE animals exhibit greater H_2O_2 production in conjunction with increased protein oxidation and decreased activity of the electron transport chain complexes. The authors suggest that these increased levels of oxidized proteins may contribute to the low-FE phenotype by increasing the amount of energy required to repair the proteins and by compromising or decreasing the function of the damaged proteins. Increased H_2O_2 production in low-FE birds and subsequent protein damage may lead to changes in mitochondrial gene expression.

Some studies have also shown that high-FE birds show increased electron transport activity and, consequently, an increased ability to synthesize ATP. This trait may be due to the decreased expression of UCP mRNA, which has also been observed in high-FE birds (Ojano-Dirain et al., 2007; Raimbault et al., 2001).

In our study, we observed that high-FE birds had lower UCP mRNA expression in both tissues analyzed, independent of temperature. This finding suggests that, consistent with a previous report (Dridi et al., 2004), UCP may be involved in animal performance by increasing energy dissipation through mitochondrial oxidation.

Several studies have shown that UCP mRNA expression changes as a function of air temperature (Dridi et al., 2008; Toyomizu et al., 2002). We observed that high- and low-FE birds had significantly higher UCP mRNA expression under cold conditions and lower UCP expression under hot conditions. Increased UCP mRNA expression in animals under cold stress can be attributed to heat generation, as birds do not have brown adipose tissue. There is strong evidence that mitochondrial uncoupling can contribute to heat production in skeletal muscle (Dridi et al., 2008; Raimbault et al., 2001; Toyomizu et al., 2002).

Animals exposed to low temperatures also show an increase in the amount of available fatty acids and circulating T3 levels, both of which may be involved in heat generation via UCP activation. Fatty acids play important roles as both muscle activity substrates and as oxidative phosphorylation uncoupling agents. These molecules may contribute to UCP expression by activating PPARs (peroxisome proliferator-activated receptors) because uncoupling protein expression depends on PPAR binding to the UCP promoter region (Solanes et al., 2003).

T3 appears to be necessary for maximum UCP mRNA expression, and it is known to promote specific mRNA accumulation through transcriptional and post-transcriptional mechanisms, including the stabilization of precursor and mature mRNA

(Rehnmark et al., 1992). Furthermore, when ducks were exposed to cold temperatures, UCP mRNA expression was significantly increased in animals treated with T3. However, this result was not observed in animals with higher circulating fatty acid levels, and therefore, the authors suggested that UPC mRNA expression is mainly controlled by the status of the thyroid (Rey et al., 2010).

Studies have shown that UCP mRNA is mainly expressed in the skeletal muscles of birds (Raimbault et al., 2001; Evock-Clover et al., 2002). We also observed higher UCP mRNA concentrations in the muscle than in the liver in our study.

The findings of this study suggest that gene-expression influences on phenotype for feed-efficiency, as that animals with high efficiency have shown greater expression of IGF-I and GHR genes, which are responsible for animal growth. Considering the UCP gene, it was observed higher UCP expression for low-efficiency animals exposed to cold stress, indicating that this gene is related to thermal-regulation. Considering distinct environmental temperatures, lower expressions of IGF-I and GHR were observed on heat stressed animals, showing that there is a higher physiological damage to animals exposed to higher temperatures. With this work, it was also possible to conclude that on heat stress situations, extrahepatic tissues might express high levels of IGF-I gene.

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IV. ANT AND COX III GENE EXPRESSION IN THE LIVER AND MUSCLE OF HIGH AND LOW FEED EFFICIENCY JAPANESE QUAIL LAYERS SUBMITTED TO DIFFERENT ENVIRONMENTAL TEMPERATURES

(Journal: Genetics and Molecular Research)

ABSTRACT: We had the objective of evaluating the ANT and COX III gene expression in the muscle and liver of Japanese quails presenting high and low feed efficiency (FE) and submitted to three different environmental temperatures: comfort, heat stress (38ºC) and cold stress (10ºC). The ANT mRNA level was lower in the liver of heat stressed animals. In the muscle lower ANT mRNA level was observed for high FE and for heat stressed animals. The greatest COX III mRNA expression in the muscle was observed for high FE animals and that were subjected to cold stress. In the liver there was a much higher gene expression of COX III mRNA in cold stressed animals. The obtained results suggest a possible relation between genes involved in energy production by mitochondria and feed efficiency phenotype, and that environmental temperature affects the ANT and COX III gene expression. Japanese quails presenting different feed efficiency levels respond differently to environmental stimuli.
KEYWORDS: ATP; cold stress; feed efficiency; heat stress; mitochondria

INTRODUCTION

The mitochondria are responsible for producing 90% of the energy required by the cells (Bottje et al., 2006), they are the organelles responsible for transforming chemical energy from metabolites into energy easily accessed by the cell. This energy is accumulated in labile compounds, the main one being ATP (Schauss et al., 2010). The

free energy generated by the transport of electrons pass through the mitochondrial respiratory chain until its ultimate acceptor, O_2, is used to produce ATP from ADP + Pi. The electron transport system is a chain of enzymatic and non-enzymatic compounds, whose function is to transport high-energy electrons. This energy is provided for the complexes of chain where the ATP will be produced. The mitochondrial enzymes involved in oxidative phosphorylation are positioned in the inner membrane of the organelle, and arranged in five multiprotein complexes (Nelson and Cox, 2008)

Recent studies have shown a bond between feed efficiency and the energy production efficiency by mitochondria. These studies show that animals that are more efficient in converting feed to body weight can display alterations in the electron transport chain gene expression, which can influence the use of nutrients and alter the body energy consumption, and that animals with higher residual feed intake may suffer failure in the transport of electrons/protons, thus reducing the efficiency of ATP production by the mitochondrial genes, which has an adverse effect on the feed conversion (Bottje and Carstens, 2009; Krueger et al., 2008; Iqbal et al., 2005).

Other proteins involved in energy synthesis are the adenine nucleotide translocator (ANT) and the cytochrome oxidase subunit III (COX III). ANT is responsible for moving the ADP from the cytosol to the mitochondrial and matrix the ATP across the inner mitochondrial membrane (Ojano-Dirain et al., 2007). The mitochondrial function may be impaired by the incapacity of ADP/ATP exchange between the cytosol and the mitochondria, thus there may be some connection between the ANT expression with the phenotypic expression of feed efficiency (Bottje et al., 2006). COX III is also present in the electron transport chain and related to the oxidative phosphorylation efficiency because it is responsible for pumping protons and transporting electrons. Lower expression of these genes can occur due to lower cellular efficiency or greater oxidative damage due to the production of reactive oxygen species (ROS) (Kemp et al., 2003).

Reactive oxygen species (ROS) are normally produced in cellular biological processes. At increased levels, ROS are frequently associated with events such as apoptosis, protein oxidation, lipid peroxidation and mitochondrial DNA damage (Lee and Wei, 2005; Moustafa et al., 2004). An imbalance between the production and elimination of reactive oxygen species in an organism leads to a state known as oxidative stress. Several studies have linked heat stress with oxidative stress (Mujahid et al., 2009; Mujahid et al., 2005), and the effects of heat stress are possibly due to an

acceleration in the rate of ROS formation and/or an increase in ROS reactivity (Bai et al., 2003).

As well as high temperatures, the increase in energy requirements when animals are submitted to low environmental temperatures results in changes in the circulatory system in order to supply these higher energy demands (Blahová et al., 2007). The blood concentration of the hormone T3 seems to be involved in the regulation of growth rate as a function of low environmental temperature. Blood T3 concentrations were negatively correlated with environmental temperature and positively with feed intake (Yahav, 2000).

This work was performed under the hypothesis that genes involved in energy production by mitochondria may be related to the feed efficiency phenotype observed in poultry, and that the environment in which these animals are exposed could affect the expression of such genes. Thus, we had the objective of evaluating the ANT and COX III gene expression in the muscle and liver of Japanese quails presenting high and low feed efficiency and submitted to three different environmental temperatures: comfort (25^{o}C), heat stress (38ºC) for 12 hours and cold stress (10ºC) for 12 hours.

MATERIAL AND METHODS

The experimental procedure was approved by the Brazilian Animal Ethics Committee.

The experiment was conducted at the Iguatemi Experimental Farm of the State University of Maringá – UEM. A total of 400 male Japanese quail layers derived from the same breeder flock were reared until 28 days of age according to conventional management practices and were submitted to the same experimental conditions. On day 28, birds were transferred to individual cages in an environmentally-controlled room and were submitted to a 7-d adaptation period. The feed efficiency was evaluated in the period of 35 to 42 days of age, and it was calculated by the increase in body weight relative to consumption of food. Birds were individually weighed in the beginning and at the end of this period to calculated body weight gain, and feed intake was calculated as the difference between the amount of feed offered and of feed residues at the end of this period. Birds were offered water and feed *ad libitum* during the entire experimental period. Feeds were formulated to supply Japanese quails' nutritional requirements, according to Rostagno et al. (2011). A two-stage feeding program was adopted, with a

starter diet offered between 1-14 days, and a grower diet offered after 15 days of age. At 42 days of age, birds were divided in groups with high (n=36) or low feed efficiency (n=36), and submitted to three different environmental temperatures: comfort (25^{o}C, according to Pinto et al., 2003), heat stress (38ºC) for 12 hours or cold stress (10ºC) for 12 hours. 12 high feed efficiency quails and 12 low feed efficiency quails were subjected to each thermal environment.

After the stress period, birds were sacrificed by neck dislocation and breast muscle (*Pectoralis superficialis*) and liver samples were collected and stored in RNA Holder® (BioAgency Biotecnologia, Brazil) at - 20ºC until RNA extraction. Birds in the comfort group were sacrificed immediately after the groups were established. Out of the 24 birds submitted to each temperature stress, only 12 (six from each feed efficiency group) were used for gene expression analysis.

