UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

MECANISMOS MOLECULARES RELACIONADOS À DEPOSIÇÃO PROTÉICA E AO ESTADO REDOX DE AVES ALIMENTADAS COM DL-METIONINA E SUBMETIDAS AO ESTRESSE TÉRMICO

Autor: Ana Paula Del Vesco
Orientador: Prof. Dra. Eliane Gasparino

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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

MARINGÁ
Estado do Paraná
junho - 2014

"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

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DEDICO ESTE TRABALHO

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BIOGRAFIA

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No dia 27 de junho de 2014, submeteu-se à banca examinadora para a defesa de tese apresentada, como parte das exigências para obtenção do título de doutor em Zootecnia.

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RESUMO

A taxa de crescimento é dependente, entre outros fatores, da deposição de massa muscular. Esta por sua vez, é dada pelo balanço entre a síntese e degradação protéica, coordenadas em parte pelos nutrientes da dieta, por hormônios ligados ao crescimento, e por complexas rotas metabólicas envolvidas na degradação muscular e lisossomal. O desempenho das aves também é afetado pelas condições ambientais a que estes animais são expostos. Sendo assim, este trabalho teve como principal objetivo avaliar o efeito do estresse térmico agudo e da suplementação de metionina sobre o desempenho, expressão gênica, e estado redox em aves: frangos e codornas. Para isto, frangos de corte de 1-21 e de 22-42 dias de idade foram divididos em três tratamentos referentes à suplementação de metionina; sem suplementação de metionina, nível recomendado de metionina (DL1), e suplementação em excesso de metionina (DL2). Os animais foram mantidos em conforto térmico ou expostos ao estresse térmico agudo (38˚C por 24 horas começando aos 20 ou aos 41 dias de idade, dependendo da fase experimental avaliada). Nestes experimentos, foram avaliados, entre outros fatores, o desempenho, alguns marcadores do estresse oxidativo, e a expressão dos genes: proteína desacopladora (UCP), betaina homocisteína metiltransferase (BHMT), cistationina β sintase (CBS), glutationa sintetase (GSS), glutationa peroxidase (GPx 7), fator de crescimento semelhante à insulina I (IGF-I), receptor do hormônio do crescimento (GHR), fosfatidilinositol 3-quinase, subunidade reguladora 1 (PI3KR1), atrogina 1, e catepsina L2 (CTSL2). Para a avaliação das codornas, estes animais foram divididos em dois tratamentos referentes à suplementação de metionina: sem suplementação de metionina, ou nível recomendado de metionina. Os animais foram mantidos em conforto térmico ou expostos ao estresse térmico agudo de 38˚C por 24 horas.

Observamos que o estresse térmico aumentou a temperatura corporal, e o consumo de água nos frangos de ambas as idades. O estresse térmico também reduziu consumo de ração e o ganho de peso dos animais. A suplementação de metionina, por sua vez, também reduziu consumo de ração, entretanto, aumentou o ganho de peso. Observamos que o estresse térmico diminuiu a atividade da enzima creatina quinase (CK), e os níveis de ácido úrico, e aumentou a atividade da enzima alanina aminotransferase (ALT), e a concentração de creatinina. A suplementação de metionina reduziu a atividade das enzimas aspartato aminotransferase (AST) e ALT, e aumentou a atividade da CK e a concentração de ácido úrico plasmático. Maior expressão dos genes GSS, CBS, GPX 7, atrogina-1, e CTSL2 foi observada em aves expostas ao estresse térmico. Já a suplementação de metionina foi responável por menor expressão dos genes CTSL2, atrogina-1, BHMT, e UCP, e maior expressão de IGF-I, GHR, GSS, CBS, e GPx 7. A expressão dos genes BHMT ($P<.0001$), CBS ($P<.0001$), GSS ($P=0.0036$), e GPx7 ($P=0.0375$) foi afetada pela interação ambiente x dieta. Maior expressão do gene BHMT foi observada em animais do conforto térmico alimentados com dieta SM. Aves do estresse, recebendo dietas DL1 e DL2 apresentaram maior expressão de CBS, GSS e GPx 7. No experimento com codornas, não foi avaliado a dieta DL2, entretanto os resultados de expressão dos genes IGF-I, GHR, e UCP, os resultados de desempenho, e dos parâmetros plasmáticos avaliados foram semelhantes aos observados nos experimentos com frangos. No experimento com codorna, também observamos maior produção significativa de peróxido de hidrogênio (H_2O_2) em animais que foram submetidos ao estresse térmico por 24 horas, e em animais recebendo dieta sem suplementação de metionina. Maior quantidade de glutationa (GSH) foi observada em animais recebendo dieta com suplementação de metionina e em animais que permaneceram em conforto térmico. Maior nível de substâncias reativas ao ácido tiobarbitúrico (TBARS) e maior atividade da enzima catalase foram observados em animais que foram submetidos a estresse térmico e alimentados com dieta sem inclusão de metionina. Maior atividade da glutationa peroxidase foi observada em animais submetidos ao estresse térmico consumindo dieta com suplementação de metionina. A análise dos nossos resultados de forma conjunta, leva à conclusão de que o estresse térmico aumenta a temperatura corporal, e induz o estresse oxidativo e maior proteólise; e que a suplementação de metionina pode atenuar os efeitos do estresse, contribuindo para maior expressão e atividade de elementos relacionados à atividade antioxidante, e pode estimular a deposição protéica, não apenas por garantir maior expressão de genes

relacionados à síntese, mas também, menor expressão dos genes relacionados à degradação.

Palavras-chave: antioxidante, deposição proteica, estresse térmico, estresse oxidativo, metionina, nutrigênomica

ABSTRACT

The growth rate is linked, among other factors, to muscle deposition. The muscle deposition is based on the balance between protein synthesis and degradation, which are partially coordinated by dietary nutrients, hormones related to growth, and complex metabolic pathways involved in muscle and lysosomal degradation. The bird's performance is also affected by environmental conditions to which these animals are exposed. Therefore, the main objective of this study was to evaluate the effect of acute heat stress and methionine supplementation on performance, gene expression, and the redox state in birds, broilers, and quail. Broilers from 1 to 21 days of age and 22 to 42 days of age were divided into three treatments regarding methionine supplementation: without methionine supplementation (MD), recommended level of methionine (DL1), and excess methionine supplementation (DL2). The animals were kept under thermal comfort or exposed to acute heat stress (38˚C for 24 hours, starting at 20 or 41 days of age, depending on the assessed experimental phase). In this experiment, we evaluated, among other parameters, animal performance, some markers of oxidative stress, and the gene expression of the following genes: uncoupling protein (UCP), betaine-homocysteine methyltransferase (BHMT), cystathionine β synthase (CBS), glutathione synthetase (GSS), glutathione peroxidase 7 (GPx 7), insulin-like growth factor 1(IGF-I), growth hormone receptor (GHR), phosphatidylinositol 3-kinase regulatory subunit 1 (PI3KR1), atrogin 1, and cathepsin L2 (CTSL2). We observed that heat stress increased the body temperature and the water intake in broilers at both ages. The heat stress also decreased the ration intake and the body weight gain in the animals. The methionine supplementation also reduced the ration intake, but it increased the body weight gain. We observed that heat stress decreased both the activity of the enzyme creatinine kinase

(CK) and the uric acid levels, and increased the activity of the enzyme alanine aminotransferase (ALT). The methionine supplementation decreased the activity of the enzymes aspartate aminotransferase (AST) and ALT, and increased the activity of CK and the uric acid plasmatic concentration. A greater expression of the genes GSS, CBS, GPX 7, atrogin 1, and CTSL2 were observed in birds exposed to heat stress. The methionine supplementation resulted in a lower gene expression of CTSL2, atrogin 1, BHMT, and UCP and also caused a greater gene expression of IGF-I, GHR, GSS, CBS, and GPx 7. The gene expression of BHMT ($P<.0001$), CBS ($P<.0001$), GSS ($P=0.0036$), and GPx 7 ($P=0.0375$) were affected by the interaction between ambience × diet. A greater BHMT gene expression was observed in animals kept under thermal comfort and fed with the WM diet. Birds kept under heat stress and fed the DL1 and DL2 diets had a greater CBS, GSS, and GPx7 gene expression. In the experiment with quail, we did not evaluate the DL2 diet; however, the results of IGF-I, GHR, and UCP gene expression, animal performance, and the other evaluated plasmatic parameters were similar to those observed in the broiler experiment. In the experiment with quail, we also observed a significantly greater H_2O_2 production by the animal kept under heat stress for 24 hours, and in animals receiving a diet without methionine supplementation. Higher glutathione (GSH) content was observed in animals that were fed a diet with methionine supplementation and in animals kept under thermal comfort. A greater TBARS level and activity of the catalase enzyme were found in animals kept under heat stress and fed a diet without methionine. A greater glutathione peroxidase activity was observed in animals kept under heat stress and fed a diet with methionine supplementation. All of the results obtained in this study suggest that under thermal comfort, methionine supplementation reduced the damage caused by ROS, which may be related to the increase in the expression and activity of antioxidants. Our results reveal that heat stress increases the body temperature, induces oxidative stress, and induces greater protein proteolysis; methionine supplementation can also attenuate the effects of stress, thereby contributing to a greater expression and activity of elements related to antioxidant activity. This may stimulate protein deposition, which not only ensures greater gene expression related to synthesis, but also ensures the increase in gene expression related to protein degradation.

Key words: antioxidant, heat stress, oxidative stress, methionine, nutrigenomics, protein deposition

I. INTRODUÇÃO

Apesar de grandes avanços, a avicultura mundial não parou de se capacitar nas últimas décadas. No Brasil, a organização do setor, aliada à qualidade do produto brasileiro, fez com que a carne de frango ganhasse espaço como escolha preferencial, e não apenas em substituição a outras carnes.

Para atender os objetivos de aumentar a produção de carne de forma mais rápida e mais eficiente, tornando o produto de melhor qualidade e capaz de atender as expectativas do mercado consumidor, pesquisas e novas tecnologias devem ser sempre implementadas. Algumas das abordagens tecnológicas dizem respeito à genética das aves, e elegem animais mais eficientes, com rápido crescimento, e alto rendimento de cortes nobres; e também à nutrição, que contribui para a expressão da genética (Calabotta, 2002).

A taxa de crescimento é dependente, entre outros fatores, da deposição de massa muscular. Esta por sua vez, é dada pelo balanço entre a síntese e degradação protéica, coordenadas em parte por hormônios ligados ao crescimento, e por complexas rotas envolvidas na degradação muscular e lisossomal.

O desempenho das aves é ainda 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). Este por sua vez, tem sido 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). Segundo estes autores, a

atividade de algumas enzimas envolvidas no sistema de defesa antioxidante também é alterada em função do estresse térmico.

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 devido a mudanças fisiológicas a nível celular / molecular.

Muitos são os estudos que avaliam o efeito do ambiente sobre a expressão de diversos genes de milhares de rotas metabólicas, entretanto, o mecanismo que atua no controle da expressão gênica, ainda não está totalmente esclarecido. Todos estes fatores tomados em conjunto demonstra como é complexo o metabolismo animal. Este trabalho trata de alguns genes envolvidos na deposição protéica: fator de crescimento semelhante a insulina I (IGF-I), receptor do hormônio do crescimento (GHR), fosfatidilinositol 3-quinase, subunidade reguladora 1 (PI3KR1), atrogina 1 e Catepsina L2 (CTSL2); avalia também a expressão da proteína desacopladora (UCP), gene envolvido na produção de ROS; a expressão de genes relacionados ao metabolismo da metionina: betaina homocisteína metiltransferase (BHMT), e cistationina β sintase (CBS); e de genes envolvidos na ação antioxidante da glutationa: glutationa sintetase (GSS), e glutationa peroxidase 7 (GPx 7).

1. Desempenho das aves: Nutrição e Deposição proteica

1.1. Metionina

A formulação das rações otimizando a utilização da proteína da dieta, aumenta a retenção protéica e diminui a excreção de nitrogênio. Desta forma, há o fornecimento exato, sem excesso ou falta de aminoácidos, que permite o perfeito metabolismo de mantença e produção (Araujo e Sobreira, 2008).

As proteínas são formadas por aminoácidos, sendo suas conformações e funções biológicas ditadas pelas sequências destes (Nelson e Cox, 2011). As proteínas da dieta são hidrolisadas no lúmen intestinal e nas células da mucosa do trato grastrointestinal, resultando em aminoácidos livres que são em grande parte transportados para o fígado, através do sangue portal. O organismo animal possui pouca capacidade de estocar proteínas, quando há consumo acima da necessidade, o que ocorre é uma maior formação de uréia ou ácido úrico, acompanhada pela conversão do esqueleto de carbono

a carboidratos, lipídeos e CO_2 para a geração de ATP, por outro lado, se a ingestão for menor que a necessidade do animal, haverá catabolismo das proteínas corpóreas (Reece, 2006).

A metionina é o primeiro aminoácido limitante para frangos de corte, devido à composição dos alimentos utilizados em suas dietas e em função da grande exigência deste aminoácido para a formação das penas. Além de ser utilizada para deposição de músculos e penas, a metionina desempenha importantes funções como doador de grupos metil (Simon, 1999), como participante da síntese de glutationa (Piovacari et al., 2008), como doador de enxofre e como participante na síntese da cisteína e da S-adenosilmetionina (SAM), sendo este composto responsável pelas metilações essenciais para a biossíntese de uma variedade de compostos celulares, como creatina, carnitina, fosfolipídeos e proteínas (Stipanuk, 2004).

Em função da exigência em metionina, a suplementação de fontes industriais na dieta de aves é importante para se alcançar desempenho adequado. Uma das fontes comerciais disponíveis em grande escala no mercado é a DL metionina (Marchizeli, 2009). A DL-metionina é uma substância em pó, composta apenas por monômeros de metionina (99%), encontrados em uma mistura racêmica entre isômeros D (dextrógiro) e L (levógiro) (Jansman et al., 2003).

1.2. Deposição protéica

1.2.1. Crescimento

O crescimento das aves é dado pela ação do eixo somatotrópico, 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 a insulina I), a presença de GH no organismo induz a síntese e a liberação deste hormônio (Becker et al., 2001).

O GH é produzido pelas células somatotróficas 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 de 620 aminoácidos, com um domínio transmembrana, um domínio extracelular, onde ocorre a

ligação do GH, e um domínio intracelular que está envolvido na sinalização do GH (Leung et al., 1987).

Para estimular a síntese de IGF-I, o GH provoca a dimerização do seu receptor, se ligando 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, pela iniciação 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 chamados de STATs. Neste processo, as STATs são fosforiladas, o que faz com que estas formem complexos com outras proteínas e se movam para o núcleo. Uma vez no núcleo as STATs se ligam a sequências regulatórias específicas para ativar ou inibir a transcrição de genes alvos, dessa forma a rota da JAK/STAT providencia um mecanismo direto para traduzir uma sinalização externa em uma resposta transcricional, como por exemplo a indução da síntese de mRNA IGF-I pela ação da ligação do GH com seu receptor (Kofoed et al., 2003).

O complexo pode ativar também as proteínas quinases MAPK, por intermédio do recrutamento da molécula adaptadora GrB2. As MAPKs fosforilam moléculas responsáveis pela transcrição no núcleo, agindo na síntese proteica (Guyoti, 2009). Outra rota bioquímica que pode ser ativada envolve as proteínas IRS-1 (receptor de insulina substrato 1), as quais quando fosforiladas servem como locais de ligação para uma variedade de moléculas sinalizadoras, envolvidas no metabolismo e crescimento (Carter-Su et al., 1996).