Total RNA was extracted using the reagent Trizol® (Invitrogen, Carlsbad CA, USA), according to the manufacturer's recommendations, at a ratio of 1mL per 100 mg of tissue. All materials were previously treated with RNase inhibitor (RNase AWAY®, Invitrogen, Carlsbad, CA, USA). The muscle (muscle + trizol) was ground using an electric homogenizer (Polytron) until its complete dissociation, after which 200 µL chloroform were added and manually stirred for one minute. Samples were then centrifuged for 15min at 12.000rpm at 4ºC, and the resulting liquid phase was collected and transferred to a clean tube where 500uL isopropanol were added. Samples were again centrifuged for 15min at 12.000rpm at 4ºC. The supernatant was discarded and the precipitate was washed with 1mL ethanol at 75%. The material was again centrifuged at 12.000rpm for 5min, and the supernatant was discarded. The resulting pellet was dried for 15min, and re-suspended in RNase-free ultrapure water.

Total RNA concentration was determined with the aid of a spectrophotometer at 260 nm wavelength. RNA integrity was evaluated in 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove possible genomic DNA residues, as recommended by the manufacturer.

The SuperScrippt^{TM} III First-Strand Synthesis Super Mix kit (Invitrogen Corporation, Brazil) was used to synthesize the complementary DNA (cDNA), according to the manufacturer's instructions. In a sterile and RNA-free tube, 6uL total RNA, 1uL de oligo (dT) (50uM oligo $(dT)_{20}$ and 1uL annealing buffer were added. The reaction was incubated for 5min at 65ºC and then placed on ice for 1min. Subsequently,

10µL of 2x First-Strand Reaction Mix solution and 2µL of the solution containing the reverse transcriptase enzyme SuperScript III and RNAse inhibitor were added. The solution was then incubated for 50min at 50ºC for the synthesis of the complementary DNA. The reaction was again incubated for 5min at 85ºC and immediately placed on ice. Samples were stored at -20ºC until subsequent analyses.

The fluorescent compound SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, USA) was used for real-time PCR. RT-PCR analyses were carried out in the StepOnePlus v.2.2 apparatus (Applied Biosystems, Carlsbad, CA, USA). All reactions were submitted to the same analysis conditions and were normalized by the signal of the passive reference dye (ROX Reference Dye; Invitrogen, Carlsbad, CA, USA) to correct reading fluctuations caused by volume variations and evaporation during reaction.

The avANT and COX III primers used in the reactions were designed according to Ojano-Dirain et al. (2007), as shown in Table 1. Two endogenous controls – ß-actin and GAPDH genes – were tested, and the ß-actin gene (access n. L08165) was used because it was shown to be more efficient in the reaction. All analyses were carried out at a final volume of 25µL and in duplicate. Gene expression values are expressed in arbitrary units (AU).

Table 1- qRT-PCR primers

Gene	Amplicom (bp)	GenBank access number	Primers sequence (5'-3')
COX III	71	NP_006921	AGGATTCTATTTCACAGCCCTACAAG
			AGACGCTGTCAGCGATTGAGA
ANT	67	AB088686	TGTGGCTGGTGTGGTTTCCTA
			GCGTCCTGACTGCATCATCA
ß -actin	136	L08165	ACCCCAAAGCCAACAGA
			CCAGAGTCCATCACAATACC

The results are shown as the mean and the standard deviation. To investigate the interactions between different temperatures and feed efficiency, the data were subjected to an analysis of variance (ANOVA). A general liner model (GLM) was used to determine significant interactions between treatments. Where significant effects were detected in the ANOVA (P = 0.05), it means they were compared using the Tukey test. Prior to analysis, data were checked for normality using the univariate procedure in SAS (SAS Inst. Inc., Cary, NC).

RESULTS

Quantitative RT-PCR was used to evaluate gene expression patterns in liver and pectoralis muscle in response to the different efficiency and environmental temperatures. The data were normalized using the b-actin gene, once its expression does not change during the treatments.

After the period of individual feed intake and weight gain evaluation we can separate the animals into two homogeneous groups, one group was considered of high feed efficiency (High FE) and the other of low feed efficiency (Low FE). At the beginning of the evaluation period of feed efficiency, the animals in both groups had statistically equal initial weight (119.55 g for animals of high FE, and 121.00 g for animals of low FE). The animals of high FE had greater final weight, greater weight gain and better feed conversion, even consuming the same amount of feed that low FE animals (107.00 g for high FE, and 106.74 g for low FE) (Figure 1).

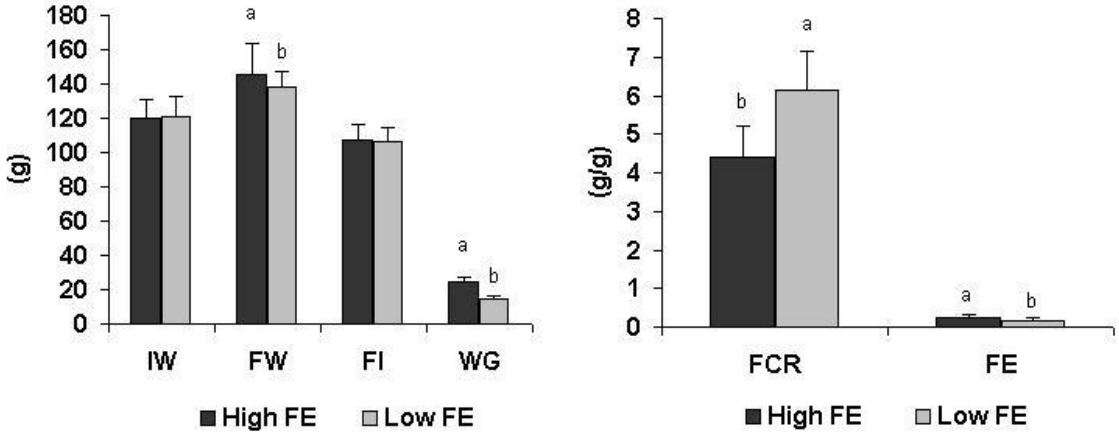

Figure 1- IW: initial weight, FW: final weight, FI: feed intake, WG: weight gain, FCR: feed conversion ratio, FE: feed efficiency of high and low- FE quails. Values are means with their standard deviations represented by vertical bars. Different letters above de bars indicate statistically significant differences ($P<0.05$).

The results of the expression of the ANT and COX III genes in the liver and muscle of high- and low-FE Japanese quails submitted to three environmental temperatures are shown in Table 2.

The ANT and COX III mRNA expression in the liver suffered only environmental temperature effect. Regarding ANT mRNA expression, we can observe lower expression of this gene in animals exposed to heat stress (4.77 AU), with no difference between animals subjected to cold stress and thermal comfort. Whereas for COX III

mRNA expression we observed that when the animals were exposed to low temperature, there was a much higher expression of this gene.

Regarding the expression of these genes in the muscle, we observed that ANT mRNA expression suffered the effects of efficiency, high FE animals showed higher expression than low FE animals (7.77 vs 6.87 AU); and they also suffered environmental temperature effect, higher expression was observed in animals subjected to cold stress, followed by thermal comfort animals, and then heat stressed animals.

We can observe a significant interaction between feed efficiency and environmental temperature effect on the expression of COX III mRNA in the muscle. The greatest COX III mRNA expression was observed for high FE animals and that were subjected to cold stress. There was no difference in the expression among high FE animals from thermal comfort and heat stress, and low FE animals from thermal comfort and cold stress. The lower COX III mRNA expression was observed in the muscle of animals with low EF subjected to heat stress.

Table 2- Mitochondrial genes expression in the liver and muscle of high- and low- FE Japanese quails submitted to three environmental temperatures

		Liver		Muscle	
		ANT	COX III	ANT	COX III
High- FE	Comfort	5.83±0.24	0.006±0.001	6.57±2.19	0.08±0.03b
	Heat stress	4.83±0.18	0.004±0.001	5.29±0.67	0.10±0.02b
	Cold stress	5.38±0.55	0.47±0.14	8.80±0.75	0.14±0.03a
Low-FE	Comfort	5.92±0.31	0.01±0.0005	8.37±0.53	0.08±0.01b
	Heat stress	4.71±0.40	0.003±0.0005	5.52±0.58	0.06±0.02c
	Cold stress	5.73±0.81	0.37±0.18	9.42±0.71	0.08±0.008b
Main Effects					
Efficiency	High	5.35±0.54	0.16±0.024	7.77±1.79a	0.11±0.04
	Low	5.46±0.75	0.13±0.020	6.87±1.98b	0.07±0.02
Environment					
	Comfort	5.88±0.29a	0.008±0.002b	7.47±1.78b	0.08±0.02
	Heat stress	4.77±0.27b	0.003±0.001b	5.40±0.61c	0.08±0.03
	Cold stress	5.55±0.68a	0.42±0.16a	9.11±0.77a	0.11±0.03
Probabilities					
Efficiency		0.4968	0.2774	0.0196	<.0001
Environment		<.0001	<.0001	<.0001	0.0006
Interaction		0.4729	0.2782	0.1974	0.0040

Values are means with their standard deviations.
[a,b,c]Within a column, it means lacking a common superscript letter differ (P<0.05).

DISCUSSION

The main objective of this study was to determine whether there was any connection between the quail FE phenotype with the mitochondrial genes expression, and to observe if the environmental temperature might influence the expression of such genes. After the period of individual feed efficiency evaluation we have been able to separate the animals into two fairly homogeneous groups in relation to feed efficiency, a group of high FE and another group of low FE, our results showed that high-FE Japanese quails gained significantly more body weight, although their feed intake was similar to those presenting low-FE. Therefore, we suggest the observed differences are partially explained by differences in the expression of important genes that activate different metabolic mechanisms.

Our results show that animals with greater feed efficiency also showed higher expression of mitochondrial genes known to play important roles in the energy production efficiency. Several studies in literature show the relationship between mitochondrial genes expression and feed efficiency phenotype. As shown in our studies increased ANT and COX III mRNA expression in higher FE animals. In addition to this result, high FE animals are also related to reduced ROS production, reduced protein oxidation, increased mitochondrial respiration rate, and increased respiratory chain complexes activity with greater coupling in the electron transport by the chain (Kelly et al., 2011; Bottje and Carstens 2009; Krueger et al. 2008; Ojano-Dirain et al., 2007; Iqbal et al., 2005; Iqbal et al., 2004).

Despite the ATP production having great influence on feed efficiency many other physiological systems are involved in the control of such feature. Metabolic pathways involved in protein deposition, and in food intake control, among others, also play a key role on feed efficiency. Recent studies on muscle growth (Zheng et al., 2011; Tesseraud et al., 2007) and about food intake control showed that the body has cellular/molecular mechanisms with a single signaling pathway that connects peripheral tissues, where energy is used or stored, with central nervous system (CNS), which regulates energy acquisition by controlling the feed intake (Richards et al., 2010).

To better understand the cellular mechanisms that define the feed efficiency phenotype, Bottje and Kong (2012) conducted an experiment with broilers separated into groups of high and low FE in order to evaluate the overall relationship between feed efficiency and gene expression. The authors found 782 genes that were

differentially expressed in high and low FE birds. The authors results suggest that high FE birds exhibited increased expression of genes associated with signal transduction pathway, anabolic activities and activities of energy coordination, metabolic pathway that are favorable to the development and cell growth. Low FE animals on the other hand, showed greater expression of genes related to actin-myosin filaments and genes related or responsive to stress. Thus, the authors suggest that the low-FE phenotype could be the product of gene expression which is modulated by oxidative stress.

Regarding the association between stress and low FE birds, other studies found that the mitochondria of low feed efficiency poultry produced more H_2O_2, which is associated to higher protein oxidation and lower activity of the complexes of the electron transport chain (Bottje et al., 2006). Those authors suggest that this higher level of oxidized proteins may contribute to the low feed efficiency phenotype due to an increase in cell energy requirements to repair those proteins, as well as because the function of the damaged proteins may be impaired or reduced, and higher H_2O_2 production in poultry with low feed efficiency followed by protein damage may also impair the mitochondrial genes activity.

The novelty of our work is the fact that we submitted high and low FE quails to cold and heat stress. We had this goal since in the literature there are several studies that show the environmental temperature effect on the birds' metabolism and even on the feed efficiency phenotype (Hangalapura et al., 2004). These studies show that changes in environmental temperature above or below the thermal comfort zone stimulate thermal-regulation physiological mechanisms that may affect animal performance at a higher or lesser degree.

Stress is known to be related to neuro-chemical and hormonal changes including alterations in adrenal and thyroid hormone levels (Hangalapura, 2006). In birds, the thyroid hormones are essential for animals normal growth, since the thyrotropic axis has profound effects on development that are closely connected to somatotropic axis. The predominant form of thyroid hormone secreted by the thyroid gland is thyroxine (T4); Triiodothyronine (T3) is an active form and is derived from hepatic monodeiodination of T4, while both T4 and T3 can be catabolized to a metabolically inactive reverse (rT3) and T2 (Kim, 2010).

Several studies show that environmental temperature can affect thyroid hormones circulating levels. The results of these studies show that heat stress generally decreases (Melesse et al., 2011; Willemsen et al., 2011), and cold stress increases the

concentration of these hormones (Venditi et al., 2010). Thyroid hormones are also known to act on birds thermogenesis. When animals are exposed to high temperatures, T4 is inactivated to rT3, while if the birds are exposed to cold stress, T4 is converted to T3, which stimulates an increase in metabolic activity (Melesse et al., 2011).

In addition to their many functions in growth, thyroid hormones have also been related to participate in the control of ANT transcription. In mice the ANT transcription regulation occurs through of a positive promoter element muscle-specific called OXBOX- it is involved in the induction coordinate of this gene in muscle; and another element, the REBOX that overlaps the promoter OXBOX. The binding of factors to the element REBOX is regulated by environmental factors, including T3 and T4 (Portman, 2002; Chung et al., 1992).

We observed that the ANT mRNA expression was significantly lower in the liver of animals exposed to heat, and that in the muscle expression of this gene occurred differentially among the three treatments, we observed greater ANT mRNA expression in quails subjected to cold stress, and lower expression in animals exposed to heat stress. Because the thyroid hormones are needed to ANT transcription control, these results of expression may be due to the already mentioned fact that higher temperatures reduce T3 and T4 levels and that lower temperatures are known to increase levels of such hormones. Thus, cold stress could have increased thyroid hormones levels, and they, in turn, contributed to greater ANT mRNA expression in cold stressed birds.

The environmental temperature is an important determinant of respiratory activity in different tissues and it has been shown to increase respiratory activity in animals subjected to low temperatures (Martin et al., 1993). Regarding to temperature, in this work, we did not observe differences in COX III mRNA expression between animals of thermal comfort and heat stress, though the highest expression was observed in liver and muscle of cold stressed animals. A result that should be highlighted, because the great importance of COX III gene for the ATP production efficiency, is that there was an interaction effect between feed efficiency and environment on the COX III mRNA expression in muscle, with the highest expression in high FE and subjected to cold stressed animals. Greater cytochrome c oxidase activity has been related to high FE birds (Iqbal et al., 2005) and to animals subjected to low temperatures (Martin et al., 1993). There are also studies in the literature showing the negative heat stress effects on cytochrome c oxidase activity (Ando et al., 1997), and studies suggesting that heat shock protein has the ability to protect the respiratory chain complexes in stressful

situations, therefore contributing to the mitochondrial activity maintenance (Vogt et al., 2011; Chen et al., 2004).

The obtained results suggest a possible correlation among genes involved in energy production and feed efficiency phenotype and that the differences observed on weight gain between high and low feed-efficiency are partially explained by the differences on expression of important genes that activates different metabolic mechanisms. It was also observed that the ANT gene mRNA expression was significantly lower on heat exposed animals and higher on the ones exposed to cold. These results in gene expression might be due the fact that lower temperatures increase T3 and T4 levels, and these hormones contribute to a higher ANT mRNA expression. Regarding environmental temperature, we observed a higher mRNA expression of ANT and COX III both on liver and muscle of cold-stressed animals.

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V. THE EFFECT OF AIR TEMPERATURE ON GHR, IGF-I, ANT, UCP AND COXIII MRNA EXPRESSION IN THE LIVER AND MUSCLE OF HIGH AND LOW FEED EFFICIENCY MALE MEAT QUAILS

(Journal: Journal of Animal Breeding and Genetics)

Summary: In this study, we have analyzed insulin-like growth factor I (IGF-I), growth hormone receptor (GHR), uncoupling protein (UCP), adenine nucleotide translocase (ANT) and cytochrome c oxidase - subunit III (COXIII) mRNA expression in the muscle and liver of high-(0.25 g/g) and low-(0.17 g/g) feed-efficiency (FE) meat quails at three different air temperatures: comfortable (25ºC), heat stress (38ºC) for 12 hours and cold stress (10ºC) for 12 hours. IGF-I mRNA expression was higher in the livers of high-FE quails than in the livers of low-FE quails under both comfortable and cold stress conditions. In the muscle, regardless of the environment, high-FE birds showed higher IGF-I mRNA expression. High-FE birds also showed higher GHR mRNA expression under comfortable conditions. Regarding the environment, higher expression was observed in birds at comfortable conditions, and lower expression in birds under heat stress. UCP mRNA expression in the liver was lower in high-FE birds and higher under heat stress compared with the other conditions. Comparisons of the low and high-FE birds revealed higher levels of UCP mRNA in low-FE birds when housed in comfortable conditions. Low and high-FE birds showed greater ANT mRNA expression in the muscle under cold stress. Comparisons of the low and high-FE birds revealed higher levels of this gene in the liver in high-FE birds when housed in comfortable conditions and when the quails were submitted to heat stress. Greater mRNA COX III expressions were observed in the liver and muscle of quails under comfortable conditions. Our results suggest that air temperature affects the expression of genes related to growth and mitochondrial energy production, and quails with different feed efficiencies respond differently to environmental stimuli.

Keywords: ATP, cold stress, feed efficiency, GHR, heat stress, IGF-I, UCP

Introduction

The scientific research development regarding nutrition and genetics improved the poultry feeding index and reduced production costs, thus enabling increased productivity. Expenses with food account for about 50-70% of total production costs in livestock, so, feed efficiency becomes an important aspect of great interest in recent researches (Lassiter *et al*. 2006).

The efficiency in converting food into body mass is related to several factors. Among these, it is the animal capacity to deposit muscle and efficiency in the energy production by mitochondria. Studies have shown that birds with lower ATP production due to lower efficiency of mitochondria to produce ATP from substrates have worse feed efficiency or feed conversion (Bottje & Carstens 2009); and the muscle deposition occurs by the balance between protein synthesis and degradation which is regulated by somatotropic action.

The efficiency in energy production is governed not just by perfect coordination between the respiratory chain complexes, but also depends on a powerful antioxidant system that protects the mitochondria from generated damaging during the ATP production; and the muscle mass deposition is product of protein synthesis, promoted in part by hormones related to growth.