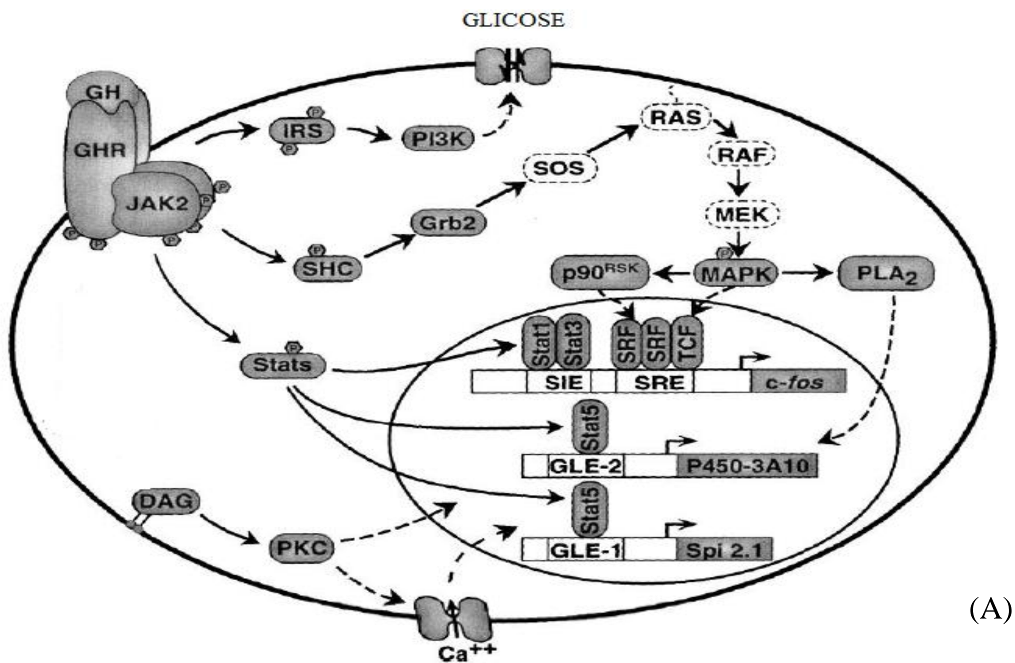
Kuhn et al. (2002) afirmam que níveis sanguíneos de GH são correlacionados inversamente com a expressão de GHR. Kim (2010) sugere que o GH inibi a expressão de GHR diretamente ou por intermédio da liberação de IGF-I, e estimula a formação do complexo GH-GRH pelas células.

O IGF-I é uma proteína de estrutura semelhante à insulina, de aproximadamente 7 kDA. É sintetizado principalmente no fígado, entretanto, sua produção ocorre também em outros tecidos, e independente do local de síntese, este hormônio é fundamental para o crescimento adequado. Como não há um local de armazenamento deste hormônio, a maioria do IGF-I está na circulação ligado a um complexo, formado por uma proteína ligadora de IGF-I (IGFBP) e por uma subunidade proteica ácido-lábil (ALS). A ligação

desta proteína ao complexo estende a meia vida deste por cerca de 15 horas (Kim, 2010).

Segundo Scanes (2009), o IGF-I possui um 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 protéica em aves com maior nível de IGF-I plasmático, o que resulta em maior deposição de músculo esquelético (Conlon e Kita, 2002). Muitos autores têm mostrado relação entre administração de aminoácidos, como a metionina, e a secreção de GH (Groschl et al., 2003; Collier et al., 2005), e ainda, o desempenho mediado pela ação do IGF-I também pode ser influenciado pela suplementação da metionina, já que estudos mostram que aves recebendo dieta deficiente em metionina apresentam menor crescimento (Carew et al., 2003).

De acordo com Stubbs et al. (2002), ainda não está bem esclarecido como mudanças na suplementação de aminoácidos pode iniciar mudanças na expressão dos genes GH e IGF-I, entretanto sugerem que a atividade da mTOR (proteína alvo da rapamicina), proteína envolvida na regulação da síntese proteica, possa mediar tal efeito. Há evidências de que o metabolismo de síntese e degradação protéica possa ocorrer pela ação da mesma rota bioquímica, alguns genes envolvidos com a atividade de degradação protéica no complexo ubiquitina-proteossoma são o Murf-1 e a atrogina-1. Denominados de atrogenes, a super expressão destes tem sido ligada à atrofia muscular (Sacheck et al., 2004). Tesseraud et al. (2007) demonstraram que a expressão da atrogina-1 no músculo esquelético de codornas pode ser reduzida não apenas por fatores de crescimento, mas também pela disponibilidade de metionina, via mecanismos que envolvem a proteína TOR (Figura 1).



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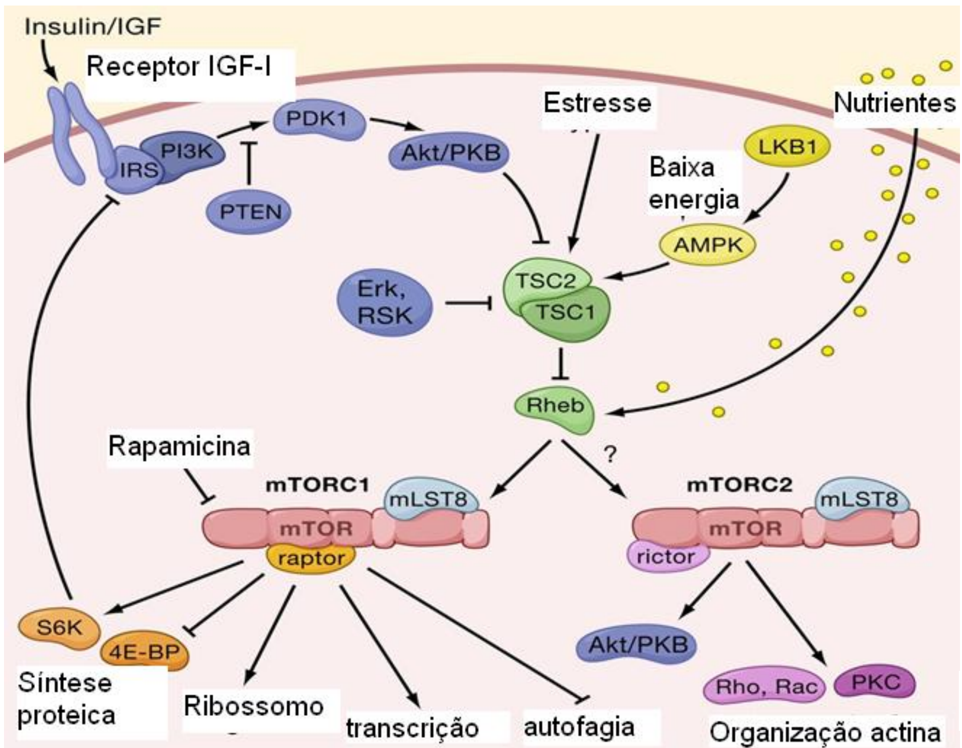


(B)

Figura 1- Crescimento: síntese proteica. Para estimular a síntese de IGF-I, o GH provoca a dimerização do seu receptor, se ligando a ele (A). Essa mudança conformacional é responsável pela ligação da proteína JAK2 (Janus Kinase 2) ao complexo, e assim, pela iniciação da sinalização intracelular, que modula as ações do GH. Com a ligação da JAK2, diversas rotas biológicas podem ser induzidas. Uma das rotas ativadas com a formação do complexo GH-GHR-JAK2, é a rota que envolve os fatores de transcrição chamados de STATs. Neste processo, as STATs são fosforiladas, o que fazem com que estas formem complexos com outras proteínas e se movam para o núcleo. Uma vez no núcleo, as STATs se ligam a sequências regulatórias específicas para ativar ou inibir a transcrição de genes alvos, como o IGF-I. A presença de fatores de crescimento e de nutrientes podem desencadear sinais que levam a maior síntese e menor degradação protéica. Proteínas como PI3K e Akt/Pkb estão envolvidas não apenas com o processo de ativação da mTOR, mas também com a inibição de componentes da família FoxO,

que quando ativados atuam no processo de transcrição de genes relacionados a degradação pela rota da ubiquitina-proteossomo. (Fonte: Adaptado de Carter-Su et al., 1996; Wullschleger, et al., 2006).

1.2.2. Síntese protéica

O crescimento é devido em grande parte à deposição protéica, esta por sua vez, é dada por um balanço entre a síntese e a degradação de proteína no metabolismo animal. Estes dois caminhos distintos são produtos da mesma rota biológica (Sacheck et al., 2004), e a concentração hormonal e a dieta são fatores que podem determinar qual destes irá prevalecer.

A mTOR é uma proteína de multi-domínios com atividade serina/treonina proteína quinase que atua como componente principal da sinalização nos mecanismos de síntese protéica. O sistema da mTOR é composto por dois complexos, o mTORC1 e mTORC2, que dependem da ligação da proteína com o raptor (complexo 1) e com o rictor (complexo 2), respectivamente. Estes dois complexos são distintos em estrutura, e em função nas células, e ainda, o complexo 2 coordena os efeitos da mTOR que são insensíveis a rapamicina, já o mTORC1, interage com proteínas que são reguladas de maneira sensível a esta droga. A atividade da mTOR está relacionada, entre outros fatores, à disponibilização de aminoácidos, ao conteúdo de energia, e à hormônios, como insulina e IGF-I, que atuam principalmente através da rota PI3K e PKB/Akt (Wu, 2013).

A ligação do IGF-I ao seu receptor leva à sua autofosforilação, e assim à mudanças conformacionais que geram locais de ancoragem para o substrato receptor de insulina (IRS), o qual também é fosforilado pelo receptor do IGF-I. O IRS agora fosforilado, serve como um local de ancoragem e ativação do fosfatidilinositol-3-quinase (PI3K). O PI3PK atua sobre fosfolipideos da membrana gerando fosfoinositídeo-3,4,5-trifosfato (PIP3), a partir do fosfoinositídeo-,4,5-bifosfato (PIP2). O PIP3 atua como local de ancoragem para duas proteínas, a fosfoinositídeo-quinase dependente 1 (PDK1) e a PKB/Akt. Após essas duas proteínas serem recrutadas pelo PIP3 ocorre a fosforilação-ativação da PKB/Akt pela PDK1. A ativação da Akt é importante, já que esta proteína tem função positiva sobre a síntese de proteína, por estimular a mTOR; e efeito negativo sobre a degradação protéica, já que age de forma direta sobre fatores de transcrição da família *Forkhead box* FoxO (Schiaffino e Mammucari, 2011).

A PKB/Akt atua de forma indireta sobre a mTOR. A Akt fosforila uma proteína chamada TSC2 (Tuberous sclerosis 2), que atua como um GAP (GTPase proteina ativador) para a proteína Rheb (Ras homolog enriched in brain). A fosforilação da TSC2 inibe sua atividade GAP, permitindo que a proteína Rheb se acumule em sua forma ativa, ligada ao GTP. Este passo da via é importante, já que dessa forma a Rheb pode interagir com complexos da mTOR e o GTP pode estimular sua atividade quinase. A mTOR controla um número grande de componentes envolvidos na iniciação e elongação, já que a síntese de proteína envolve muitos fatores que são independentes do ribossomo, e suas regulações geralmente envolvem alteração por fosforilação (Wang e Proud, 2006).

Após a ativação da mTOR pela Rheb, esta atua sobre fatores envolvidos na síntese protéica, como o 4EBP, e a S6K1. A primeira etapa da tradução, síntese proteica, é a formação do complexo de iniciação. Para que isso ocorra, um fator de tradução chamado de eIF2 (fator de iniciação eucariótico 2) deve estar ligado a um GTP. Os próximos passos são a ligação de um tRNA associado a uma metionina e do complexo eIF2-GTP à subunidade 40S do ribossomo. No fim deste processo, o GTP é hidrolisado e o eIF2 liberado na forma inativa (Brown, 1999).

O eIF2 pode estar novamente em sua forma ativa (eIF2-GTP), desde que seja reciclado pelo eIF2B (fator de troca de guanina nucleotídeo para o eIF2). A atividade do eIF2B pode ser regulada pela fosforilação da subunidade alfa e também pela fosforilação da subunidade ε do eIF2B (Fafournoux et al., 2000). A fosforilação da eIF2α converte o eIF2 de um substrato a um competidor do eIF2B e reprime a tradução da maioria dos mRNAs (Kimball e Jefferson, 2004).

A segunda etapa do início da tradução é a ligação do mRNA ao complexo formado. Este passo é mediado por fatores de iniciação eucarióticos comumente chamados de eIF4, são eles: eIF4A, eIF4E e a eIF4G. A proteína eIF4E se liga à estrutura Cap do mRNA e através disso ocorre a ligação da eIF4G e eIF4A e do complexo de iniciação formado. A fosforilação da eIF4E faz com que aumente a afinidade pela Cap do mRNA. Já a ligação desta com uma proteína de ligação do fator de iniciação eucariótico, a 4EBP, faz com que não ocorra a ligação eIF4E-eIF4G-Cap. A mTOR é conhecida por reprimir a ação do 4EBP (Fafournoux et al., 2000).

A elongação conta com dois fatores, fator de elongação eucariótico eEF1 e eEF2. O eEF2 é regulado pela MTOR, e seu papel é promover o translocação do ribossomo pelo mRNA. O eEF2 pode sofrer uma fosforilação que impede sua ligação ao

ribossomo, prejudicando assim sua atividade. A fosforilação do eEF2 é dada pela proteína eEF2 quinase. O controle da eEF2 quinase pela MTOR envolve sua fosforilação em vários locais, entre eles a serina da posição 366 pela proteína S6 quinase (S6K1) (Wang et al., 1998; Wang et al., 2001; Wang et al., 2005).

Estas proteínas descritas acima são uma ínfima parte de todos os complexos envolvidos no controle da síntese proteica pela mTOR, vale salientar ainda que vários componentes desta rota existem em múltiplas isoformas (Schiaffino e Mammucari, 2011).

1.2.3. Degradação protéica

As proteínas intracelulares são degradadas por rotas metabólicas altamente seletivas, entre estas estão a rota de degradação lisossomal, e as rotas proteolíticas não-lisossomais, que compreende o sistema das calpaínas, as caspases, o sistema proteolítico dependente de ATP, o sistema da ubiquitina-proteossoma (Wu, 2013).

O proteossoma 26S é uma protease multicatalítica composta pela subunidade 20S, reconhecido como o local proteolítico para a quebra dos peptídeos, e a subunidade 19S, responsável pela retirada da cadeia de ubiquitina (Ub) e por translocar a proteína alvo para dentro da subunidade 20S. A rota de degradação da ubiquitina-proteossoma consiste de ações enzimáticas que culminam na liberação de aminoácidos após a quebra das proteínas ubiquitinizadas. Três componentes enzimáticos são necessários para tal ação: E1, enzima ativadora da Ub; E2, enzima carreadora da Ub; E3: enzima ligante da Ub. As E3 são consideradas as enzimas chaves, já que são elas as responsáveis por reconhecer a proteína que será substrato da degradação, e por transferir a ubiquitina para ela (Lecker et al., 2006). Entre as E3 estão o MURF-1 e atrogina-1, devido a seus papéis na degradação, os genes codificantes destas enzimas tem sido chamados de atrogenes, e suas expressões têm se mostrado elevada em situações de estresse, deprivação de energia ou aminoácidos (Lecker et al., 2006; Tesseraud et al., 2007). Acredita-se que modificações estruturais que ocorrem nas moléculas de proteínas, devido a causas ambientais, ou proveniente de um defeito inicial, podem expor parte da molécula e assim, direcioná-la à ubiquitinação.

Para ocorrer a degradação das proteínas alvos, a E1 se liga a ubiquitina e a ativa. A E1, então, tranfere a ubiquitina ativada para a E2, que é quem vai carregar a Ub. A E3 reconhece de forma seletiva a proteína marcada e se liga a ela. Ocorre então, a ligação dos complexos E2-Ub ao complexo E3-substrato. A ubiquitina é tranferida para o

substrato, e a E2 deixa o complexo. Outro complexo E2-Ub se liga, e novamente a ubiquitina é tranferida para a proteína, e E2 deixa o complexo. Esse processo se repete até formar uma cadeia de ubiquitina. Quando a proteína já esta ubiquitinizada, a E3 se desliga, a proteína é capturada pela subunidade 19S, onde ocorre a liberação da proteína da cadeia de Ub, e esta por fim, é direcionada para subunidade 26S do proteossoma e é quebrada em aminoácidos (Rothman, 2010; Jackman e Kandarian, 2004).