Bird performance is also affected by environmental conditions. The production can be impaired due to high temperatures, which trigger heat stress to the animals. Heat stress has been associated with metabolic changes involving oxidative stress. According to Yang *et al*. (2010), birds subjected to stress by high temperatures may have reduced activity of the mitochondrial respiratory chain followed by increased production of reactive oxygen species (ROS).

Similar to high temperatures, animals exposed to low temperatures undergo cardiovascular system changes in order to meet their increased energy requirements (Blahová *et al*. 2007). The hormone T3 appears to be involved in regulating the growth rate at low air temperatures. The circulating level of T3 has been negatively correlated with temperature and positively correlated with feed ingestion in chicken (Yahav 2000).

In this study, we hypothesized that feed efficiency could be related to the expression of genes from somatotropic axis, and mitochondrial genes involved in energy production, and that the environment to which the birds are exposed may influence the expression of such genes and thus contribute for the feed efficiency phenotype. This way it was done a trial dividing the animals into high feed efficiency

(FE) and low feed efficiency (FE) groups, and maintaining these quails under comfort, heat or cold stress to evaluated changes in IGF-I, GHR, ANT, UCP and COX III mRNA expression in liver and muscle tissues.

Materials and Methods

The experimental procedure was approved by the Brazilian Animal Ethics Committee and the experiment was conducted at the Iguatemi Experimental Farm at the State University of Maringá. First, 400 male meat quails (*Coturnix coturnix coturnix*), born from the same incubation, were conventionally raised for 28 days under the same experimental conditions. At this time, the birds were transferred to individual cages and underwent an adaptive period for seven days. Feed efficiency was calculated as the increase in body weight relative to feed intake from 35 to 42 days of age. Feed consumption and weight gain during the test period were measured individually. During this time, the birds were kept at a comfortable temperature (25 ± 0.9°C with 60 ± 1.2% relative humidity (RH)). The animals had free access to food and water throughout the experiment. The feed was formulated for the two phases of the birds' lives according to Rostagno *et al*. (2011). Starter feed was provided during the first 14 days of life, and growth feed was provided from 15 days of age. At 42 days of age, the animals were separated into two groups: the 36 birds with the highest FE (high-FE) and the 36 birds with the lowest FE values (low-FE) (Table 1). These groups were then divided into three environmental conditions: comfortable (25ºC, according to Pinto *et al*. 2003), heat stress (38ºC) for 12 hours and cold stress (10ºC) for 12 hours, with 12 animals in each group.

After the stress period, the animals were euthanized by cervical dislocation, and tissue from the breast muscle (*pectoralis superficialis*) and liver were collected and stored in RNA Holder® (BioAgency Biotecnologia, Brazil) at -20ºC until RNA extraction. Animals in comfortable conditions were sacrificed immediately after the groups were separated. Only 12 (6 high-FE and 6 low-FE) of the 24 animals submitted to each experimental condition were used for gene-expression analysis.

Total RNA was extracted using Trizol® (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions (1 mL per 100 mg of tissue). All the materials used had been previously treated with the RNase inhibitor RNase AWAY® (Invitrogen, Carlsbad, CA, USA). The tissue and Trizol mixture were triturated with a Polytron electric homogenizer until completely dissociated. Next, 200 µL of chloroform

was added to the sample, and the mixture was manually homogenized for 1 minute. The samples were then centrifuged for 15 minutes at 12,000 rpm and 4ºC. The aqueous phase was collected and transferred to a clean tube containing 500 µL of isopropanol per tube and again homogenized and centrifuged for 15 minutes at 12,000 rpm and 4ºC. The supernatant was discarded, and the precipitate was washed in 1 mL of 75% ethanol. The material was once again centrifuged at 12,000 rpm for 5 minutes, and the supernatant was discarded. The pellet was dried for 15 minutes and re-suspended in ultrapure RNase-free water.

The total RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. RNA integrity was analyzed using a 1% agarose gel stained with 10% ethidium bromide and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions to remove possible genomic DNA contamination.

A SuperScript^{TM} III First-Strand Synthesis Super Mix (Invitrogen Corporation, Brazil) kit was used for cDNA synthesis according to the manufacturer's instructions. For this reaction, 6 µL of total RNA, 1 µL of oligo dT (50 µM oligo $(dT)_{20}$) and 1 µL of annealing buffer were added to a sterile RNA-free tube. The reaction was then incubated for 5 minutes at 65ºC and placed on ice for 1 minute. Subsequently, 10 µL of 2× First-Strand Reaction Mix and 2 µL of solution containing SuperScript III reverse transcriptase enzyme and RNase inhibitor were added to the tubes. The solution was incubated for 50 minutes at 50ºC for the synthesis of complementary DNA. Next, the reaction was incubated for 5 minutes at 85ºC and immediately placed on ice. The samples were stored at -20ºC until they were used.

Real-time PCR reactions were performed using the fluorescent dye SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, USA) in a StepOnePlus v.2.2 PCR machine (Applied Biosystems, Carlsbad, CA, USA). All of the reactions were analyzed under the same conditions and normalized to the ROX Reference Dye (Invitrogen, Carlsbad, CA, USA) to correct for fluctuations in the readings due to evaporation during the reaction.

The primers used in the GHR and IGF-I amplification reactions were designed based on the gene sequences deposited at www.ncbi.nlm.nih.gov (accession numbers NM001001293.1 and FJ977570.1, respectively) using the website www.idtdna.com. The ANT, UCP and COX III primers were described by Ojano-Dirain *et al*. (2007) (Table 1). Two endogenous controls, ß-actin and GAPDH were tested and ß-actin

(accession number L08165) was selected because its amplification was shown to be more efficient. All of the analyses were performed in duplicate, each in a volume of 25 µL.

Table 1. qRT-PCR primers

Gene	Amplicon (bp)	Annealing Temperature (ºC)	Primer sequence (5'-3')
GHR	145	60ºC	AACACAGATACCCAACAGCC
			AGAAGTCAGTGTTTGTCAGGG
IGF-I	140	60ºC	CACCTAAATCTGCACGCT
			CTTGTGGATGGCATGATCT
ANT	67	60ºC	TGTGGCTGGTGTGGTTTCCTA
			GCGTCCTGACTGCATCATCA
UCP	41	60ºC	GCAGCGGCAGATGAGCTT
			AGAGCTGCTTCACAGAGTCGTAGA
COX III	71	60ºC	AGGATTCTATTTCACAGCCCTACAAG
			AGACGCTGTCAGCGATTGAGA
ß-actin	136	60ºC	ACCCCAAAGCCAACAGA
			CCAGAGTCCATCACAATACC

The experimental design was a completely randomized factorial set up with two environments (comfortable and heat stress) and two feed efficiencies (high and low). Data were analyzed using GLM procedures of the SAS statistical package (SAS Inst. Inc., Cary, NC). The Univariate procedure was applied to verify the normality of gene expression residues (expressed as $2^{-\Delta CT}$) and production data. All evaluated genes were log-transformed [ln(x+1)] (Voge *et al*. 2004) because they did not comply with normality assumptions. Means were compared by Tukey test ($P<0.05$). The results are shown as averages with standard deviations.

Results

After the period of individual feed intake and weight gain evaluation we can separate the animals into two homogeneous groups, one group was considered of high feed efficiency (High-FE) and the other of low feed efficiency (Low-FE). High-FE birds had a higher final weight, increased weight gain and a better feed conversion ratio than quails with low-FE (Table 2).

Table 2. Performance data for the animals separated into high- and low-FE groups

	IW (g)	FW (g)	WG (g)	FI (g)	FCR (g/g)	FE (g/g)
High-FE	216.00±30.2	257.11±22.3a	41.11±5.8a	168.12±20.2	3.85±0.7b	0.25±0.01a
Low-FE	206.44±28.9	233.44±20.9b	27.04±3.7b	165.84±17.7	6.12±0.9[a]	0.17±0.01b

IW: initial weight, FW: final weight, WG: weight gain, FI: feed intake, FCR: feed conversion ratio, FE: feed efficiency.
Lowercase letters indicate a significant difference by the Tukey test ($P<0.05$).

The gene expression results for IGF-I and GHR in the liver and muscle of the high and low-FE birds are shown in Table 3.

When we evaluated the IGF-I mRNA expression in the liver, we observed that high-FE birds in comfortable and cold conditions showed higher expression than low-FE birds. This expression pattern was maintained in muscle: regardless of the environment, high-FE birds showed higher IGF-I mRNA expression.

Regarding the environment, low and high-FE birds in comfortable conditions showed higher IGF-I mRNA expression in liver, compared to birds that were exposed to cold and heat stress. In the muscle, lower expression was observed in high-FE birds under cold stress, compared to those birds housed in comfortable, or under heat stress.
In the liver, we can observe that feed efficiency had effect only on the mRNA GHR expression in thermal comfort birds: High-FE animals showed higher expression than low-FE animals. In the muscle, in comfortable conditions, high-FE birds showed higher GHR mRNA expression than low-FE birds; and under heat stress, low-FE birds had higher expression than high-FE birds.