A Akt, proteína relacionada à estimulação das ações da mTOR, também atua de forma positiva na deposição protéica, por inibir a rota de degradação ubiquitina-proteossoma. A Akt fosforila (inibe) os FoxO, fatores de transcrição da família *forkhead*, sendo estes reconhecidos como necessário para ativação das enzimas E3: MURF-1 e atrogina-1 (Nakashima et al., 2006). A expressão destes genes tem se mostrado elevada em condições de estresse e deprivação de nutrientes, entre outros (Tesseraud et al., 2007).

A degradação proteíca também pode ocorrer por enzimas presentes no interior dos lisossomos. Neste caso a degradação é conhecida como autofagia, e em função de como o material a ser degradado chega ao interior do lisossomo, a autofagia pode ser dividida em: macroautofagia, onde a organela ou proteína é separada do citoplasma pelo autofagossomo; microautofagia, a membrana do própio lisossomo capta o material a ser degradado; e autofagia mediada pelas chaperona, nesse caso, as chaperonas formam complexos com proteínas marcadas com uma sequência específica - sequência KFERQ - e então as entregam aos lisossomos (Benbrook e Long, 2012; Eskelinen e Saftig, 2009).

Independente da forma que o substrato é levado até o interior dos lisossomos, todos serão denaturados em função do baixo pH lisossomal (3-5) e degradados pelas mesmas proteases lisossomais. Entre elas estão, as catepsinas B, D, K, e uma das principais delas, a maior cisteína protease lisossomal, a catepsina L (Wu, 2013).

A autofagia tem a função não apenas de eliminar, mas também de servir como um sistema de reciclagem que produz novas matérias primas para serem utilizadas como fonte de energia, e para a renovação celular (Mizushima e Komatsu, 2011).

2. Desempenho das aves: Estresse térmico e estresse oxidativo

2.1 Estresse térmico

As aves são animais endotérmicos, capazes de produzir calor internamente, e dependem de conforto térmico para expressar o máximo desempenho permitido por seus potenciais genéticos (Miragliotta, 2004). 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 se termorregular, 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 4 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 e Macari, 2002).

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

Quando as aves estão sofrendo por estresse térmico, 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 respiratória, e assim, desequilíbrio eletrolítico relacionado com redução na ingestão de alimentos, e portanto, pior desempenho (Silva, 2004).

Oliveira et al. (2006) observaram que altas temperaturas 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, e portanto, as respostas fisiológicas ao estresse também dependerão destes fatores (Abreu e Abreu, 2012).

Estresse agudo (Lin et al., 2006) ou crônico (Yang et al., 2010) por calor, também tem sido relacionado a alterações metabólicas envolvendo o estresse oxidativo, de acordo com os autores, maior produção de espécies reativas de oxigênio (ROS) e menor atividade da cadeia respiratória mitocondrial podem ser respostas ao estresse induzido por altas temperaturas. Anteriormente, Mager e Kruijff (1995), já haviam observado que células expostas a altas temperaturas apresentavam menor atividade de enzimas envolvidas na desintoxicação por oxigênio e menor manutenção dos níveis de glutationa.

Salo et al. (1991) observaram que os níveis de mRNA da proteína HSP70 foram semelhantemente induzidos pelo estresse por calor ou pelo estresse oxidativo provocado pelo exercício. Essa similaridade no padrão de expressão gênica pode indicar relação entre os dois fatores. Os autores sugerem ainda, que a hipertermia pode causar estresse oxidativo, já que com altas temperaturas houve maior produção de ROS.

2.2. Espécies reativas de oxigênio

Radical livre é o termo utilizado para definir qualquer espécie química que tenha um ou mais elétrons desemparelhados. O elétron não pareado de um radical livre é representado como um ponto em negrito (˙), e estes radicais livres, quando relacionados ao oxigênio, são chamados de espécies reativas de oxigênio (ROS) (Gutteridge e Mitchell, 1999). Durante o processo de obtenção de energia, 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 intermediários neste processo, que ainda são oxidantes potentes, as espécies reativas de oxigênio (ROS).

Essas substâncias são capazes de oxidar macromoléculas biológicas como proteínas e lipídeos. Trabalhos que estudam a oxidação de proteínas mostram que o O_2 pode oxidar seletivamente resíduos de aminoácidos ou agregados proteicos, podendo levar à destruição dos mesmos. Os peróxidos proteicos podem reagir com outras biomoléculas, como DNA, gerando um dano adicional (Ronsein et al., 2006). O nome ROS é dado ao conjunto de produtos intermediários formado pelas espécies reativas: radical superóxido ($O_2^{\cdot -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\cdot}OH$) (Vieira, 2008).

O superóxido é produzido em diversos processos biológicos, entre eles, como dito anteriormente, na cadeia transportadora de elétrons das mitocôndrias. A síntese de $O_2^{\cdot-}$ ocorre quando um elétron é adicionado a uma molécula de oxigênio, e a partir desta espécie reativa, as demais são sintetizadas. O peróxido de hidrogênio é sintetizado através de uma reação de dismutação catalisada pela enzima superóxido dismutase (SOD) (Gutteridge e Mitchell, 1999), este por sua vez, em reações com metais reativos, como íons de ferro, pode participar na síntese de $^{\cdot}OH$ (Fenton, 1894 citado por Gutteridge e Mitchell, 1999). Outro destino para o peróxido de hidrogênio é ser convertido em água, pela ação das enzimas catalase e glutationa peroxidase (Droge, 2002).

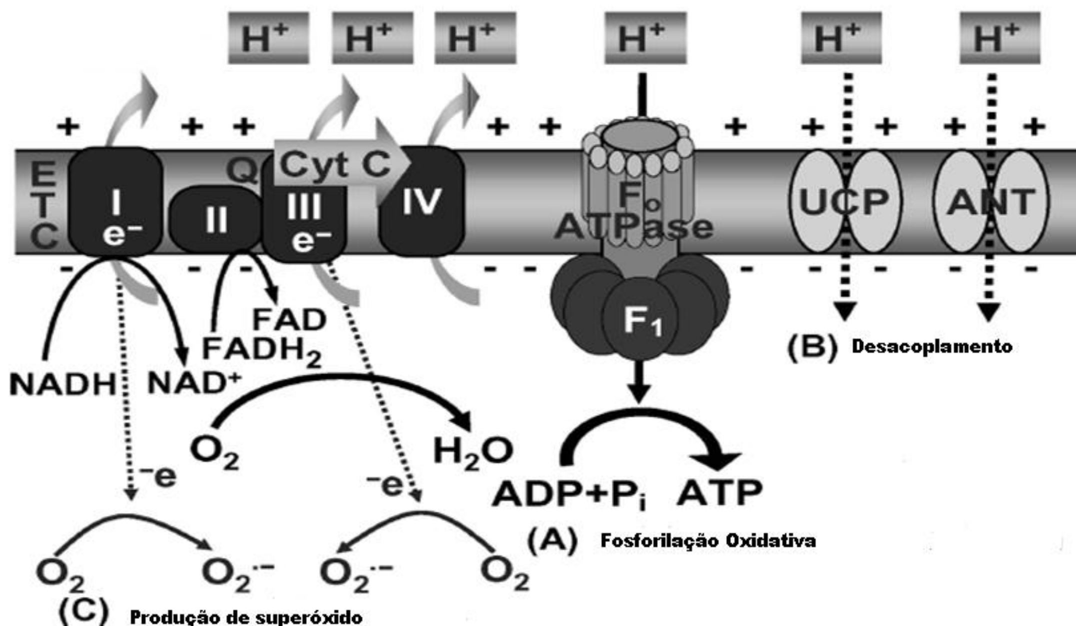
As espécies reativas de oxigênio são produzidas normalmente nos processos biológicos celulares. Entretanto, quando em maiores quantidades, são frequentemente associadas a distúrbios, como apoptose (Moustafa et al., 2004), oxidação de proteínas (Ronsein et al., 2006), peroxidação de lipídeos (Maia e Bicudo, 2009), danos ao DNA mitocondrial (Lee e Wei, 2005), e a diversas doenças (Silva et al., 2011; Reis et al., 2008; Barbosa et al., 2006).

A produção aumentada de ROS tem sido relacionada ao avanço da idade, no qual escapes de elétrons, devido a distúrbios na cadeia respiratória, aumentam a produção de radicais livres, que provocam mais lesão no mtDNA, ocasionando produção de ROS adicional (Tengan et al., 1998), e prejudica a eficiência alimentar das aves. Trabalhos recentes mostram que animais com menor produção de energia, em função de menor eficiência das mitocondrias em produzir ATP, apresentam pior conversão alimentar (Bottje et al., 2009; Parker et al., 2008). A disfunção mitocondrial pode ocorrer devido ao acúmulo no conteúdo de ROS, danos no mtDNA bem como pela ação conjunta desses.

Outro tema de pesquisas recentes é a influência do estresse por calor na produção de ROS. Autores verificaram que aves expostas a altas temperaturas podem apresentar redução na atividade da cadeia respiratória mitocondrial e redução da proteína desacopladora (UCP), ligada à maior produção das espécies reativas de oxigênio, o que pode gerar estado de estresse oxidativo (Tan et al., 2010; Yang et al., 2010; Mujahid et al., 2009; Mujahid et al., 2005).

O desequilíbrio entre a produção e eliminação das espécies reativas de oxigênio pelo organismo, leva a um estado biológico conhecido como estresse oxidativo. Nesta condição, grandes quantidades de ROS estão presentes, e isto se deve não apenas a

superprodução destes, mas também à deficiência nos sistemas de defesas antioxidantes (Halliwell e Gutteridge, 2001) (Figura 2).



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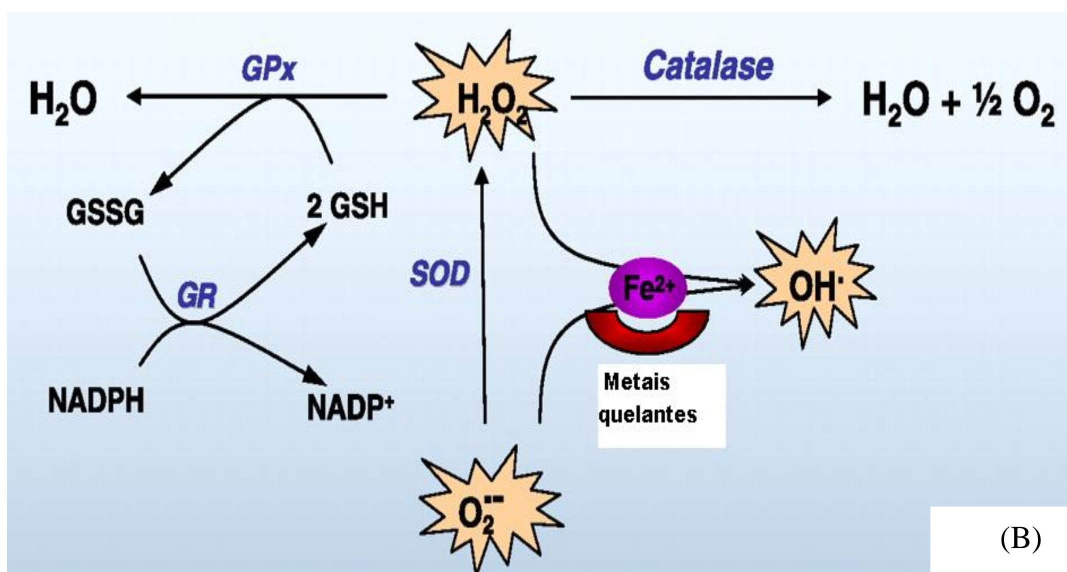


(B)

Figura 2- Produção (A) e combate às espécies reativas de oxigênio (B). O O_2 atua como aceptor final de elétrons durante a fosforilação oxidativa, entretanto, nem todo O_2 é convertido em H_2O. Estes O_2 que receberam elétrons e não foram convertidos à H_2O, formam a espécie reativa de oxigênio chamada de superóxido (O_2^-). O O_2^- pode ser convertido em peróxido de hidrogênio (H_2O_2) pela enzima superóxido dismutase (SOD), e o H_2O_2, por sua vez, convertido à água, pela ação das enzimas glutationa peroxidase (GPx) e catalase. No sistema antioxidante da glutationa (GSH), atuam além da GPx, a enzima glutationa redutase. (Fonte: Adaptado de Mujahid et al., 2007; Bashan et al., 2009).

2.3. Sistemas antioxidantes

Uma definição ampla de um antioxidante é, qualquer substância que, quando presente em baixas concentrações em relação a de um substrato oxidável, promove atrasos ou impede a oxidação do referido substrato. Portanto, em um sistema biológico, um antioxidante é uma substância que é facilmente oxidada pelo ROS, diminuindo a velocidade à qual o ROS poderá reagir com componentes celulares cruciais, como as membranas lipídicas, DNA ou proteínas (Stinefelt, 2003).

A defesa dos organismos contra o ROS pode ser mediada por antioxidantes não enzimáticos, sendo os principais, as vitaminas lipossolúveis, vitaminas hidrossolúveis, oligoelementos (Zinco, cobre, selênio, magnésio) e bioflavonóides (derivados de plantas); e por antioxidantes enzimáticos, representados principalmente pelas enzimas superóxido dismutase e catalase, e pelo sistema de defesa da glutationa (Kuss, 2005).

A glutationa (GSH) está envolvida em uma variedade de ações biológicas, entre elas, proteção contra compostos tóxicos, redução de ligações dissulfetos em proteínas, síntese de precursores de DNA, reservatório de cisteína, e a principal, defesa contra os radicais livres (Morand et al., 1997).

O sistema da glutationa é composto, além da GSH, pelas enzimas glutationa oxidase (GO), glutationa peroxidase (GPx) e pela glutationa redutase (GR). Sendo assim, a ação de defesa antioxidante, depende da atividade de todo o conjunto. Para reduzir as espécies oxidantes, e assim cumprir seu papel, a glutationa se oxida à glutationa dissulfeto (GSSG), pela ação das enzimas GO e GPx. Na forma de GSSG, a glutationa está incapacitada para nova reação. A enzima glutationa redutase é a responsável por regenerar a GSH, a partir de GSSG, permitindo assim, que a mesma molécula seja usada mais de uma vez no combate às espécies reativas de oxigênio (Huber et al., 2008).

A família das enzimas glutationa peroxidases, pode ser dividida em duas classes: enzimas dependente de selênio e enzimas selênio independente, estas chamadas de glutationa S-tranferases e com atividade relacionada à hidroperóxidos orgânicos (Arthur, 2000).

De acordo com Mézes et al. (2003), cinco isoformas de GPx dependente de selênio já foram descritas, estas enzimas estão presentes em quase todas as células

animais, entretanto, a distribuição das isoformas entre os tecidos ocorre de forma variada.

3. Estresse térmico e metionina sobre fatores do estresse oxidativo a da deposição proteica

3.1. Estresse térmico e metionina sobre fatores do estresse oxidativo

Muitos trabalhos na literatura associam o estresse térmico ao estresse oxidativo (Mujahid et al., 2009; Mujahid et al., 2005), sendo a influência do estresse térmico dada possivelmente pela aceleração na formação de ROS e/ou por aumentar a reatividade destes (Bai et al., 2003). Apesar dos extensivos estudos sobre este assunto, os mecanismos pelos quais altas temperaturas podem influenciar no estado redox da célula ainda não são bem consolidados. A menor expressão de proteínas desacopladoras (UCP) que ocorre em aves expostas ao estresse térmico (Del Vesco and Gasparino, 2012) pode ser um dos mecanismos envolvidos na maior produção de ROS, já que proteínas como UCP e ANT (adenina nucleotídeo translocase) podem despolarizar a membrana interna das mitocôndrias, representando com demais fatores um controle fisiológico da fosforilação oxidativa (Sack, 2006).

Altas temperaturas estão relacionadas não apenas com maior produção de ROS, mas também com alterações nas atividades de enzimas antioxidantes. Entre os diversos fatores que afetam a atividade da GPx, autores (Tan et al., 2010; Yang et al., 2010; Pamok et al., 2009) têm mostrado a influência do estresse térmico. Estes autores verificaram que animais expostos a altas temperaturas, em estresse térmico agudo, apresentam maior atividade desta enzima, e sugerem que isto pode ser uma tentativa do organismo de combater o ROS, que também tem sua produção aumentada nessa condição.

A GPx é uma enzima que atua em conjunto com a glutationa (GSH). A biossíntese da GSH ocorre na maioria dos tecidos a partir de três aminoácidos precursores. Entre estes está a cisteína que durante o metabolismo pode ser sintetizado a partir da metionina através da rota de transsulfuração da homocisteína (Shoveller et al., 2005).

A metionina está envolvida no metabolismo da homocisteína através de duas rotas biológicas, a remetilação e a transsufulração. A homocisteina é convertida em metionina

pela remetilação por intermédio de duas enzimas, a metionina sintase (MS) e betaína:homocisteína metiltransferase (BHMT). Pela ação da metionina sintase, enzima dependente de vitamina B12, o metiltetrahidrofolato doa um grupo metil para a homocisteína, que é convertida então em metionina. Já, na reação catalisada pela betaína:homocisteína metiltransferase, o processo se dá de maneira similar, entretanto, neste caso o grupo metil é doado pela betaína. A transsulfuração ocorre em duas etapas, na primeira, ocorre à reação da homocisteína com a serina, pela ação da cistationina B-sintase (CBS), resultando em cistationina. Na segunda, a cistationina é metabolizada, com ação da cistationina B-liase, ocorrendo a síntese da cisteína (Stipanuk, 2004). Estas rotas são consideradas de grande importância já que o excesso de homocisteína no organismo está relacionado a doenças cardiovasculares (Laurenti, 2005).

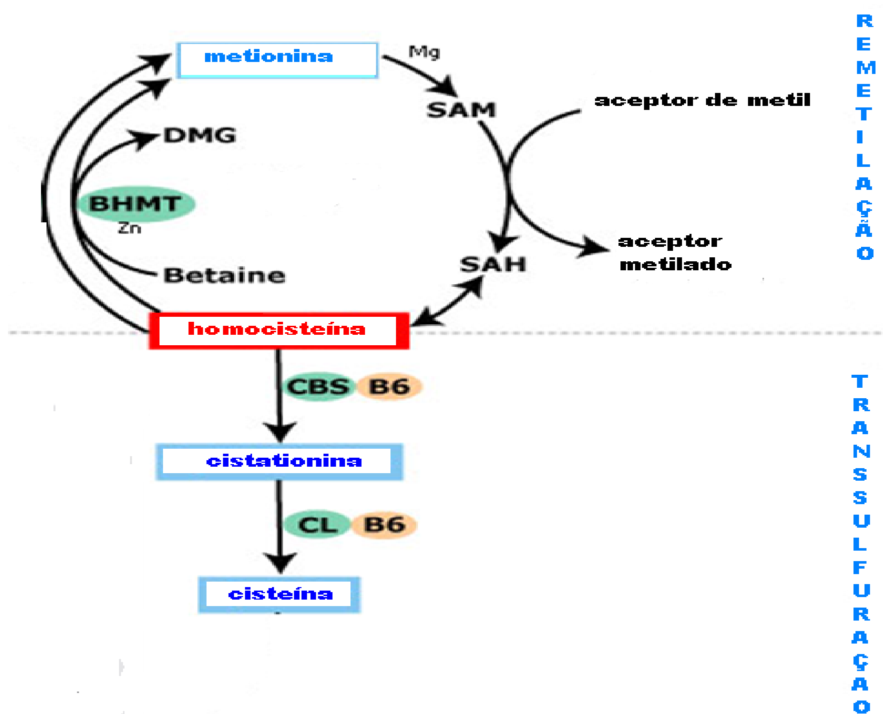
A remetilação é favorecida quando há baixas concentrações de metionina ou de S-adenosil metiltransferase (SAM), já, quando há maiores quantidade de metionina ou de SAM, a rota de transsulfuração é a mais ativada, e há maior produção de cisteína (Finkelstein, 1998).

Para síntese da GSH, na primeira etapa, ocorre uma ligação entre os aminoácidos ácido glutâmico e cisteína, por intermédio da enzima γ- glutamilcisteína sintetase, resultando em γ-L-glutamil-L-cisteína. A segunda fase consiste na ligação desse dipeptídeo com a glicina, pela ação da enzima glutationa sintetase (GSS). Para prevenção de síntese excessiva de glutationa, a γ-glutamilcisteína sintetase, pode sofrer um feedback negativo apartir da GSH, este mecanismo também confere que não haja acúmulo do intemediário, γ-glutamilcisteína (Huber et al., 2008).

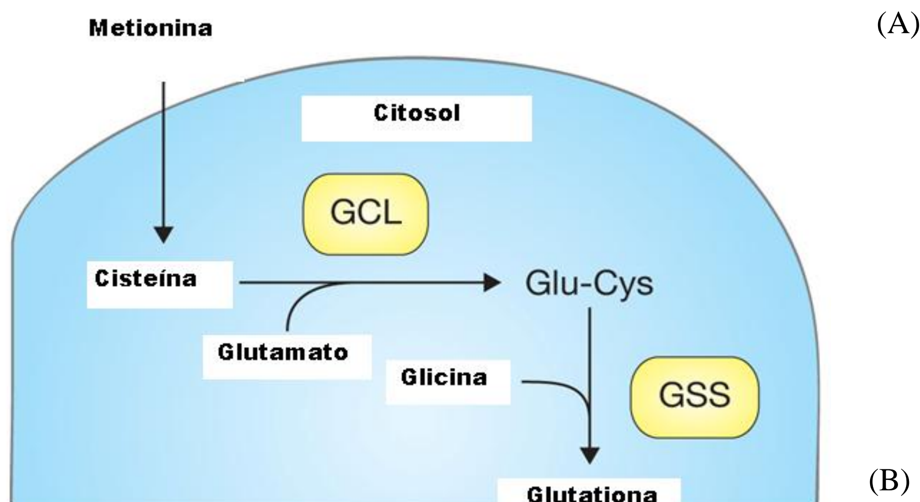
Há uma estimativa de que cerca de 50% da produção da glutationa é de origem da homocisteína, cisteína proveniente da homocisteína, através da rota de transsulfuração, e que sob condições de estresse oxidativo, na qual é requisitada maior produção de glutationa, e assim, maior atividade da cistationina ß-sintase, ocorre maior expressão desta enzima (Mosharov et al., 2000). Os resultados de Persa et al. (2004) confirmam que a presença de radicais livres podem induzir a super expressão de CBS e inibir a metionina sintase, estimulando assim a transsulfuração e maior produção de cisteína e glutationa (Figura 3). Além de síntese de novo, através de aminoácidos precursores, a homeostase da glutationa, é mantida também pela regeneração da glutationa oxidada (GSSG) e pela absorção de glutationa extracelular intacta (Shoveller et al., 2005).

Além da importante participação da metionina na síntese da glutationa (revisado por Stipanuk, 2004), alguns estudos na literatura mostram o efeito antioxidante direto de

proteção que este aminoácido tem sobre o estresse oxidativo (Stadtman et al., 2002; Moskovitz et al., 2001; Levine et al., 2000). Nas células, uma variedade de espécies reativas de oxigênio reage com resíduos de metionina formando metionina sulfoxido. Entretanto, a maioria das células conta com a presença da enzima metionina sulfóxido redutase, a qual cataliza, em uma reação dependente da tiorredoxina, a metionina sulfóxido de volta à metionina. Desta forma, os resíduos de metionina atuam diretamente como antioxidantes, protegendo do ROS as proteínas onde estas estão localizados e também outras diversas moléculas (revisado por Luo and Levine, 2009).



(A)



(B)

Figura 3- Metabolismo da metionina (A) e síntese de glutationa (B). A glutationa pode ser produzida biologicamente a partir dos aminoácidos cisteína, glutamato e glicina. Na primeira

etapa de produção ocorre a ligação da cisteína e do glutamato pela ação da enzima γ - glutamilcisteína sintetase, já na segunda fase, ao dipepitideo resultante, é incorporado a glicina pela ação da enzima glutationa sintetase. A cisteína, por sua vez, pode ser produzida durante a rota metabólica da metionina. A metionina pode ser convertida em homocisteína, e esta convertida em cisteína em uma reação de dois passos catalizados pelas enzimas cistationina β sintetase e cistationina β liase. A homocisteína também pode ser convertida de volta em metionina, esta reação pode ocorrer pela ação da enzima metionina sintetase ou da enzima betaína:homocisteína metiltransferase. (Fonte: Kadhim e Clement, 2011; deBarardinis, 2012).

3.2. Estresse oxidativo e metionina sobre deposição protéica

Diversas pesquisas são realizadas para estudar efeitos do ambiente (Mujahid et al., 2009, Yang et al., 2010) e de nutrientes da dieta (Morand, 1997; Willemsen et al., 2011) sobre a expressão gênica e o metabolismo. Sobre a deposição proteica, de acordo com Wu (2013), algumas conclusões gerais podem ser tomadas: 1) aumento da ingestão de aminoácidos ou das concentrações extracelulares destes promove a síntese e inibe a degradação de proteína intracelular; 2) fontes energéticas apresentam pouco efeito sobre a síntese, mas previnem a degradação em diversos tecidos; 3) hormônios anabólicos estimulam a síntese, e hormônios catabólicos, citocinas inflamatórias e acidose metabólica promovem a quebra; 4) Por estimular a síntese e inibir a degradação, a ingestão de quantidade adequada e a disponibilização de proteínas de alta qualidade são o melhor caminho para otimizar a deposição protéica corporal.

A suplementação de metionina tem efeito positivo sobre a síntese protéica por atuar em diversos fatores. Entre eles, está o efeito que a suplementação deste aminoácido tem sobre a expressão de genes relacionados ao crescimento. Maior expressão de mRNA, e maiores níveis de IGF-I circulantes são frequentemente associados a animais que receberam dieta com suplementação de metionina (Stubbs et al., 2002; Scanes, 2009; Del Vesco et al., 2013).

Além do efeito que o IGF-I pode apresentar sobre a mTOR, aminoácidos como a metionina também tem sido relacionados ao estímulo deste complexo. Ainda não está bem definido como é dada a ação dos aminoácidos, mas estudos sugerem que a rota metabólica pela qual os aminoácidos atuam é distinta daquela dos hormônios como insulina e IGF-I, e ocorre não através do PI3K, mas por meio da Vps34 (Nobukuni et al., 2005; Yoon e Chen, 2013).

Ainda na rota metabólica de síntese, abaixo da mTOR também pode ocorrer ação direta dos aminoácidos. De acordo com Kimball e Jefferson (2004), os aminoácidos

desempenham papel chave na regulação de alguns processos celulares, como a regulação da expressão gênica através da modulação do mRNA com efeito sobre proteínas e fatores de tradução. Ainda de acordo com estes autores, as células são capazes de reconhecer a disponibilidade de aminoácidos e gerar alterações nas rotas de sinal de tradução, que são também reguladas por hormônios e fatores de crescimento.

Quando ocorre deprivação de aminoácidos, ocorre a desfosforilação da 4EBP, o que leva ao sequestro da eIF4E, impedindo, assim, a tradução (Fafournoux et al., 2000), e ainda, a disponibilidade de aminoácidos também influencia na proteína ribossomal S6. A falta de aminoácidos leva a uma perda de atividade da proteína p70 S6 quinase ($P70^{S6K}$), responsável pela fosforilação da S6K. Desta forma, a síntese de proteínas fica comprometida (Kimball et al., 1999).

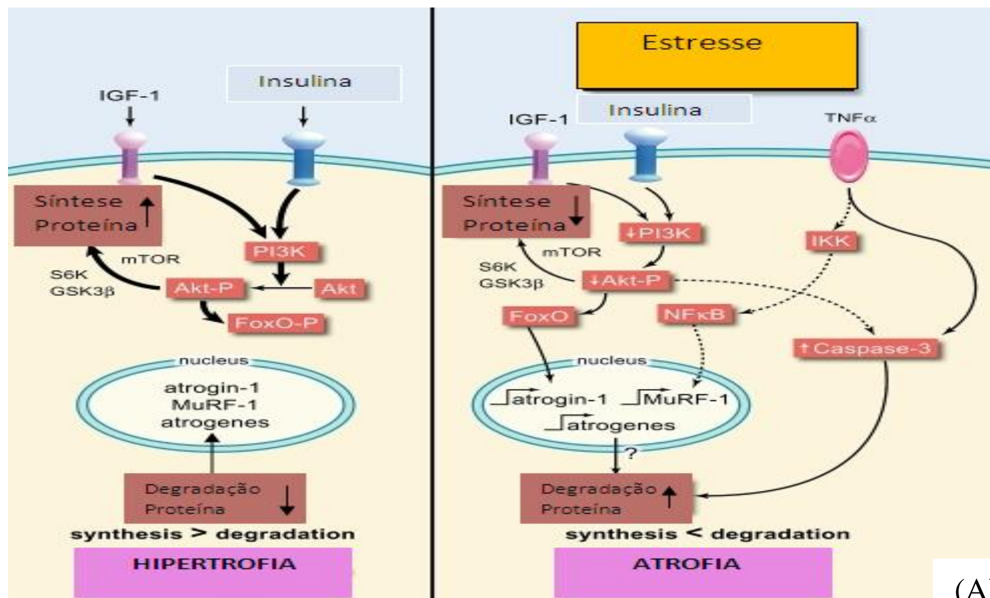
A falta de aminoácidos também tem sido associada à maior expressão de alguns genes envolvidos com a atividade de degradação proteica no complexo ubiquitina-proteossoma. Tesseraud et al. (2007) demonstraram que a expressão da atrogina-1 no músculo esquelético de codornas pode ser reduzida não apenas por fatores de crescimento, mas também pela disponibilidade de metionina, via mecanismos que envolvem a proteína mTOR.

A deficiência de nutrientes tem efeito não só sobre a via ubiquitina-proteossoma, mas também sobre a autofagia. A falta de aminoácidos induz a autofagia, não apenas por não estimular a ação da mTOR, mas também, por estar relacionada a formação de complexos essenciais para a formação do autofagossomo. A mTOR também regula a autofagia por controlar a biogênese dos lisossomos através da fosforilação do fator de transcrição EB (TFEB), o qual está envolvido na transcrição de genes relacionados à autofagia (Mizushima e Komatsu, 2011). Por outro lado, os aminoácidos gerados durante a quebra lisossomal inibem a autofagia justamente por inibir a formação dos autofagossomos (Jewell e Guan, 2013; Jewell et al., 2013).