Table 3- IGF-I and GHR mRNA expression in the liver and muscle of high- and low-FE quails under three environmental conditions

	Liver			
	IGF-I		GHR	
ENV	High-FE	Low-FE	High-FE	Low-FE
Comfortable	0.5322aA	0.2562bA	0.3132aA	0.2197bA
Cold	0.2435aAB	0.1353bB	0.2264aAB	0.2299aA
Hot	0.1197aB	0.1520aB	0.1791aB	0.1798aB
CV (%)	27.8		25.1	
P value	<.0001		0.0027	
	Muscle			
	IGF-I		GHR	
ENV	High-FE	Low-FE	High-FE	Low-FE
Comfortable	0.3899aA	0.0965bA	1.411aA	0.0934bB
Cold	0.2021aB	0.0516bB	0.6004aB	0.7447aA
Hot	0.4095aA	0.072bAB	0.2946bB	0.5257aA
CV (%)	32.8		25.9	
P value	<.0001		<.0001	

Identical lowercase letters in the same row indicate no significant difference by the Tukey test (P<0.05).
Identical uppercase letters in the same column indicate no significant difference by the Tukey test (P<0.05).

The gene expression results for UCP, ANT and COX III in the liver and muscle of the high and low-FE birds are shown in Table 4.

Regarding feed efficiency, UCP mRNA expression in the liver was higher in low-FE birds in comfortable conditions than in high-FE birds. High-FE birds had significantly lower UCP mRNA expression under heat stress, compared to the other temperatures. No significant difference in expression of this gene between the cold and comfortable conditions was observed for low-FE birds; however, low-FE birds exposed to the high temperature had significantly lower UCP mRNA expression.

In the muscle, higher expression levels for this gene were observed in high-FE birds under cold and heat stress; and in low-FE birds under comfortable conditions. Comparisons of the low and high-FE birds revealed higher levels of UCP mRNA in low-FE birds when housed in comfortable conditions.

Regarding ANT mRNA expression in the liver, comparisons of low and high-FE birds revealed higher levels of this gene in high-FE birds when housed in comfortable conditions and when the quails were submitted to heat stress; however, in the muscle, higher levels of this gene were observed in low-FE birds.

About the environment, lower mRNA ANT level in the liver was observed in high-FE quails under cold stress. Unlike liver, in the muscle, low and high-FE birds showed greater expression of this gene under cold stress.

Comparing the low and high-FE birds in the liver we can observe higher levels of mRNA COX III in low-FE birds under cold stress, and greater levels in high-FE birds under heat stress; and in the muscle we can observe higher levels of mRNA COX III in high-FE birds under comfortable conditions. Regarding environment, greater expressions were observed in the liver and muscle of quails under comfortable conditions.

Table 4. Mitochondrial genes expression in the liver and muscle of high and low-FE quails under three environmental conditions

	Liver					
	UCP		ANT		COX III	
ENV	High-FE	Low-FE	High-FE	Low-FE	High-FE	Low-FE
Comfortable	0.00029bA	0.00147aA	6.180aA	5.782bA	0.0075aA	0.00872aA
Cold	0.0020aA	0.00356aA	5.206aB	5.350aA	0.0013bB	0.0040aB
Hot	0.00046aB	0.00051aB	6.176aA	5.258bA	0.00677aA	0.0039bB
CV (%)	41.5		8.5		25.8	
P value	<.0001		0.0160		<.0001	
	Muscle					
	UCP		ANT		COX III	
ENV	High-FE	Low-FE	High-FE	Low-FE	High-FE	Low-FE
Comfortable	0.1209bB	0.3503aB	6.078bB	7.89aAB	0.137aA	0.0496bA
Cold	0.4624aA	0.5804aA	8.646aA	8.332aA	0.1322aA	0.1040aA
Hot	0.4200aA	0.4187aA	5.030aB	5.236aB	0.0680aB	0.0768aA
CV (%)	20.3		7.3		28.2	
P value	<.0001		0.0196		0.0040	

Identical lowercase letters in the same row indicate no significant difference by the Tukey test (P<0.05).
Identical uppercase letters in the same column indicate no significant difference by the Tukey test (P<0.05).

Discussion

The endocrine mechanisms that control birds´ growth are not as well understood as in the case of mammals. The relationship between GH, GHR and IGF-I in birds, and other hormones like T3 and T4, need further studies, especially in its molecular basis, at levels not only translational but also transcriptional.

The IGF-I function in birds is similar to mammals function (McMurtry 1998) affecting the organism growth and development. Studies have been conducted to evaluate the influence of energy and protein nutrients suppliers in plasma concentration

of this hormone (Carew et al. 2003), however few studies have been conducted to evaluate the relationship between the use of these nutrients by the animal when these are subjected to heat or cold stress conditions.

The action of GH on IGF-I is mediated by GH receptor, the GHR stimulates the IGF-I synthesis and release. Thus, it is expected a coordinated relationship between the IGF-I and GHR mRNA expression. In dwarf birds that do not have GHR, the growth is decreased and this reduction seems to be associated with lower concentrations of circulating IGF-I (Hybrechts et al. 1985).

The interaction of GH with its receptor, and subsequent increased IGF-I production is a critical step for somatotropic activation (Kim 2010). In mammals, GH stimulates growth, milk production and inhibits fat deposition (Etherton ε Bauman 1998). The plasma IGF-I concentration and IGF-I mRNA expression in liver are positively correlated with weight gain, feed efficiency and protein synthesis rate (Tesseraud et al. 2000). In our results, it has been observed that high-FE quails in the comfortable conditions showed higher IGF-I mRNA in both liver and muscle.

Although the main site of IGF-I expression is the liver, high-FE birds under heat stress, showed greater values of expression in the muscle. High levels of IGF-I mRNA expression in extrahepatic tissues occur in animals under stress (Katsumata et al. 2002); stress can alter food intake, thus it can promote a energy and/or protein restriction which could reduce plasma IGF-I levels, encouraging increased IGF-I mRNA expression in other tissues. This reduction in plasma level is associated, probably, with the uncoupling of GH and its receptor (Scanes et al. 2009).

Under heat stress conditions, changes in performance and carcass composition are expected. Chronic stress alters the weight gain probably due to reduced feed intake and increased water consumption, and due to others biological mechanisms. High- FE quails under heat stress showed lower GHR mRNA expression, regardless of tissue. It has been shown that acute heat stress affects protein synthesis and changes in gene expression pattern caused by stress can alter the protein synthesis ability (Jacob 1995). However, heat stress for longer periods can result in some degree of thermotolerance and so it can affect differently the proteins synthesis and degradation. High temperatures can reduce the T3 concentration and increase the plasma corticosterone concentration, factors known to reduce protein deposition through protein turnover in birds (Yunianto et al. 1997).

Regarding the mitochondrial genes expression, differences in poultry feed efficiency have been associated with the ability of mitochondria to generate energy with minimum production of ROS. Among the protein from complex mitochondrial, the ANT, the UCP and COXIII have received special attention (Ojano-Dirain et al. 2007).

The expression pattern of the genes of these proteins apparently affects the ability of birds to better convert dietary nutrients into muscle. In our study, high-FE quails showed higher mRNA ANT expression than low-FE quails. Increased expression of ANT has also been observed in high-FE chickens muscle (Ojano-Dirain et al. 2007). In addition to the feed efficiency, ANT expression was also influenced by the environment. Our results in the muscle showed higher expression in birds under cold stress, and lower expression in birds under heat stress.

Stress is known to be related to neuro-chemical and hormonal changes including alterations in adrenal and thyroid hormone levels (Hangalapura 2006). Several studies show that environmental temperature can affect thyroid hormones circulating levels. The results of these studies show that heat stress generally decreases (Melesse et al. 2011; Willemsen et al. 2011), and cold stress increases the concentration of these hormones (Venditi et al. 2010). Thyroid hormones are also known to act on birds´ thermogenesis. When animals are exposed to high temperatures, T4 is inactivated to rT3, while if the birds are exposed to cold stress, T4 is converted to T3, which stimulates an increase in metabolic activity (Melesse et al. 2011).

In addition to their many functions in growth, thyroid hormones have also been related to participate in the control of ANT transcription. Because the thyroid hormones are needed to ANT transcription control, our results of mRNA ANT expression may be due to the already mentioned fact that higher temperatures reduce T3 and T4 levels and that lower temperatures are known to increase levels of such hormones. Thus, cold stress could have increased thyroid hormones levels, and they, in turn, contributed to greater ANT mRNA expression in cold stress birds.

The uncoupling proteins are related to the thermogenesis in mammals. The UCP is located in the inner mitochondrial membrane and is part of the anion carrier proteins family. The UCP dissipates the proton-motive force, uncoupling mitochondrial respiration from ATP synthesis; thereby it possibly increases the heat production and reduces the ATP production. In our study, we observed that high-FE birds under comfortable conditions had lower UCP mRNA expression in both tissues analyzed. This finding suggests that, consistent with a previous report (Dridi et al. 2004), UCP may be

involved in animal performance by increasing energy dissipation through mitochondrial oxidation.

Several isoforms have been described and in many tissues it is already found its expression. Birds UCP is homologous to mammals UCP 1, and it was named avUCP. Its expression is predominantly found in skeletal muscle (Raimbault et al. 2001). Our results confirm this statement: the expression was about 100 times higher in the muscle than in the liver.

Uncoupling proteins (UCPs) have been described as elements that are capable of decreasing ROS production, as they contribute to the depolarization of the internal mitochondrial membrane (Sack 2006). Studies have shown that UCP-encoding mRNA expression may vary with age (Gasparino et al. 2012) and environmental conditions (Del Vesco ε Gasparino, 2012), among other factors. The same studies suggest that decreased expression levels of UCP-encoding mRNA are associated with increased ROS production; in 28 days old quails, the expression of UCP-encoding mRNA is decreased in comparison to seven days old birds, and the expression was also decreased in meat quails that were exposed to acute heat stress.