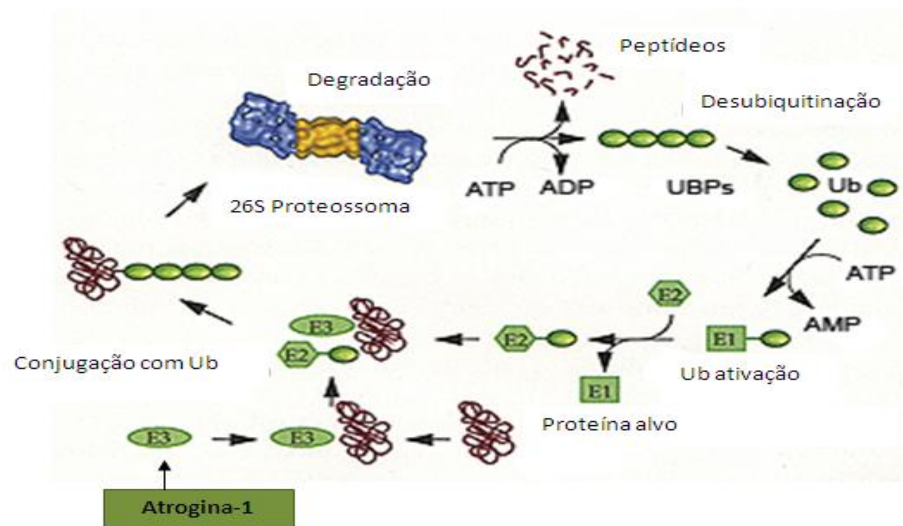
A mTOR atua como um sensor de nutrientes e energia para coordenar a deposição protéica, sendo os complexos da mTOR grandes inibidores da autofagia. Em condições de baixa quantidade de energia para utilização celular, a proteína AMPK fosforila (ativa) a TSC2, o que faz com que a proteína Rheb deixe de estimular a mTOR (Eskelinen e Saftig, 2009). Dessa forma a quantidade de energia celular pode aumentar, tanto pela redução na síntese protéica (Wang e Proud, 2006), quanto pela energia obtida através da degradação de moléculas pelos lisossomos (Mizushima e Komatsu, 2011).

Assim como a falta de aminoácidos e outros nurientes, o estresse também é responsável por desencadear ações que promovem a proteólise. Além de todos os efeitos da maior produção de ROS sobre o metabolismo, o estresse ainda é associado à menor expressão de IGF-I, maior expressão de componentes da via ubiquitina-proteossoma (Gomes-Marcondes e Tisdale, 2002) e ativação da sinalização do FoxO (Furukawa-Hibi et al., 2002) (Figura 4).

Na figura 4, podemos observar que a presença de fatores de crescimento e de nutrientes podem desencadear sinais que levam a maior síntese e menor degradação protéica. A degradação protéica pode ocorrer através da rota ubiquitina-proteossomo, e também pela a ação dos lisossomos, isto ocorre não apenas quando ocorrem eventos que desencadeiam a degradação, mas também quando deixa de haver estímulos para a síntese. Fatores de crescimento, como o IGF-I, e nutrientes, como a metionina, exercem papeis fundamentais em ambos os processos. Proteínas como PI3K e Akt/Pkb estão envolvidas não apenas com o processo de ativação da mTOR, mas também com a inibição de componentes da família dos fatores de transcrição FoxO, que quando ativados atuam no processo de transcrição de genes relacionados a degradação pela rota da ubiquitina-proteossomo. Nesta rota, atuam três classes de enzimas: as E1, que são responsáveis pela ativação da ubiquitina, as E2, que transportam a ubiquitina ativada, e as E3, como a atrogina-1, que são responsáveis por reconhecer e se ligar a proteína substrato, para que essas proteínas passem pela ubiquitinação, e então possam entrar no complexo proteossomo para enfim serem degradadas. A mTOR além de favorecer a síntese proteica, ainda reprime sua degradação pelos lisossomos, já que a mTOR é conhecida por inibir a formação do autofagossomo, estrutura essencial para a autofagia (Rajan e Mitch, 2008; Mizushima e Komatsu, 2011; Gordon, 2013).



(A)



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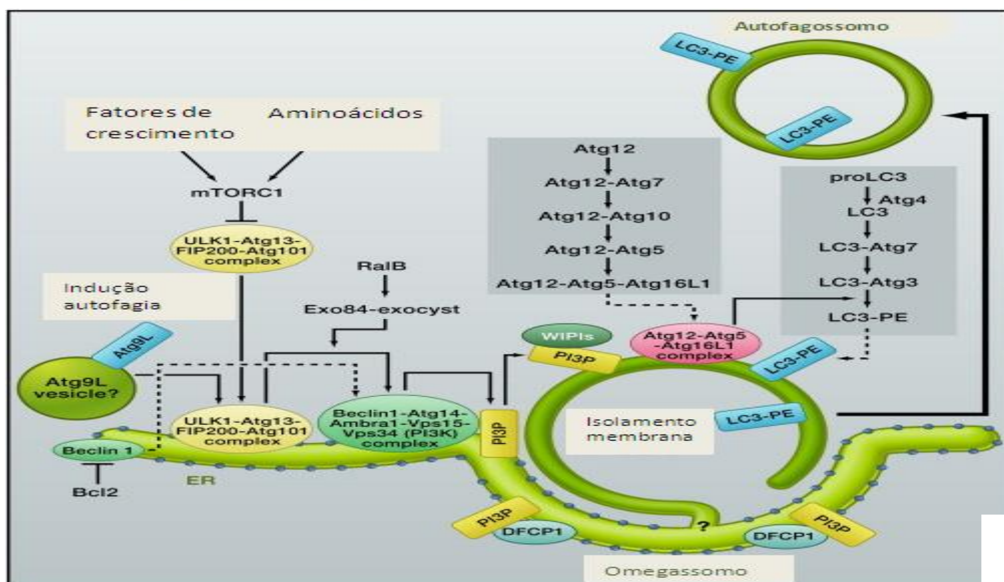

(C)

Figura 4- Deposição: Síntese (A) e degradação proteica (B) e (C). (Fonte: Adaptado de Rajan e Mitch, 2008; Mizushima e Komatsu, 2011; Gordon, 2013).

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II. OBJETIVOS

Objetivos Gerais:

Os objetivos gerais deste trabalho foram avaliar o desempenho de frangos e codornas, a expressão gênica, produção de ROS, atividade enzimática, e marcadores do estresse oxidativo em aves alimentadas com diferentes níveis de suplementação de metionina e submetidas a diferentes ambientes.

Objetivos Específicos:

Capítulo III: Avaliar a expressão de genes envolvidos no metabolismo da metionina: betaina homocisteina metil transferase (BHMT), e cistationina ß sintase, e genes envolvidos nos sistemas antioxidantes: UCP, glutationa sintetase e glutationa peroxidase em frangos de corte;

Capítulo III: Avaliar níveis sanguíneos de homocisteína, ácido úrico, e creatinina, e a atividade das enzimas alanina aminotransferase, aspartato aminotransferase e creatina quinase em frangos de corte;

Capítulo IV: Avaliar o desempenho de frangos que receberam dietas distintas em níveis de suplementação de metionina e que foram submetidas a diferentes temperaturas ambientais;

Capítulo IV: Avaliar a expressão de genes responsáveis pelo crescimento corporal: fator de crescimento semelhante a insulina I (IGF-I), receptor do hormônio do crescimento (GHR), fosfatidilinositol-3-quinase, subunidade reguladora 1 (PIK3R1), e genes ligados à degradação proteica: atrogina-1 e catepsina L2 (CTSL2) em frangos de corte;

Capítulo V: Avaliar o efeito do estresse térmico agudo e da suplementação de metionina sobre o desempenho, sobre a produção de peróxido de hidrogênio (H_2O_2), sobre a atividade das enzimas antioxidantes: glutationa peroxidase e catalase, e sobre marcadores biológicos do estresse oxidativo: níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), GSH, níveis sanguíneos de ácido úrico, creatinina, e a atividade das enzimas alanina aminotransferase, aspartato aminotransferase em codornas;

Capítulo VI: Avaliar o efeito do ambiente e da suplementação de metionina sobre a expressão de IGF-I, GHR e UCP em codornas.

III. EFFECTS OF METHIONINE SUPPLEMENTATION ON THE EXPRESSION OF OXIDATIVE STRESS-RELATED GENES OF ACUTE HEAT STRESS-EXPOSED BROILERS

(Journal of Animal Science)

ABSTRACT- This study aimed to evaluate the effect of heat stress and methionine supplementation on markers of stress as well as on uncoupling protein (UCP), betaine homocysteine methyltransferase (BHMT), cystathionine β synthase (CBS), glutathione synthetase (GSS), and glutathione peroxidase 7 (GPx 7) gene expression. Broilers from 1-21 and from 22-42 days of age were divided into three treatments related to methionine supplementation: without methionine supplementation (**MD**); recommended level of methionine (**DL1**), and excess methionine supplementation (**DL2**). The animals were either kept at a comfortable thermal temperature or exposed to heat stress (**HS**) (38˚C for 24 hours starting on day 20 or day 41, depending on the experimental phase evaluated). Heat stress increased body temperature in both experimental phases (P<0.0001). *Starter period*: We observed effects from the interaction between diet and the environment on the gene expression of UCP (P=0.0095), BHMT (P<0.0001), and GSS (P=0.0012). Greater UCP and BHMT gene expression was observed in animals that were maintained in thermal comfort and received MD diet. HS animals fed with DL1 and DL2 diets had the greatest GSS expression. The expression of the CBS and GPx 7 genes was influenced by the environment (P<0.0001 and P=0.0004, respectively) and by methionine supplementation (P=0.0167 and P=0.0042, respectively). It was observed that the environment x diet interaction had an effect (P=0.0022) on homocysteine concentration, such that a greater concentration was found in the birds that remained in thermal comfort and on the DL2 diet. *Grower period*: Gene expression of BHMT (P<0.0001), CBS (P<0.0001), GSS (P=0.0036), and GPX7 (P=0.0375) was affected by the diet x environment interaction. Greater expression of BHMT was observed in animals maintained at thermal comfort and on the MD diet. Heat stress induced higher expression of CBS, GSS, and GPx 7 in animals on the DL1 and DL2 diets. UCP gene expression was lower in animals on the MD diet (P=0.0063) and in HS animals (P<0.0001). Our results suggest that under HS conditions, in which body temperature is greatest, methionine supplementation was able to mitigate the stress

effects, since methionine contributed to increased expression of genes related to antioxidant activity.

Key Words: antioxidant system, homocysteine, methionine metabolism, oxidative stress

INTRODUCTION

In the literature, oxidative stress is the subject of several lines of research. Among these are the studies associated with diseases and metabolic disorders (Moustafa et al., 2004), changes and damage to mitochondrial DNA (Lee and Wei, 2005), stress effects on autophagy (Lee et al., 2012), on protein degradation (Aiken et al., 2011), and on DNA methylation (Gu et al., 2013), as well as research about the relationship of heat stress and oxidative stress (Mujahid et al., 2006; Mujahid et al., 2009; Yang et al., 2010).

Heat stress causes damage to a bird's performance and the yield of parts, which may be explained by physiological changes to the bird's body (Geraert et al., 1996; Yunianto et al., 1997). These physiological changes might partly be caused by oxidative stress that occurs in animals kept under heat stress (HS). It was not entirely known how oxidative and heat stress were related; however, studies show that heat stress can induce common mechanisms of oxidative stress such as increased lipid peroxidation (Willemsen et al., 2011), decreased activity of the enzyme creatine kinase (Del Vesco et al., 2014), and increased protein oxidation (Ronsein et al., 2006). Heat stress is also related to a decrease in the gene expression of uncoupling proteins (UCP) (Mujahid et al., 2006; Gasparino et al., 2013). The UCPs are proteins that are found in the inner mitochondrial membrane, and their main function in mammals is related to heat production (Ledesma et al., 2002). This uncoupling mechanism in ATP production has also been described as an agent that enables a reduction in the production of reactive oxygen species (ROS) (Abe et al., 2006). Thus, lower UCP production can contribute to oxidative stress induction.

Large amounts of ROS are present when there is an oxidative stress state; this occurs not only due to overproduction of reactive oxygen species, but also due to the deficiency in the antioxidant defense systems (Halliwell and Gutteridge, 2001). The antioxidant system of glutathione (GSH) is composed of glutathione oxidase (GO), glutathione peroxidase (GPx), and glutathione reductase (GR); the effectiveness of the defense system depends on the coordinated activity of the whole (Huber et al., 2008).

GSH biosynthesis occurs in most tissues based on three precursor amino acids. Among these is cysteine, which during metabolism, can be synthesized from methionine by the transsulfuration pathway (Shoveller et al., 2005). Methionine is involved in homocysteine metabolism via two metabolic pathways: remethylation, in which homocysteine is converted to methionine through the enzyme methionine synthase or betaine:homocysteine methyltransferase (BHMT), and transsulfuration, where homocysteine is converted to cysteine by the action of two enzymes, cystathionine B-synthase (CBS) and cystathionine β-lyase. Estimates have suggested that approximately 50% of glutathione is generated from homocysteine, and that under conditions of oxidative stress, which requires higher amounts of GSH, the production rate increases through the stimulation of the transsulfuration pathway via the increased expression and activity of cystathionine ß-synthase (Mosharov et al., 2000). Results confirm that the presence of free radicals can induce the overexpression of CBS and can also inhibit methionine synthase, thereby stimulating an increased production of cysteine and glutathione (Persa et al., 2004).

This study was developed under the hypothesis that heat stress induces oxidative stress, and that methionine supplementation may contribute to the production and action of antioxidant components, thereby reducing the damage caused by stress. We assumed that remethylation is the main alternative when there is a deficiency of methionine, and that transsulfuration mediated by cystathionine ß-synthase is the main, when methionine is available for synthesis of cellular components as glutathione. Thus, we aimed to evaluate the effect of heat stress and methionine supplementation on markers of stress, on plasma homocysteine concentration, and on the expression of genes related to ROS production (uncoupling protein, UCP), genes involved in methionine metabolism (betaine:homocysteine methyltransferase, BHMT; and cystathionine β synthase, CBS), and genes related to combating oxidative stress (glutathione synthetase, GSS; and glutathione peroxidase 7, GPx 7) in broilers from 1-21 and 22-42 days of age.

MATERIALS AND METHODS

The procedures in this experiment were approved by the Committee on Animal Care of the Universidade Estadual de Maringá - Brazil.

Experimental Design and Animals

Experiment 1- Starter period- 1-21 days old

180 male broilers (Cobb 500) (*Gallus gallus*) were used for the starter period experiment. The animals were divided into three treatments related to methionine supplementation: without supplementation (**MD**, n = 60), supplementation of the recommended level of methionine (**DL1**, n = 60) (Rostagno et al., 2011), and excess supplementation of methionine (**DL2**, n = 60) (Table 1). The animals were distributed in a completely randomized design with four replications (pens) per treatment, and each replicate consisted of 15 birds. Throughout the experimental period, the animals had free access to food and water.

The 180 birds distributed among treatments were raised in a climatized room at thermal comfort (according Cobb guide) until 20 days of age, when 90 animals (30 from each diet) were subjected to acute heat stress of 38°C for 24 hours. During the stress period, the remaining 90 animals (30 from each diet) were removed from the chamber and kept in a thermoneutral environment throughout the period. After 24 hours of stress, the animals from both groups (comfort and heat stress- **HS**) were slaughtered by cervical dislocation at 21 days. Rectal temperature was measured in the thermal comfort and HS birds (n = 20).

Table 1- Experimental diets centesimal composition (expressed as-fed basis)

	Starter period			Grower period		
Ingredients	MD[1]	DL1	DL2	MD	DL1	DL2
Corn 7.8% CP	550.75	548.80	542.70	600.00	598.20	592.05
Soy bean meal 46.0% CP	373.00	373.00	374.00	324.00	324.00	325.00
Soy oil	39.00	38.00	36.00	46.00	45.00	43.00
Salt	4.50	4.50	4.50	4.30	4.30	4.30
Calcareous 38%	11.60	11.60	11.60	9.30	9.30	9.25
Dicalcium phosphate 20%	15.25	15.25	15.30	10.65	10.70	10.70
DL- Methionine 99%	-	2.95	10.00	-	2.75	10.00
L- Lysine HCl 78%	1.55	1.55	1.55	1.55	1.55	1.50
L-Treonine 78%	0.35	0.35	0.35	0.20	0.20	0.20
Premix[2]	4.00	4.00	4.00	4.00	4.00	4.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
Composition analysis (%)						
CP	21.61	21.77	22.191	19.73	19.88	20.36
Lysine digestible	1.19	1.19	1.20	1.08	1.08	1.08
Met+Cis digestible	0.58	0.88	1.57	0.54	0.81	1.53
Treonine digestible	0.78	0.78	0.78	0.70	0.70	0.70
Triptophane digestible	0.24	0.24	0.24	0.22	0.22	0.22
Valine digestible	0.92	0.92	0.92	0.84	0.84	0.84
Isoleucine digestible	0.86	0.86	0.86	0.77	0.77	0.77
Arginine digestible	1.38	1.38	1.38	1.24	1.24	1.24
Composition Calculated (%)[3]						
Ca	0.88	0.88	0.88	0.68	0.68	0.68
P	0.45	0.45	0.45	0.35	0.35	0.35
Na	0.20	0.20	0.20	0.19	0.19	0.19
AME (kcal/kg)	3052.51	3051.94	3051.38	3169.60	3168.53	3168.60

[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, excess methionine supplementation. [2]Supplied by kilogram of diet: retinyl-acetate, 3.44 mg; cholecalciferol, 50 µg; DL-α-tocopherol, 15 mg; thiamine, 1.63 mg; riboflavin, 4.9 mg; pyridoxine, 3.26 mg; cyanocobalamin, 12 µg; D-pantothenic acid, 9.8 mg; D-biotin, 0.1 mg; menadione, 2.4 mg; folic acid, 0.82 mg; niacinamide, 35 mg; selenium, 0.2 mg; iron, 35 mg; copper, 8 mg; manganese, 60 mg; zinc, 50 mg; I, 1 mg; choline, 650 mg; salinomycin, 60 mg; avilamycin, 5 mg; butyl hydroxy toluene, 80 mg. [3]Feed formulations were made based on the total amino acids of corn and soybean meal as analyzed by Evonik Degussa (Hanau, Germany). The digestibility coefficient suggested by Rostagno et al. (2011) was used to obtain digestible amino acids. AME: apparent metabolizable energy. Amino acids, crude protein and dry matter were analyzed by Evonik Degussa (Hanau, Germany).

Experiment 2- Grower period- 22-42 days old

180 male broilers (Cobb 500) (*Gallus gallus*) were used for the grower period experiment. The animals were raised conventionally until 21 days of age and were fed a balanced diet to meet their nutritional demands (Rostagno et al., 2011). After 21 days, the animals were divided into three treatments related to methionine supplementation: without supplementation (MD, n = 60), supplementation at the recommended level of

methionine (DL1, n = 60) (Rostagno et al., 2011), and excess supplementation of methionine (DL2, n = 60) (Table 1). The animals were distributed in a completely randomized design with four replications (pens) per treatment, and each replicate consisted of 15 birds. Throughout the experimental period, the animals had free access to food and water.

The 180 birds distributed among treatments were raised in a climatized room at thermal comfort (according Cobb guide) until 41 days of age, when 90 animals (30 from each diet) were subjected to acute heat stress of 38°C for 24 hours. During the stress period, the remaining 90 animals (30 from each diet) were removed from the chamber and kept in a thermoneutral environment throughout the period. After 24 hours of stress, the animals from both groups (comfort and heat stress- HS) were slaughtered by cervical dislocation at 42 days. Rectal temperature was measured in the thermal comfort and HS birds (n = 20).

Plasma analyses

Blood was collected from five animals per each starter and grower period treatment for the analyses of homocysteine and uric acid content, plasma creatine kinase (**CK**), alanine aminotransferase (**ALT**), and aspartate aminotransferase (**AST**) activity. Blood was collected from the jugular vein into heparin tubes and was kept on ice. After centrifugation (3.024 × g, 10 min, 4°C), plasma was collected and stored at -20ºC until further analyzed.

Plasma homocysteine was determined by chemiluminescence. Uric acid content, and ALT, AST, and creatine kinase activity analyses were performed according to colorimetric methods with the following kits: uric acid - MS 80022230171, ALT - MS 80022230086, AST - MS80022230083, and CK-NAC-PP - MS 80022230088, respectively, according to the manufacturer's recommendations (Gold Analisa, Belo Horizonte, Minas Gerais, Brazil). The enzymatic activity of ALT and AST in the sample was calculated based on the decrease in absorbance at 340 nm when NADH becomes NAD+. One unit (U) of creatine kinase activity was defined as the amount of enzyme needed to convert 1 mmol of creatine into CrP/min at 37°C, pH 9.0.

Gene expression

For the analysis of gene expression, samples of breast muscle (*Pectoralis superficialis*) were collected from five animals per each starter and grower period

treatment, and were stored in RNA Holders (BioAgency Biotecnologia, São Paulo, Brasil) at -20ºC until total RNA extraction.

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 was triturated with a Polytron electric homogenizer until complete dissociation was achieved. 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 was 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 in order to remove possible genomic DNA contamination.

A SuperScript™ III First-Strand Synthesis Super Mix (Invitrogen Corporation, Brasil) 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 was immediately placed on ice. The samples were stored at -20ºC until further use.

Real-time PCR reactions were performed using the fluorescent dye SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, USA). All of the reactions were analyzed under the same conditions and normalized to the ROX

Reference Dye (Invitrogen, Carlsbad, CA, USA) in order to correct for fluctuations in the readings due to evaporation during the reaction.

The primers used in the UCP, BHMT, CBS, GPx 7, and GSS amplification reactions were designed based on the gene sequences deposited at www.ncbi.nlm.nih.gov (accession numbers AF433170.2, XM_414685.3, XM_416752.3, NM_001163245.1, XM_425692.3, respectively) using the site www.idtdna.com (Table 2). 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 2- Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Amplicom (bp)	Annealing Temperature (ºC)	Primer sequence (5'- 3')
UCP	41	60°C	GCAGCGGCAGATGAGCTT AGAGCTGCTTCACAGAGTCGTAGA
CBS	146	60°C	GAGTATGGAGAAGGTGGATGTC GGTCTAAGATATGTGCGTTGGG
BHMT	96	60°C	AGAGATTGTGATTGGAGATGGG TGTTCTACTGTTGCTTCGGG
GSS	108	60°C	GTGCCAGTTCCAGTTTTCTTATG TCCCACAGTAAAGCCAAGAG
GPx7	140	60°C	TTGTAAACATCAGGGGCAAA TGGGCCAAGATCTTTCTGTAA
β- actin	136	60°C	ACCCCAAAGCCAACAGA CCAGAGTCCATCACAATACC

Statistical analysis

Statistical analysis was performed separately for each period. The $2^{-\Delta CT}$ method was used to analyze the relative expression. The results are expressed as averages and standard deviations. The UNIVARIATE procedure was applied to evaluate data normality. The experiment was conducted in a completely randomized factorial design, with 2 environments (thermal comfort and heat stress) and 3 methionine supplementation levels (MD, DL1, and DL2). The averages were compared using Tukey's test ($P < 0.05$) (SAS Inst. Inc., Cary, NC, USA).

RESULTS

Regardless of the experimental period (1-21 or 22-42 days of age), we observed that acute heat stress (38°C for 24 hours) was sufficient to increase the birds' body temperature: 40.21ºC ± 0.30 (thermal comfort) *vs* 41.99ºC ± 0.12 (HS animals) ($P<0.0001$) for animals in the starter period, and 41.51ºC ± 0.33 *vs* 42.87ºC ± 0.21 ($P<0.0001$) for animals in the grower phase.

Gene expression

Table 3 shows the recorded gene expression of birds from the starter period for the three diets and two environments that were studied.

UCP (P=0.0095), BHMT ($P<0.0001$), and GSS (P=0.0012) gene expression in the muscle was influenced by the interaction between temperature and diet. Animals that remained at thermal comfort and were fed with the MD diet presented higher UCP (3.25 AU) and BHMT (0.67 AU) gene expression. Regarding GSS gene expression, higher values were observed in HS animals on DL1 and DL2 diets.

The CBS and GPx 7 gene expression was influenced by both methionine supplementation (P=0.0167 and P=0.0042, respectively) and heat stress ($P<0.0001$ and P=0.0004, respectively). Higher CBS and GPx 7 gene expression levels were observed in animals that received DL1 and DL2 diets in comparison to animals on the MD diet, and in HS animals in comparison to animals kept at thermal comfort.

Table 3- UCP, BHMT, CBS, GSS, and GPx7 gene expression in the muscle of broilers from the starter period

		UCP		BHMT		CBS		GSS		GPx7[2]	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	3.25[a]	1.40	0.67[a]	0.21	0.74	0.23	0.99[b]	0.13	1.08	0.37
	DL1	1.56[b]	0.83	0.11[b]	0.04	2.30	0.68	1.15[ab]	0.31	1.80	0.19
	DL2	1.51[b]	0.76	0.10[b]	0.04	2.08	1.21	0.94[bc]	0.23	1.56	0.27
Stress	MD	1.29[b]	0.48	0.10[b]	0.05	2.73	1.51	0.54[c]	0.15	1.70	0.70
	DL1	1.50[b]	0.57	0.09[b]	0.02	3.28	0.48	1.58[ab]	0.37	2.46	0.57
	DL2	1.49[b]	0.48	0.08[b]	0.03	3.31	0.83	1.79[a]	0.22	2.45	0.50
Main effects											
Environment	Comfort	2.11	1.28	0.29	0.03	1.70[b]	0.90	1.03	0.24	1.48[b]	0.44
	Stress	1.43	0.49	0.09	0.01	3.11[a]	1.01	1.30	0.43	2.20[a]	0.74
Diet	MD	2.28	1.44	0.39	0.03	1.74[b]	0.46	0.76	0.19	1.39[b]	0.46
	DL1	1.53	0.68	0.10	0.04	2.79[a]	0.76	1.36	0.48	2.13[a]	0.53
	DL2	1.50	0.61	0.09	0.01	2.70[a]	1.18	1.37	0.49	2.00[a]	0.60
Probabilities											
Environment		0.0196		<0.0001		<.0001		0.0438		0.0004	
Diet		0.0464		<0.0001		0.0167		0.0007		0.0042	
Interaction		0.0095		<0.0001		NS		0.0012		NS	

[a, b, c] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, excess methionine supplementation.
[2]Expressed as arbitrary unity (AU).

Similar to the starter period, in the grower phase, there was an interaction effect on almost all of the genes. BHMT (P<0.0001), CBS (P<0.0001), GSS (P=0.0036), and GPx7 gene expression was influenced by the interaction between temperature and methionine supplementation (Table 4).

The BHMT expression was higher in animals that were kept at thermal comfort and fed with the MD diet (0.16 AU). There was no difference among the other treatments for BHMT expression.

The HS broilers on the DL1 and DL2 diets had an increase in CBS gene expression (6. 57 and 7.12 AU, respectively). The lowest value was observed in animals that remained at comfort and were fed with the MD diet (0.79 AU).

Regarding GSS expression, we observed higher levels of expression in HS animals on DL1 and DL2 diets. There was no difference among the other treatments.

The highest GPx 7 gene expression was found in HS animals fed with greater levels of methionine (DL1 and DL2). Animals subjected to heat stress on the MD diet presented higher GPx 7 expression than all animals kept at thermal comfort. No

difference was observed among the three diets in animals that remained in thermal comfort.

No effect of the interaction between diet and temperature was observed on UCP expression. However, the expression was influenced by both of the study variables. Decreased UCP expression was observed in the HS animals in comparison to animals kept at thermal comfort (0.27 *vs.* 0.50 AU; P=0.0010). Regarding methionine supplementation, it was observed that animals on DL1 and DL2 diets presented higher UCP expression than birds fed with the MD diet (P=0.0063).

Table 4- UCP, BHMT, CBS, GSS, and GPx7 gene expression in the muscle of broilers from the grower period

		UCP		BHMT		CBS		GSS		GPx7[2]	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD	0.78	0.33	0.16^{a}	0.05	0.79^{c}	0.25	0.70^{b}	0.30	1.20^{c}	0.46
	DL1	0.37	0.19	0.03^{b}	0.01	2.45^{b}	0.72	1.24^{b}	0.45	1.95^{c}	0.20
	DL2	0.36	0.18	0.02^{b}	0.02	1.78^{bc}	0.59	1.22^{b}	0.32	1.54^{c}	0.36
Stress	MD	0.31	0.11	0.01^{b}	0.01	1.80^{bc}	0.56	0.97^{b}	0.19	3.79^{b}	1.53
	DL1	0.25	0.12	0.02^{b}	0.01	6.57^{a}	0.96	2.75^{a}	1.20	5.03^{a}	1.17
	DL2	0.25	0.09	0.01^{b}	0.01	7.12^{a}	1.52	3.25^{a}	0.52	6.02^{a}	0.76
Main effects											
Environment	Comfort	0.50^{a}	0.30	0.07	0.01	1.67	0.87	1.05	0.42	1.57	0.46
	Stress	0.27^{b}	0.10	0.02	0.01	5.16	2.66	2.33	1.23	4.95	1.46
Diet	MD	0.54^{a}	0.34	0.09	0.01	1.29	0.67	0.84	0.27	2.49	1.73
	DL1	0.31^{b}	0.16	0.03	0.01	4.50	2.30	1.99	1.17	3.49	1.79
	DL2	0.30^{b}	0.14	0.02	0.02	4.45	1.00	2.23	1.03	3.79	1.41
Probabilities											
Environment		0.0010		<0.0001		<0.0001		<0.0001		<0.0001	
Diet		0.0063		<0.0001		<0.0001		<0.0001		0.0032	
Interaction		NS		<0.0001		<0.0001		0.0036		0.0375	

[a, b, c] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, excess methionine supplementation.
[2]Expressed as arbitrary unity (AU).

Plasma Analyses

Homocysteine content was also influenced by interaction of environment x methionine supplementation (P=0.0022). The highest homocysteine levels were observed in animals that were on the DL2 diet and at a thermal comfort temperature, and the lowest value was found in HS animals that were fed with MD and DL1 diets (Figure 1).

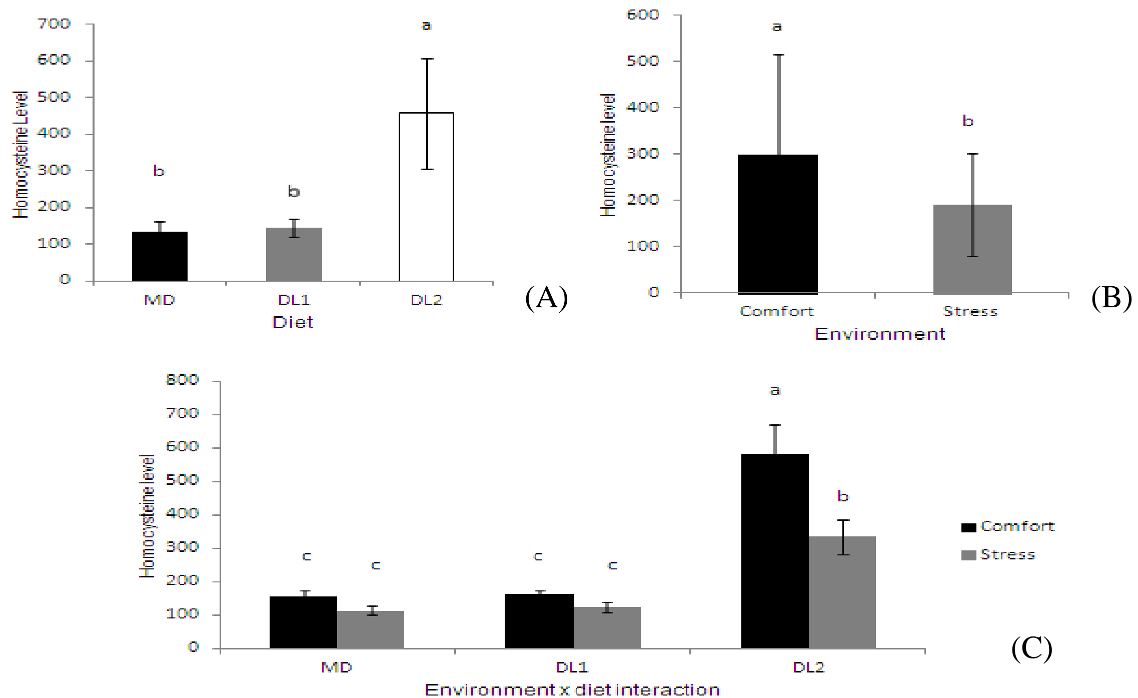

Figure 1- Methionine supplementation (A), environment (B), and methionine supplementation x environment interaction effects (C) on plasma homocysteine content of birds from the starter period. The results are expressed as umol/L. The results are shown as the average, and the standard deviation is represented by the vertical bars. The different letters between the treatment groups represent a significant difference (P < 0.05). MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, excess methionine supplementation.