Several studies have shown that UCP mRNA expression changes as a function of air temperature (Dridi et al. 2008). We observed that in the liver high- and low-FE birds had significantly lower UCP mRNA expression under heat stress. Again this result may be due to reduced T3 levels that occur in animals under heat stress, since T3 appears to be necessary for maximum UCP mRNA expression, and it is known to promote specific mRNA accumulation through transcriptional and post-transcriptional mechanisms, including the stabilization of precursor and mature mRNA (Rehnmark *et al.* 1992). Rey *et al.* (2010) suggested that UPC mRNA expression is mainly controlled by the status of the thyroid.

Different tissues respond differently to oxidative and environmental stress, thus the differences in the mRNA expression levels of mitochondrial genes in liver and muscle of quails subjected to the cold and heat stress are expected. The COXIII is responsible for transporting electrons to the complex IV of the respiratory chain, a fact that makes it essential in energy generation. We observe in both, liver and the muscle, higher COX III values for high-FE birds in almost all environments. The COX III expression level was also higher in high-FE birds in the results of Ojano- Dirain *et al*. (2007). Animals that have lower feed efficiency have been associated with lower efficiency in the ATP production. The reduction in mitochondrial energy production in

low-FE birds may be associated with oxidative damage produced by the increased presence of ROS, such as H_2O_2 and O_{2-}.

Conclusion

The obtained results suggest that the expression of somatropic axis genes and genes involved in mitochondria energy production is correlated with the environmental temperature in which the animals were housed. Our results demonstrate that in comfortable conditions high feed-efficiency animals show higher IGF-I mRNA expression both on liver and muscle tissues and, when submitted to heat-stress, lower GHR mRNA expression was observed.

Regarding mitochondrial genes, it was possible to observe a higher ANT mRNA expression on high feed-efficiency animals, and, at the muscle tissue, a higher expression was observed in cold-stressed animals. Another important result is that the UCP mRNA expression was lower on high feed-efficiency animals. This result suggests that UCP might be related to animal performance through the increase of energy dissipation by mitochondrial oxidation.

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VI. THE EFFECT OF HEAT STRESS ON GHR, IGF-I, ANT, UCP AND COXIII MRNA EXPRESSION IN THE LIVER AND MUSCLE OF HIGH AND LOW FEED EFFICIENCY JAPANESE FEMALE MEAT QUAIL

(Journal: Livestock Science)

Abstract: In this study, it was done a trial dividing the animals into high feed efficiency (FE) and low feed efficiency (FE) groups, and maintaining these quails under comfort and heat stress (38ºC for 12 hours) environment to evaluate changes in insulin-like growth factor I (IGF-I), growth hormone receptor (GHR), adenine nucleotide translocator (ANT), uncoupling protein (UCP) and cytochrome oxidase subunit III (COX III) gene expression in liver and muscle tissues. High-FE quails (0.28g/g) presented higher final body weight, higher weight gain, and better feed conversion ratio than low FE birds (0.18g/g). We observed that high-FE birds showed greater IGF-I expression in the liver and muscle, and greater GHR expression in the muscle. We observed environment effect only on GHR expression in the liver, which quails under comfortable conditions had greater expression than quails subjected to heat stress. We can observe a significant interaction effect between feed efficiency and environmental temperature on the ANT expression in the liver. The greatest ANT expression was observed for high FE-birds that remained under comfortable conditions. In the liver, UCP expression was statistically similar among the quails, independent of environment and efficiency. However, comparisons of the low- and high-FE birds, in the muscle, revealed higher levels of UCP in low-FE birds. The COX III expression in the liver suffered environmental temperature and feed efficiency effects. Higher expression was observed in animals that remained under comfortable conditions; and high FE-birds showed higher expression than low FE- birds. The obtained results suggest a possible correlation between genes from somatotropic axis and genes involved in energy

production by mitochondria with feed efficiency phenotype, and that environmental temperature could affect some of these genes.

Keywords: ATP, feed efficiency, growth, heat stress, mRNA

Introduction

The efficiency in converting food into body mass is related to several factors. Among these is the animal capacity to deposit muscle and efficiency in the energy production by mitochondria.

The muscle deposition occurs by the balance between protein synthesis and degradation which is regulated by somatotropic action. It is suggested that these two distinct pathways are products of the same biological route, and that the hormonal concentration is a factor that can determine which of these pathways will prevail (Sacheck et al., 2004). The presence of growth hormone (GH) in the body leads to the synthesis and release of insulin-like growth factor I (IGF-I) through GHR pathway. Specific GH receptor binding site is followed by conformational changes that stimulates various signalling pathways, including the route that involves Janus kinase 2 (JAK2), which leads to different cellular responses such as, synthesis and release of IGF-I (Kita et al., 2005). IGF-I plays an important role in poultry's growth metabolism, thus, lowest levels of IGF-I decrease growth rates (Scanes, 2009).

As important as the tissue deposition is the efficiency in energy production. Recent studies have shown that animals which convert food into body mass with more efficiency can display alterations in the electron transport chain gene expression, which can influence the use of nutrients and alter the body energy consumption. Such research suggests that animals with a higher consumption of residual animal feed suffer failure in the transport of electrons/protons, thus reducing the efficiency of ATP production by the mitochondria, which has an adverse effect on the feed conversion (Bottje and Carstens, 2009; Krueger et al., 2008; Iqbal et al., 2005; Johnson et al., 2003). Thus, the efficiency in energy production depends on the perfect coordination among the respiratory chain complexes. The uncoupling protein (UCP), the adenine nucleotide translocator (ANT) and cytochrome oxidase subunit III (COX III) are a few of many proteins present in the inner mitochondria membrane, and therefore related to efficiency in the ATP production.

The UCPs are transporters in the inner mitochondrial membrane which divert ATP synthesis energy to heat production. The UCP provides a new route for the protons, rather than through ATP synthase (Vidal-Puig, 2000). The ANT is responsible for exchange the ADP from the cytosol to the mitochondria matrix ATP across the inner mitochondrial membrane (Ojano-Dirain et al., 2007). Therefore, the ANT has the function of increasing the quantity of ADP to be transformed into ATP by means of ATP synthase. The mitochondrial function may be impaired by the incapacity of ADP/ATP exchange between the cytosol and the mitochondrial matrixne, thus there may be some connection between the ANT expression with the phenotypic expression of feed efficiency (Bottje et al., 2006). Another one, COX III is present in the electron transport chain and related to the oxidative phosphorylation efficiency. COX III is a subunit of protein complex IV of the mitochondria, responsible for pumping protons and transporting electrons. Ojano-Dirain et al. (2007) suggest that because these proteins are involved in energy production, there might be some connection between the expression of these genes and the feed efficiency phenotype.

The literature describes various factors influencing the expression of genes related to growth and mitochondrial genes, including diet (Gasparino et al., 2012; Katsumata et al., 2002), tissue type and developmental stage (Berishvili et al., 2006), breed (Beccavin et al., 2001), and environmetal temperatures (Toyomizu et al., 2002).

Heat stress causes damage on birds´ performance and also on the parts yield.This can be explained by physiological changes in the birds' body (Geraert et al., 1996; Yunianto et al., 1997). These physiological changes might partly be caused by the oxidative stress that occurs in animals kept under heat stress (HS) environment. Birds that are subjected to high temperatures may display a reduction in the activity of the mitochondrial respiratory chain, which may induce a greater production of reactive oxygen species (ROS) (Yang et al., 2010), and thus, due to a vicious cycle, influence the mitochondrial (Tengan et al., 1998) and other genes expression.

In this study, we hypothesized that feed efficiency could be related to the expression of genes from somatotropic axis, and mitochondrial genes involved in energy production, and that the environment to which the birds are exposed may influence the expression of such genes and thus contribute for the feed efficiency phenotype. This way it was done a trial dividing the animals into high feed efficiency (FE) and low feed efficiency (FE) groups, and maintaining these quails under comfort

and heat stress environment to evaluate changes in IGF-I, GHR, ANT, UCP and COX III mRNA expression in liver and muscle tissues.

Materials and Methods

The experimental procedure was approved by the Brazilian Animal Ethics Committee and the experiment was conducted at the Iguatemi Experimental Farm at the State University of Maringá. First, 300 female meat quails (*Coturnix coturnix coturnix*), born from the same incubation, were conventionally raised for 28 days under the same experimental conditions. At this time, the birds were transferred to individual cages and underwent an adaptive period for seven days. Feed efficiency was calculated as the increase in body weight relative to feed intake from 35 to 42 days of age. Feed consumption and weight gain during the test period were measured individually. During this time, the birds were kept at a comfortable temperature (25 ± 0.9°C with 60 ± 1.2% relative humidity (RH)). The animals had free access to food and water throughout the experiment.

The feed was formulated for the two phases of the birds' lives according to Rostagno et al. (2011). Starter feed was provided during the first 14 days of life, and growth feed was provided from 15 days of age. At 42 days of age, the animals were separated into two groups: the 24 birds with the highest FE (high-FE) and the 24 birds with the lowest FE values (low-FE) (Table 1). These groups were then divided into two environmental conditions: comfortable (25ºC, according to Pinto et al., 2003) and heat stress (38ºC) for 24 hours, with 12 animals in each group.

After the stress period, the animals were euthanized by cervical dislocation, and tissue from the breast muscle (*pectoralis superficialis*) and liver were collected and stored in RNA Holder® (BioAgency Biotecnologia, Brazil) at -20ºC until RNA extraction. Animals in comfortable conditions were sacrificed immediately after the groups were separated.