The effects of supplementation and high temperature on plasma analyses from the starter period are shown in Table 5.

An interaction between the factors on uric acid (P<0.0001) and ALT activity (P=0.0024) was observed. The highest value of uric acid was found in animals that remained at the thermal comfort temperature on the DL1 diet (10.97 mg/dL), and the lowest values were found in HS animals, regardless of diet. The highest value of ALT was observed in animals that were subjected to heat stress and the MD diet (24.67 U/L).

We observed an environmental effect on CK activity (P=0.0002), as lower activity was found in HS animals; meanwhile, methionine supplementation had an effect on AST activity (P=0.0190), with the greatest activity occurring in animals on the MD diet, and the lowest in animals on the DL2 diet.

Table 5- Plasma analyses of uric acid, creatine kinase (CK), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activity of broilers from the starter period

		Uric acid, mg/dL		CK, U/L		AST, U/L		ALT, U/L	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	6.47[b]	1.71	543.03	96.56	295.83	36.13	4.83[c]	0.98
	DL1	10.97[a]	1.65	697.63	137.45	298.50	83.61	4.67[c]	0.81
	DL2	5.07[b]	1.03	460.07	58.93	245.67	24.64	4.67[c]	1.37
Stress	MD	1.73[c]	0.61	323.80	113.54	292.50	50.88	24.67[a]	6.02
	DL1	1.62[c]	1.20	281.97	109.02	260.67	16.68	16.50[b]	7.42
	DL2	2.03[c]	0.45	257.68	103.36	228.40	35.00	12.17[b]	0.41
Main effects									
Environment	Comfort	7.50	2.94	567.5[a]	265.09	280.00	56.96	4.72	1.01
	Stress	1.79	0.79	287.8[b]	105.92	262.41	43.64	17.78	7.44
Diet	MD	4.10	2.76	434.41	153.12	294.17[a]	42.11	14.75	4.98
	DL1	6.29	1.07	489.80	173.51	279.58[ab]	60.79	10.58	2.75
	DL2	3.55	1,75	358.87	132.68	237.82[b]	29.58	8.41	1.90
Probabilities									
Environment		<0.0001		0.0002		NS		<0.0001	
Diet		<0.0001		NS		0.0190		0.0018	
Interaction		<0.0001		NS		NS		0.0024	

[a, b, c] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, excess methionine supplementation.

The effects of supplementation and high temperature on plasma analyses from the grower period are shown in Table 6.

Plasma CK (P<0.0001) and ALT (P=0.0004) activity were influenced by the interaction between temperature and diet. The CK activity was higher in animals that remained at the thermal comfort temperature and were fed with the DL1 diet (1908.00 U/L). The highest ALT activity was observed in HS animals on the MD diet (10.00 U/L), and the lowest in animals a thermal comfort on the DL2 diet (5.17 U/L).

Uric acid content was influenced by both of the study variables. Increased uric acid was observed in animals that remained at thermal comfort (4.36 vs. 3.20 mg/dL; P=0.0002). Regarding diet, uric acid increased in animals on the DL1 diet (P=0.0017).

The treatments did not influence the AST activity in birds between 22-42 days of age.

Table 6- Plasma analyses of uric acid, creatine kinase (CK), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activity of broilers from the grower period

		Uric acid, mg/dL		CK, U/L		AST, U/L		ALT, U/L	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD	3.92	0.89	1608.83^{b}	119.3	299.00	179.60	5.67^{bc}	0.52
	DL1	5.45	0.13	1908.00^{a}	214.6	397.33	27.49	9.67^{a}	4.22
	DL2	3.70	0.15	1602.30^{b}	262.0	340.17	106.44	5.17^{c}	0.41
Stress	MD	2.62	0.51	1120.80^{c}	180.8	409.83	34.00	10.00^{a}	0.00
	DL1	3.59	1.33	1520.62^{b}	118.4	447.00	23.85	7.33^{b}	1.03
	DL2	3.40	1.01	388.57^{d}	144.5	331.17	89.93	5.33^{bc}	0.52
Main effects									
Environment	Comfort	4.36^{a}	0.90	1706.37	243.8	345.50	121.07	6.83	3.11
	Stress	3.20^{b}	1.05	1009.99	502.5	389.63	74.85	7.55	2.06
Diet	MD	3.26^{b}	0.97	1364.72	122.3	354.42	135.55	7.83	2.28
	DL1	4.52^{a}	1.32	1714.40	452.5	417.20	35.59	8.50	3.17
	DL2	3.55^{b}	0.70	995.43	265.2	335.67	94.06	5.25	0.45
Probabilities									
Environment		0.0002		<0.0001		NS		NS	
Diet		0.0017		<0.0001		NS		0.0003	
Interaction		NS		<0.0001		NS		0.0004	

[a, b, c, d] Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, excess methionine supplementation.

DISCUSSION

In this study, it was observed that acute heat stress (38°C for 24 hours) increased the body temperature even of the birds in the starter period (1-21 days old). The higher body temperature observed in HS animals can induce metabolic changes such as increased ROS production and increased lipid peroxidation; thus, increased body temperature can be involved in the induction of oxidative stress by heat stress (Lin et al., 2006). Reactive oxygen species are produced mainly as a function of proton leakage during phosphorylative oxidation; however, the mechanism of ROS production in heat-stressed birds is not yet fully known (Tan et al., 2010). The effects of HS are possibly due to an accelerated rate of ROS formation or an increase in ROS reactivity (Bai et al., 2003).

The production of ROS in animals exposed to high temperatures has also been correlated with the potential of the mitochondrial membrane and the expression of the UCP gene (Fink et al., 2005). A greater membrane potential is related to greater ROS production, and greater UCP mRNA production is associated with a lower production

of free radicals, and therefore less cellular damage as a result of the decreased ROS content. A greater membrane potential and decreased UCP mRNA expression is observed in animals subjected to heat stress (Mujahid et al., 2009).

The UCP can reduce ROS production by affecting decoupling during the production of ATP (Abe et al., 2006); therefore, maintaining the appropriate levels of UCP mRNA transcripts could help to combat the overproduction of ROS and the oxidative stress that is caused by acute heat stress. The expression of UCP is influenced by environmental factors such as heat stress (Mujahid et al., 2006) and nutritional status (Evock-Clover et al., 2002). As found in the literature (Mujahid et al., 2006; Mujahid et al., 2007; Gasparino et al., 2013), in our study, we also observed lower UCP expression in animals exposed to heat stress. Regarding methionine supplementation, we observed that animals fed with MD presented with a higher UCP expression. This result is most easily understood when we keep in mind that animals fed diets without methionine supplementation present worse feed conversion/feed efficiency (Del Vesco et al., 2013). Despite the beneficial effect of UCP in reducing damage to the DNA and cell proteins, as it reduces the production of free radicals, a higher expression of mRNA UCP may worsen the feed conversion ratio, as it reduces ATP production (Ojano-Diran et al., 2007; Raimbault et al., 2001).

The organism's defense against reactive oxygen species may be mediated by non-enzymatic antioxidants such as the liposoluble and water-soluble vitamins, trace elements, bioflavonoids, and by enzymatic antioxidants, which are mainly represented by the superoxide dismutase and catalase enzymes and by the glutathione defense system (Kuss, 2005).

Glutathione (GSH) is involved in a variety of biological actions including protection against toxic compounds, protein disulfide bond reduction, synthesis of DNA precursors, acting as a cysteine reservoir, and the main action, defense against free radicals (Morand et al., 1997). In the organism, glutathione can be biosynthesized by three amino acids: glutamic acid, glycine, and cysteine. Cysteine in turn can be synthesized in the body through the methionine metabolic pathway, which is composed of methylation, remethylation, and transsulfuration. In the remethylation pathway, homocysteine is converted to methionine by the action of two enzymes: methionine synthase and betaine: homocysteine methyltransferase. Through methionine synthase, a B12 dependent enzyme, methyltetrahydrofolate donates one methyl group to homocysteine, which is then converted to methionine; meanwhile, in the reaction

catalyzed by betaine: homocysteine methyltransferase, the methyl group is donated by betaine. Transsulfuration occurs in two stages; in the first one, homocysteine reacts with serine through the action of cystathionine B-synthase, resulting in cystathionine. In the second, cystathionine is metabolized by cystathionine B-lyase, thus resulting in cysteine synthesis (Stipanuk, 2004).

The GSH synthesis also occurs in two steps; in the first step, a link between the amino acids cysteine and glutamic acid occurs through the action of the γ-glutamylcysteine synthetase enzyme. This reaction results in the γ-L-glutamyl-L-cysteine. In the second phase, the dipeptide is linked to glycine by glutathione synthetase (Huber et al., 2008).

Estimates have suggested that approximately 50% of glutathione is generated from homocysteine, and that under oxidative stress conditions, the production rate increases through stimulation of the transsulfuration pathway via the increased expression and activity of cystathionine ß-synthase and the inhibition of methionine synthase, which are the enzymes responsible for cysteine and methionine synthesis, respectively (Mosharov et al., 2000; Persa et al., 2004).

In our study, we evaluated the gene expression of CBS, BHMT, GSS, and GPx 7, and we observed that acute heat stress and higher methionine content increased the CBS, GSS, and GPx 7 expression. These results suggest that birds subjected to heat stress attempt to avoid increased ROS production by increasing the expression of genes that are part of or that contribute to the glutathione antioxidant system. Because adequate methionine levels are required for greater efficiency of the antioxidant system, better results were observed in animals that were fed with DL1 and DL2.

We found that BHMT gene expression was lower in HS animals. Since remethylation was inhibited, the amount of BHMT mRNA was lower even when the HS birds were fed with the MD diet, thereby indicating that the organism under stress can stimulate glutathione production even in diets that are poor in methionine. On the other hand, when we observe only animals from comfort, we can see that birds fed with the MD diet had higher BHMT gene expression. This result was expected, because in normal metabolism, remethylation is favored when there is a low methionine or S-adenosylmethionine (SAM) concentration (Finkelstein, 1998; Stipanuk, 2004).

Homocysteine is an endogenous amino acid product intermediary of methionine metabolism. In the body, most of homocysteine is linked to proteins, and most of the portion that is in the free form is either oxidized and forms dimers (homocysteine) or

combines with cysteine (Muszalska-Dietrich et al., 2012). A high concentration of homocysteine in the blood is known as hyperhomocysteinemia and is associated with several diseases. When the normal metabolism of transsulfuration and remethylation is disturbed, usually by a CBS enzyme deficiency, the cysteine levels are decreased and there is a lower antioxidant capacity; meanwhile, the methionine levels may increase dramatically, which may cause a disease known as homocystinuria type I (Ramakrishnan et al., 2006).

Because of the importance of homocysteine in methionine metabolism and its involvement in the synthesis and action of enzymes that are treated in this study, we evaluated the plasma homocysteine concentration in animals between 1-21 days of age. We observed that in general, HS animals showed a lower homocysteine concentration. The highest concentration of homocysteine was present in animals that remained in thermal comfort on the DL2 diet. Animals fed with the DL2 diet and exposed to heat stress presented higher homocysteine concentrations than animals fed with MD and DL1 diets, regardless of the environment. These results are consistent with other results of our study. A lower homocysteine concentration in HS animals was expected, because higher amounts of cysteine are being produced from homocysteine through the increased action of the CBS enzyme. In addition, higher homocysteine concentrations in animals fed with the DL2 diet correspond to our hypothesis, because with higher methionine levels in the diet, requirements of this amino acid are more easily met; therefore, there is less BHMT gene expression, resulting in an increased plasma homocysteine concentration.

Uric acid, like so many other metabolites, has been reported in the literature as one of many elements that exhibit antioxidant activity. At the physiological pH range, it is commonly found in the form of urate, a powerful ROS scavenger released into the bloodstream by deleterious reactions such as hemoglobin auto-oxidation or peroxide production by macrophages. Urate can inactivate an oxidant before they can react with biological molecules such as DNA, proteins, and lipid membranes (Sautin and Johnson, 2008).

Birds possess specific mechanisms that contribute to increased urate concentrations in the blood, such as the absence of the enzyme uricase and the ability to encapsulate uric acid with proteins. Studies have indicated a relationship between higher uric acid concentrations and the decreased presence of oxidative stress markers (Klandorf et al., 2001; Simoyi et al., 2002).

In our study, we observed that HS animals presented lower uric acid contents than animals from comfort. This result indicates that under stress conditions, a higher concentration of uric acid was used to combat ROS, which thereby decreased its concentration in these animals. Animals fed with methionine supplementation diets showed a higher uric acid concentration. This suggests that stress demands an increased concentration of this antioxidant in the plasma, and that this increased level results from methionine supplementation, because the presence of this amino acid supplement can increase feed intake, and concomitantly glycine intake, which is a necessary element for uric acid synthesis. Methionine supplementation (Bunchasak et al., 2006) and increased feed intake have been linked to increased plasma uric acid concentration levels in broiler chickens (Machin et al., 2004).

The activity of the enzyme creatine kinase can be considered as a certain kind of oxidative stress marker, since previous studies in the literature link oxidative stress to decreased creatine kinase activity (Glaser et al., 2010; Aksenova et al., 2002), possibly via thiol group oxidation. The activity of the enzyme can be preserved by endogenous glutathione, which serves as a protective agent during the half-life of the enzyme in circulation; the loss of activity under certain conditions cannot be recovered when the extracellular GSH concentration is decreased, even in the presence of thiol-reducing agents (Gunst et al., 1998). In our study, we observed that animals exposed to heat stress demonstrated lower activity of this enzyme; and we also observed that in stress conditions, animals fed the DL1 diet demonstrated greater activity than animals fed the MD diet. This result may be due to a protective role of glutathione in the CK activity.

Similar to CK activity, the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes in the plasma have been consistently associated with stress. This is due to the fact that these enzymes are released into the blood when the body suffers some kind of injury (Khan et al., 2013). However, unlike CK, increased activity of AST and ALT are observed in animals under stress (Kumar Das et al., 2007; Mokondjimobe et al., 2012; Ismail et al., 2013). In our study increased activity of these enzymes was observed in HS animals fed with the MD diet. A deficiency in methionine, as we have seen, may have contributed to decreased action of the glutathione system components, and thus the birds were affected by greater damage.

These results allow us to suggest that under heat stress conditions in which the body temperature was greatest, methionine supplementation could mitigate the stress effects, since the supplementation contributed to increased expression of genes related

to cysteine and glutathione production as well as increased expression of the GPx 7 gene. Animals subjected to stress and fed with methionine supplementation diets showed better results in the activities of enzymes used as stress markers, which could be due to a higher antioxidant capacity.