Total RNA was extracted using Trizol® (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions (1 mL per 100 mg of tissue). All of the materials used had been previously treated with the RNase inhibitor RNase AWAY® (Invitrogen, Carlsbad, CA, USA). The tissue and Trizol mixture were triturated with a Polytron electric homogenizer until completely dissociated. Next, 200 µL of chloroform was added to the sample, and the mixture was manually homogenized for 1 minute. The samples were then centrifuged for 15 minutes at 12,000 rpm and 4ºC. The aqueous

phase was collected and transferred to a clean tube containing 500 µL of isopropanol per tube and again homogenized and centrifuged for 15 minutes at 12,000 rpm and 4ºC. The supernatant was discarded, and the precipitate was washed in 1 mL of 75% ethanol. The material was once again centrifuged at 12,000 rpm for 5 minutes, and the supernatant was discarded. The pellet was dried for 15 minutes and re-suspended in ultrapure RNase-free water.

The total RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. RNA integrity was analyzed using a 1% agarose gel stained with 10% ethidium bromide and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions to remove possible genomic DNA contamination.

A SuperScript^{TM} III First-Strand Synthesis Super Mix (Invitrogen Corporation, Brazil) kit was used for cDNA synthesis according to the manufacturer's instructions. For this reaction, 6 µL of total RNA, 1 µL of oligo dT (50 µM oligo $(dT)_{20}$) and 1 µL of annealing buffer were added to a sterile RNA-free tube. The reaction was then incubated for 5 minutes at 65ºC and placed on ice for 1 minute. Subsequently, 10 µL of 2× First-Strand Reaction Mix and 2 µL of solution containing SuperScript III reverse transcriptase enzyme and RNase inhibitor were added to the tubes. The solution was incubated for 50 minutes at 50ºC for the synthesis of complementary DNA. Next, the reaction was incubated for 5 minutes at 85ºC and immediately placed on ice. The samples were stored at -20ºC until they were used.

Real-time PCR reactions were performed using the fluorescent dye SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, USA) in a StepOnePlus v.2.2 PCR machine (Applied Biosystems, Carlsbad, CA, USA). All the reactions were analyzed under the same conditions and normalized to the ROX Reference Dye (Invitrogen, Carlsbad, CA, USA) to correct fluctuations in the readings due to evaporation during the reaction.

The primers used in the GHR and IGF-I amplification reactions were designed based on the gene sequences deposited at www.ncbi.nlm.nih.gov (accession numbers NM001001293.1 and FJ977570.1, respectively) using the website www.idtdna.com. The ANT, UCP and COX III primers were described by Ojano-Dirain et al. (2007) (Table 1). Two endogenous controls, ß-actin and GAPDH, were tested and ß-actin (accession number L08165) was selected because its amplification was shown to be more efficient. All the analyses were performed in duplicate, each in a volume of 25 µL.

Table 1: qRT-PCR primers

Gene	Amplicon (bp)	Annealing Temperature (ºC)	Primer sequence (5'-3')
GHR	145	60ºC	AACACAGATACCCAACAGCC AGAAGTCAGTGTTTGTCAGGG
IGF-I	140	60ºC	CACCTAAATCTGCACGCT CTTGTGGATGGCATGATCT
ANT	67	60ºC	TGTGGCTGGTGTGGTTTCCTA GCGTCCTGACTGCATCATCA
UCP	41	60ºC	GCAGCGGCAGATGAGCTT AGAGCTGCTTCACAGAGTCGTAGA
COX III	71	60ºC	AGGATTCTATTTCACAGCCCTACAAG AGACGCTGTCAGCGATTGAGA
ß-actin	136	60ºC	ACCCCAAAGCCAACAGA CCAGAGTCCATCACAATACC

The experimental design was a completely randomized factorial set up with two environments (comfortable and heat stress) and two feed efficiencies (high and low). Data were analyzed using the GLM procedures of the SAS statistical package (SAS Inst. Inc., Cary, NC). The Univariate procedure was applied to verify the normality of gene expression residues (expressed as $2^{-\Delta CT}$) and of production data. All evaluated genes were log-transformed [ln(x+1)] (Voge et al., 2004) because they did not comply with normality assumptions. Means were compared by Tukey test ($P<0.05$). The results are shown as averages with standard deviations.

Results

After the period of individual feed intake and weight gain evaluation we can separate the animals into two homogeneous groups, one group was considered of high feed efficiency (High FE) and the other of low feed efficiency (Low FE). At the beginning of the evaluation period of feed efficiency, the animals in both groups had statistically equal initial weight (233.00 g for animals of high FE, and 234.00 g for low FE animals). The animals of high FE had greater final weight, greater weight gain and better feed conversion, even consuming the same amount of feed that low FE animals (188.70 g for high FE, and 185.43 g for low FE) (Table 2).

Table 2. Performance data for the female japanese quails separated into high- and low-FE groups

	IW (g)	FW (g)	WG (g)	FI (g)	FCR (g/g)	FE (g/g)
High-FE	233.00±19.3	287.00±10.9[a]	54.00±6.4[a]	188.70±13.1	3.54±0.8[b]	0.28±0.02[a]
Low-FE	234.00±15.6	267.00±12.3[b]	33.00±4.2[b]	185.43±12.8	5.63±0.3[a]	0.18±0.01[b]

IW: initial weight, FW: final weight, WG: weight gain, FI: feed intake, FCR: feed conversion ratio, FE: feed efficiency.
[a,b]Lowercase letters indicate a significant difference by the Tukey test (P<0.05).

The gene expression results for IGF-I and GHR in the liver and muscle of the high- and low-FE birds in the two environments are shown in Table 3. Significant interaction between feed efficiency and environment was not observed.

Table 3. IGF-I and GHR mRNA expression in the liver and muscle of high- and low-FE female japanese quails under two environmental conditions

		Liver		Muscle	
		IGF-I	GHR	IGF-I	GHR
High FE	Comfortable	0.16±0.07	0.30±0.06	0.50±0.14	0.50±0.04
	Heat stress	0.15±0.04	0.11±0.01	0.29±0.11	0.58±0.23
Low FE	Comfortable	0.12±0.03	0.33±0.02	0.14±0.07	0.27±0.17
	Heat stress	0.11±0.03	0.10±0.04	0.17±0.08	0.25±0.14
Main Effects					
Efficiency	High	0.16±0.05 a	0.20±0.11	0.39±0.16 a	0.54±0.16 a
	Low	0.11±0.03 b	0.22±0.12	0.16±0.07 b	0.26±0.11 b
Environment temperature	Comfortable	0.14±0.05	0.31±0.04 a	0.32±0.20	0.39±0.22
	Heat stress	0.13±0.04	0.10±0.02 b	0.23±0.11	0.41±0.25
Probabilities					
	Efficiency	0.0368	0.3645	0.0006	0.0096
	Environment temperature	0.6663	<0.0001	0.1286	0.7705
	Interaction	0.9337	0.2715	0.0547	0.6145

Values are means with their standard deviations.
[a,b] Identical letters in the same column indicate no significant difference by the Tukey test (P<0.05).

Our analysis of gene expression in the liver and muscle showed that high-FE birds had higher IGF-I mRNA levels than low-FE birds. We observed no significant effect of the environment on the IGF-I mRNA expression in both tissues.

Regarding GHR gene expression in the liver, we observed higher expression in quails under comfortable conditions. In the muscle, no environment effect was observed on the GHR mRNA expression; however, high FE- birds showed greater expression than low FE-birds. The results of mitochondrial genes expression in the liver and muscle of the high- and low-FE birds in the two environments are shown in Table 4.

Table 4. Mitochondrial genes expression in the liver and muscle of high- and low-FE female japanese quails under two environmental conditions

		LIVER		
		ANT	UCP	COX III
High FE	Comfortable	4.25±0.16 a	0.00013±0.00009	0.0036±0.0003
	Heat stress	2.74±0.16 b	0.00012±0.00006	0.0023±0.0006
Low FE	Comfortable	3.04±0.91 b	0.00011±0.00002	0.0023±0.0009
	Heat stress	2.70±0.45 b	0.00011±0.00001	0.0014±0.0003
Main Effects				
Efficiency	High	3.49±0.80	0.00012±0.00008	0.0029±0.0008 a
	Low	2.87±0.71	0.00011±0.00002	0.0018±0.0008 b
Environment temperature	Comfortable	3.65±0.88	0.00012±0.00006	0.0029±0.0009 a
	Heat stress	2.72±0.32	0.00011±0.00004	0.0018±0.0006 b
Probabilities				
	Efficiency	0.0084	0.7207	0.0002
	Environment temperature	0.0003	0.8143	0.0002
	Interaction	0.0127	0.9543	0.3731
		MUSCLE		
		ANT	UCP	COX III
High FE	Comfortable	2.33±0.14	0.47±0.04	0.074±0.010
	Heat stress	2.25±0.17	0.46±0.08	0.071±0.023
Low FE	Comfortable	2.23±0.39	0.91±0.03	0.034±0.018
	Heat stress	2.30±0.23	0.14±0.05	0.032±0.012
Main Effects				
Efficiency	High	2.29±0.15	0.46±0.06 a	0.073±0.017 a
	Low	2.27±0.30	0.12±0.05 b	0.033±0.011 b
Environment temperature	Comfortable	2.28±0.28	0.30±0.19	0.054±0.029
	Heat stress	2.27±0.18	0.28±0.17	0.051±0.027
Probabilities				
	Efficiency	0.8183	<0.0001	0.0001
	Environment temperature	0.9464	0.3886	0.7433
	Interaction	0.4815	0.2074	0.9654

Values are means with their standard deviations. [a,b] Identical letters in the same column indicate no significant difference by the Tukey test ($P<0.05$).

We can observe a significant interaction effect between feed efficiency and environmental temperature on the ANT expression in the liver. The greatest ANT mRNA expression was observed for high FE-birds that remained under comfortable conditions. There was no difference in the expression among the other treatments. ANT expression in the muscle was not affected by any of the evaluated treatments.

In the liver, UCP mRNA expression was statistically similar among the quails, independent of the environment and efficiency. However, comparisons of the low- and high-FE birds, in the muscle, revealed higher levels of UCP mRNA in low-FE birds.

The COX III expression in the liver suffered environmental temperature and feed efficiency effects. Higher expression was observed in animals that remained under comfortable conditions; and high FE-birds showed higher expression than low FE-birds. Regarding expression of this gene in the muscle, only feed efficiency affected the COX III mRNA expression. Our analysis showed that high-FE birds had higher COX III mRNA expression than low-FE birds.

Discussion

After the period of individual feed efficiency evaluation we have been able to separate the animals into two fairly homogeneous groups considering feed efficiency, a group of high FE and another group of low FE. Our results showed that high-FE Japanese quails gained significantly more body weight, although their feed intake was similar to those presenting low-FE, and since all animals need to adapt their metabolism to physiological and environmental conditions, which are continuously changing, and therefore, metabolic responses coordinated by the expression of specific genes are required, we suggest the observed differences are partially explained by differences in the expression of important genes that activate difference metabolic mechanisms.

However, in animal production, performance (e.g., feed efficiency and reproduction efficiency) is expressed not only according to the animal's genetic background, but also as a function of the environment it is exposed to, and to the interaction between genetics and the environment. Thus, after separating the animals into high and low FE, we kept the quails in comfortable or heat stress temperature.

Regarding somatotropic axis genes expression, we observe that high FE birds showed greater IGF-I mRNA expression in the liver and muscle, and greater GHR mRNA expression in the muscle. We observed environment effect only on GHR expression in the liver, which quails under comfortable conditions had greater expression than quails subjected to heat stress (Table 3).

Studies showed that IGF-I has an important role in broiler growth rate, and that lower IGF-I levels determine slower body growth (Scanes, 2009). Higher protein synthesis and lower protein breakdown are observed in broilers with higher IGF-I blood levels, and consequent higher skeletal muscle deposition (Conlon and Kita, 2002). Supporting the hypothesis that GH effects on growth are mainly mediated by IGF-I (Vasilatos-Younken, 1999), studies published in literature have shown that lower growth rates in broilers may be associated with higher GH levels (Burke and Marks

1982, Goddard et al. 1988). This suggests that broiler growth rates are not directly explained by GH levels; rather, more information may be obtained by evaluating IGF-I (Beccavin et al., 2001). These authors observed that broilers selected for high growth rate presented higher IGF-I mRNA expression in the liver and higher IGF-I blood levels than those selected for low growth rate.

In the study of Tesseraud et al. (2000) with broilers selected for fast or slow growth rate, fast-growing broilers presented higher weight gain, relative Pectoralis major muscle weight (g/kg body weight), higher protein content in this muscle, and higher protein synthesis and deposition. The authors suggest that this higher protein deposition is in fact due to the lower protein breakdown in the muscles of these broilers. Fast-growing poultry also seem to present more and larger muscle fibers (Remignon et al., 1994), which may be due to the fact that satellite cells in these birds are more responsive to blood IGF-I, contributing for muscle fiber hypertrophy (Duclos et al., 1996).

We did not find any published studies on the GHR expression in birds subjected to heat stress, however, reduced protein synthesis capacity and rate, lower growth rate, and lower efficiency of protein deposition, as well as lower RNA levels have been observed in animals exposed to heat stress (Temim et al., 2000). Therefore, there may be some connection in the results found by these authors, with lower expression of GHR mRNA observed at high temperatures, since GHR is essential for IGF-I action.

Despite the ATP production has great influence on feed efficiency many other physiological systems are involved in the control of such feature. Metabolic pathways involved in protein deposition, and in the food intake control, among others, also play a key role on feed efficiency. Recent studies on muscle growth (Zheng et al., 2011; Tesseraud et al., 2007) and about the food intake control showed that the body has cellular/molecular mechanisms with a single signaling pathway that connects peripheral tissues, where energy is used or stored, with the central nervous system (CNS), which regulates energy acquisition by controlling the feed intake (Richards et al., 2010).

To better understand the cellular mechanisms that define the feed efficiency phenotype, Bottje and Kong (2012) conducted an experiment with broilers separated into groups of high and low FE in order to evaluate the overall relationship between feed efficiency and gene expression. The authors found 782 genes that were differentially expressed in high and low FE birds. The authors results suggest that high FE birds exhibited increased expression of genes associated with signal transduction

pathway, anabolic activities and activities of energy coordination, metabolic pathway that are favorable to the development and cell growth. Low FE animals on the other hand, showed greater expression of genes related to actin-myosin filaments and genes related or responsive to stress. Thus, the authors suggest that the low-FE phenotype could be the product of gene expression which is modulated by oxidative stress.

Regarding the association between stress and low FE birds, others studies found that the mitochondria of low feed efficiency poultry produced more H_2O_2, which is associated to higher protein oxidation and lower activity of the complexes of the electron transport chain (Bottje et al., 2006). Those authors suggest that this higher level of oxidized proteins may contribute to the low feed efficiency phenotype due to an increase in cell energy requirements to repair those proteins, as well as because the function of the damaged proteins may be impaired or reduced, and higher H_2O_2 production in poultry with low feed efficiency followed by protein damage may also impair the mitochondrial genes activity.

Literature reports also indicate that high-FE poultry presents higher electron transport couplings, and consequently, higher ATP synthesis capacity. This may be due to lower UCP mRNA and greater COX III mRNA expression, which is also observed in poultry with high feed efficiency (Ojano-Dirain et al., 2007; Raimbault et al., 2001).

In the present study, it was found that UCP mRNA expression was greater in the muscle of low FE qualis. This suggests that, consistent with literature (Dridi et al., 2004), UCP may affect animal performance by increasing energy dissipation by mitochondrial oxidation. Contrary to the situation in the literature - several studies showed that UCP mRNA expression may change as a function of environmental temperature (Dridi et al., 2008; Toyomizu et al., 2002) - we did not observe any environment effect on UCP expression.

Regarding COX III expression, greater cytochrome c oxidase activity has been related to high FE birds (Iqbal et al., 2005) and to animals subjected to lower temperatures (Martin et al., 1993). There are also studies in the literature showing the negative heat stress effects on cytochrome c oxidase activity (Ando et al., 1997), and studies suggesting that heat shock protein could protect the respiratory chain complexes in stressful situations, therefore contributing to the mitochondrial activity maintenance (Vogt et al., 2011; Chen et al., 2004).

A result that should be highlighted, because the great importance of ANT gene for the ATP production efficiency, is that there was an interaction effect between feed

efficiency and environment on the ANT mRNA expression in liver, with the highest expression in high FE-birds that remained under comfortable conditions. Several studies show that environmental temperature can affect thyroid hormones circulating levels. The results of these studies show that heat stress generally decreases (Melesse et al., 2011; Willemsen et al., 2011), and cold stress increases the concentration of these hormones (Venditi et al., 2010). Besides their many functions in growth, thyroid hormones have also been related to participate in the control of ANT transcription. About environment, we observed that the ANT mRNA expression was significantly lower in the liver of animals exposed to heat. Because the thyroid hormones are needed to ANT transcription control, these results of expression may be due to the already mentioned fact that higher temperatures reduce T3 and T4 levels and that lower temperatures are known to increase levels of such hormones. Thus, heat stress could have reduced thyroid hormones levels, and they, in turn, contributed to reduce ANT mRNA expression in heat stress birds.

Our results suggest that the environmental temperature influences on the expression of studied genes, once a higher gene expression was observed in comfortably housed animals. It was also shown that high feed-efficiency animals kept a higher expression pattern of such genes, suggesting that the differences observed between high and low feed-efficiency animals are, partially, explained by the differences of such genes expression.

Acknowledgments

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VII. CONCLUSÕES GERAIS

A metodologia aplicada permitiu eficiente na seleção dos dois grupos de codornas de diferentes eficiências alimentares, alta e baixa eficiência alimentar.

A exposição dos animais a diferentes temperaturas ambientais (conforto, calor e frio) influenciou na expressão de genes relacionados ao crescimento, GHR e IGF I, e a produção de energia, UCP, COX III e ANT, no fígado e no músculo.

Os resultados sugerem que a temperatura influencia na expressão dos genes já mencionados, uma vez que foram observadas taxas mais elevadas de expressão nos animais submetidos ao ambiente de conforto. Foi possivel evidenciar que animais de alta EA mantiveram padrão maior de expressão desses genes, sugerindo que as diferenças observadas entre animais de alta e baixa EA, são parcialmente explicadas pelas diferenças na expressão desses genes.

Foi possível observar que a expressão gênica, influencia no fenótipo para eficiência alimentar, sendo que animais com alta eficiência apresentaram maior expressão para os genes IGF-I e GHR, que são responsáveis pelo crescimento.

Para o gene UCP, houve maior expressão gênica para animais de baixa eficiência e expostos ao frio, indicando que este gene está ligado à termorregulação.

Os resultados das análises de expressão no músculo e fígado das codornas de corte são semelhantes aos encontrados para as de postura.

ANEXO I. NORMAS DA REVISTA LIVESTOCK SCIENCE

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ANEXO II. NORMAS DA REVISTA GENETICS AND MOLECULAR RESEARCH

GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

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General information of GMR style

ANEXO III: NORMAS DA REVISTA ANIMAL BREEDING AND GENETICS

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Books

Weir, B. S. (1996) *Genetic Data Analysis II*. Sinauer, Sunderland, MA, USA.

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