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IV. EFFECTS OF METHIONINE SUPPLEMENTATION ON THE EXPRESSION OF PROTEIN DEPOSITION-RELATED GENES OF ACUTE HEAT STRESS-EXPOSED BROILERS

(Journal of Animal Science)

ABSTRACT- This study aimed to evaluate the effect of heat stress and of methionine supplementation on broilers' performance, and on the gene expression of insulin-like growth factor I (IGF-I), growth hormone receptor (GHR), phosphatidylinositol 3-kinase, regulatory 1 (PI3KR1), atrogin 1, and cathepsin L2 (CTSL2). Broilers from 1-21 and from 22-42 days of age were divided into three treatments related to methionine supplementation: without methionine supplementation (**MD**); recommended level of methionine (**DL1**), and excess supplementation of methionine (**DL2**). The animals were either kept at a thermal comfort temperature or exposed to heat stress (**HS**) (38˚C for 24 hours, starting on day 20 or day 41, depending on the experimental phase that was evaluated). The heat stress increased water intake in both experimental phases. *Starter period:* HS animals presented decreased weight gain, increased plasma creatinine content, and the highest CTSL2 gene expression (P<0.0001). The methionine supplementation increased the weight gain (P=0.0169), the IGF-I (P=0.0144), and GHR (P=0.0011) gene expression, and decreased CTSL2 (P=0.0004) and atrogin 1 (P=0.0012) gene expression. *Grower period:* HS induced a decrease in weight gain (P<0.001), decreased GHR gene expression (P=0.0053), and increased CTSL2 gene expression (P<0.0001). Birds on methionine supplementation diets presented higher weight gain (P=0.0457), lower feed intake (P=0.0016), the highest GHR (P=0.0113) gene expression, and the lowest CTSL2 (P<0.0001) and atrogin 1 (P<0.0001) gene expression. Significant effects of the interaction between supplementation and environment were observed on GHR gene expression, which was higher in animals kept at comfort and fed with the DL2 diet (P=0.0252), and on CTSL2 gene expression, which was higher in HS animals on the MD diet (P<0.0001). Our results suggest that heat stress can induce higher protein degradation and that methionine supplementation induces protein deposition, since methionine induced higher expression of genes related to protein synthesis and lower expression of genes related to protein breakdown.

Key words: heat stress, methionine, protein deposition

INTRODUCTION

Growth is due to protein deposition that occurs based on a balance between protein synthesis and breakdown. Studies have shown that in the control center, the protein target of rapamycin (mTOR) acts as a principal component of the signaling mechanisms of protein synthesis. The activity of mTOR is related, among other factors, to the amino acid availability, energy content, and hormones such as insulin-like growth factor I (IGF-I) and insulin, which operate mainly through the PI3K and PKB / Akt pathway (Wu, 2013). In addition, degradation can occur via ubiquitin-proteasome (Wu, 2013) and by lysosomal activity (Benbrook and Long, 2012; Eskelinen and Saftig, 2009).

Authors state that these two distinct pathways are products from the same biological route (Sacheck et al., 2004), and that hormone concentration, diet, and the environment are factors that can determine which of these two pathways will prevail.

Methionine supplementation has a positive effect on protein synthesis by acting on several factors, just as methionine supplementation affects the expression of genes related to growth. Higher mRNA expression and increased levels of circulating IGF-I are often associated with animals on supplemented diets (Stubbs et al., 2002; Scanes, 2009). Methionine has also been reported to be an inhibitor of the action of enzymes that participate in degradation by the ubiquitin-proteasome pathway. Among these enzymes are the MURF-1 (Muscle RING-finger protein-1) and atrogin-1; due to their roles in degradation, the genes encoding these enzymes have been called atrogenes, and their expression increases in conditions of stress, and in deficiency energy and amino acid conditions (Lecker et al., 2006; Tesseraud et al., 2007).

Moreover, similar to when there is a lack of amino acids and other nutrients, stress has also been blamed for initiating actions that promote proteolysis. In addition to all of the effects of the increased ROS production on metabolism, stress is also associated with lower IGF-I expression, increased expression of components of the ubiquitin-proteasome pathway (Gomes-Marcondes and Tisdale, 2002), activation of FoxO signaling (Furukawa-Hibi et al., 2002), and autophagy induction (Lee et al., 2012).

Therefore, this study was developed under the hypothesis that heat stress can stimulate the body to reach higher levels of proteolysis, and that methionine

supplementation may contribute not only to less degradation, but also to higher protein synthesis, thereby reducing the damage caused by stress. Thus, we aimed to evaluate the effect of heat stress and methionine supplementation on performance, and on the gene expression of insulin-like growth factor I (IGF-I), growth hormone receptor (GHR), phosphatidylinositol 3-kinase, regulatory 1 (PI3KR1), atrogin 1, and cathepsin L2 (CTSL2) in broilers from 1-21 and 22-42 days of age.

MATERIAL AND METHODS

The procedures in this experiment were approved by the Committee on Animal Care of the Universidade Estadual de Maringá - Brazil.

Experimental Design and Animals

Experiment 1- Starter period- 1-21 days old

180 male broilers (Cobb 500) *(Gallus gallus)* were used for the starter period experiment. The animals were divided into three treatments related to methionine supplementation: without supplementation (**MD**, n = 60), supplementation of the recommended level of methionine (**DL1**, n = 60) (Rostagno et al., 2011), and excess supplementation of methionine (**DL2**, n = 60) (Table 1). The animals were distributed in a completely randomized design with four replications (pens) per treatment, and each replicate consisted of 15 birds. Throughout the experimental period, the animals had free access to food and water.

The 180 birds distributed among treatments were raised in a climatized room at thermal comfort until 20 days of age, when 90 animals (30 from each diet) were subjected to acute heat stress of 38°C for 24 hours. During the stress period, the remaining 90 animals (30 from each diet) were removed from the chamber and kept in a thermoneutral environment throughout the period. After 24 hours of stress, the animals from both groups (comfort and heat stress- **HS**) were slaughtered by cervical dislocation at 21 days.

To calculate the daily weight gain of the broilers under thermal comfort, the animals were weighed at the beginning (day 1) and the end (day 21) of the thermal comfort period. The daily weight gain was calculated as (final weight – initial weight)/21. To calculate the weight gain of the animals under stress conditions, the specimens were weighed at the beginning and the end of the stress period. Feed intake

was calculated as the difference between the amount of feed offered and the feed residue at the end of the trial in both environments. Feed intake and weight gain were corrected for mortality. The daily feed intake of broilers under thermal comfort was calculated as (feed offered – feed residue)/21.

Water intake was measured during the 24 hours of the thermal comfort period and during the 24 hours of the stress period.

Table 1- Experimental diets centesimal composition (expressed as-fed basis)

	Starter period			Grower period		
Ingredients	MD[1]	DL1	DL2	MD	DL1	DL2
Corn 7.8% CP	550.75	548.80	542.70	600.00	598.20	592.05
Soy bean meal 46.0% CP	373.00	373.00	374.00	324.00	324.00	325.00
Soy oil	39.00	38.00	36.00	46.00	45.00	43.00
Salt	4.50	4.50	4.50	4.30	4.30	4.30
Calcareous 38%	11.60	11.60	11.60	9.30	9.30	9.25
Dicalcium phosphate 20%	15.25	15.25	15.30	10.65	10.70	10.70
DL- Methionine 99%	-	2.95	10.00	-	2.75	10.00
L- Lysine HCl 78%	1.55	1.55	1.55	1.55	1.55	1.50
L-Treonine 78%	0.35	0.35	0.35	0.20	0.20	0.20
Premix[2]	4.00	4.00	4.00	4.00	4.00	4.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
Composition analysis (%)						
CP	21.61	21.77	22.191	19.73	19.88	20.36
Lysine digestible	1.19	1.19	1.20	1.08	1.08	1.08
Met+Cis digestible	0.58	0.88	1.57	0.54	0.81	1.53
Treonine digestible	0.78	0.78	0.78	0.70	0.70	0.70
Triptophane digestible	0.24	0.24	0.24	0.22	0.22	0.22
Valine digestible	0.92	0.92	0.92	0.84	0.84	0.84
Isoleucine digestible	0.86	0.86	0.86	0.77	0.77	0.77
Arginine digestible	1.38	1.38	1.38	1.24	1.24	1.24
Composition Calculated (%)[3]						
Ca	0.88	0.88	0.88	0.68	0.68	0.68
P	0.45	0.45	0.45	0.35	0.35	0.35
Na	0.20	0.20	0.20	0.19	0.19	0.19
AME (kcal/kg)	3052.51	3051.94	3051.38	3169.60	3168.53	3168.60

[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, excess methionine supplementation. [2]Supplied by kilogram of diet: retinyl-acetate, 3.44 mg; cholecalciferol, 50 µg; DL-α-tocopherol, 15 mg; thiamine, 1.63 mg; riboflavin, 4.9 mg; pyridoxine, 3.26 mg; cyanocobalamin, 12 µg; D-pantothenic acid, 9.8 mg; D-biotin, 0.1 mg; menadione, 2.4 mg; folic acid, 0.82 mg; niacinamide, 35 mg; selenium, 0.2 mg; iron, 35 mg; copper, 8 mg; manganese, 60 mg; zinc, 50 mg; I, 1 mg; choline, 650 mg; salinomycin, 60 mg; avilamycin, 5 mg; butyl hydroxy toluene, 80 mg. [3]Feed formulations were made based on the total amino acids of corn and soybean meal as analyzed by Evonik Degussa (Hanau, Germany). The digestibility coefficient suggested by Rostagno et al. (2011) was used to obtain digestible amino acids. AME: apparent metabolizable energy. Amino acids, crude protein and dry matter were analyzed by Evonik Degussa (Hanau, Germany).

Experiment 2- Grower period- 22-42 days old

180 male broilers (Cobb 500) (*Gallus gallus*) were used for the grower period experiment. The animals were raised conventionally until 21 days of age and were fed a balanced diet to meet their nutritional demands (Rostagno et al., 2011). After 21 days, the animals were divided into three treatments related to methionine supplementation: without supplementation (MD, n = 60), supplementation of the recommended level of methionine (DL1, n = 60) (Rostagno et al., 2011), and excess supplementation of methionine (DL2, n = 60) (Table 1). The animals were distributed in a completely randomized design with four replications (pens) per treatment, and each replicate consisted of 15 birds. Throughout the experimental period, the animals had free access to food and water.

The 180 birds distributed among treatments were raised in a climatized room at thermal comfort until 41 days of age, when 90 animals (30 from each diet) were subjected to acute heat stress of 38°C for 24 hours. During the stress period, the remaining 90 animals (30 from each diet) were removed from the chamber and kept in a thermoneutral environment throughout the period. After 24 hours of stress, the animals from both groups (comfort and heat stress- **HS**) were slaughtered by cervical dislocation at 42 days.

To calculate the daily weight gain of the broilers under thermal comfort, the animals were weighed at the beginning (22 days) and the end (42 days) of the thermal comfort period. The daily weight gain was calculated as (final weight – initial weight)/21. To calculate the weight gain of the animals under stress conditions, the specimens were weighed at the beginning and the end of the stress period. Feed intake was calculated as the difference between the amount of feed offered and the feed residue at the end of the trial in both environments. Feed intake and weight gain were corrected for mortality. The daily feed intake of broilers under thermal comfort was calculated as (feed offered – feed residue)/21.

Water intake was measured during the 24 hours of thermal comfort period and during the 24 hours of stress period.

Performance and plasma analysis

The livers, hearts, legs, breasts, and abdominal fat of 12 specimens from each treatment group (comfort MD, comfort DL1, comfort DL2, stress MD, stress DL1, and

stress DL2) from each experimental phase were weighed to obtain the proportional organ weights, which were calculated as (organ weight/bird weight) x 100.

Blood from five animals from each starter and grower period treatment was collected for creatinine content analysis. Blood was collected from the jugular veins into heparin tubes and was kept on ice. After centrifugation (3.024 × g, 10 min, 4°C), plasma was collected and stored at -20ºC until it was analyzed. Creatinine analyses were performed according to colorimetric methods with the kit, creatinine-PP MS80022230066, following the manufacturer's recommendations (Gold Analisa, Belo Horizonte, Minas Gerais, Brazil).

Gene expression

For gene expression analysis, samples of liver and breast muscle (*Pectoralis superficialis*) were collected from five animals from each treatment of starter and grower periods, and stored in RNA Holders (BioAgency Biotecnologia, São Paulo, Brasil) at -20ºC until total RNA extraction.

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 was 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™ III First-Strand Synthesis Super Mix (Invitrogen Corporation, Brasil) 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 use.

Real-time PCR reactions were performed using the fluorescent dye SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, 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 IGF-I, GHR, PIK3R1, atrogin-1, and CTSL2 amplification reactions were designed based on the gene sequences deposited at www.ncbi.nlm.nih.gov (accession numbers: FJ977570.1, NM001001293.1, XM_424759.3, NM_001030956, NM_001168009.1, respectively) using the site www.idtdna.com (Table 2). IGF-I, GHR, and PI3KR1 gene expression was evaluated in the liver. Atrogin-1, and CTSL2 gene expression was evaluated in the muscle. 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 2- Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Amplicom (bp)	Annealing Temperature (ºC)	Primers sequence (5'- 3')
GHR	145	60°C	AACACAGATACCCAACAGCC AGAAGTCAGTGTTTGTCAGGG
IGF-I	140	60°C	CACCTAAATCTGCACGCT CTTGTGGATGGCATGATCT
PI3KR1	145	60°C	GCCCTCTCCTTTTCAAAT ACAGTATTAGGTTTCGGTGGC
CTSL2	80	60°C	GAAGTCAGAAAGGAAGTACAGAGG CTCTCCAGTCAACAGATCGTG
Atrogin-1	174	60°C	CCAACAACCCAGAGACCTGT GGAGCTTCACACGAACATGA
β- actin	136	60°C	ACCCCAAAGCCAACAGA CCAGAGTCCATCACAATACC

Statistical analysis

Statistical analysis was performed separately for each period. The $2^{-\Delta CT}$ method was used to analyze the relative expression. The experiment was conducted in a completely randomized factorial design, with 2 environments (thermal comfort and heat stress) and 3 methionine supplementation levels (MD, DL1, and DL2). Data from weight gain during the grower period (22-42 days of age) was analyzed using the GENMOD procedure. The distribution of the variables was considered to be normal with the identity link function. Means were compared by contrasts. The averages of the other variables were compared using Tukey's test (P < 0.05) (SAS Inst. Inc., Cary, NC, USA). The results are expressed as averages and standard deviations.

RESULTS

Performance and plasma analysis

HS animals presented higher water intake than animals that remained in thermal comfort temperature: 0.28L±0.02 *vs* 0.20L±0.01 (P <.0001), in the starter period, and 0.66 L±0.16 *vs* 0.51L±0.11 (P=0.0173), in the grower phase.

Daily weight gain (DWG), daily feed intake (DFI), and creatinine content of animals from starter period are shown in Table 3. HS animals presented lower weigh gain (P=0.0018), lower feed intake (P=0.0016), and higher plasma creatinine content than animals kept at thermal comfort. Regarding methionine supplementation, animals on DL1 diet had higher DWG than animals fed with MD diet (P=0.0169).

Table 3- Daily weight gain (DWG), daily feed intake (DFI), and plasma creatinine of broilers from the starter period

		DWG, Kg		DFI, Kg		Creatinine, mg/dL	
		Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	0.040	0.003	0.070	0.006	0.66	0.10
	DL1	0.046	0.002	0.066	0.001	0.74	0.00
	DL2	0.044	0.002	0.064	0.004	0.74	0.00
Stress	MD	0.016	0.006	0.061	0.001	0.88	0.05
	DL1	0.034	0.011	0.063	0.003	0.91	0.05
	DL2	0.026	0.013	0.059	0.003	0.87	0.10
Main effects							
Environment	Comfort	0.044^{a}	0.003	0.066^{a}	0.004	0.71^{b}	0.07
	Stress	0.025^{b}	0.011	0.060^{b}	0.003	0.89^{a}	0.07
Diet	MD	0.030^{b}	0.014	0.066	0.007	0.77	0.13
	DL1	0.041^{a}	0.010	0.065	0.002	0.82	0.09
	DL2	0.036^{ab}	0.010	0.062	0.004	0.81	0.10
Probabilities							
Environment		<.0001		0.0025		<.0001	
Diet		0.0260		NS		NS	
Interaction		NS		NS		NS	

$^{a,\,b}$ Mean values within a column with unlike superscript letters were significantly different (P<0.05).

[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, methionine supplementation in excess.

No effect of the interaction between methionine supplementation and temperature was observed on DWG, DFI, and creatinine content in animals from grower period (Table 4).

Animals kept at thermal comfort presented higher weight gain (P<.0001), and higher feed intake (P<.0001) than HS animals.

Regarding methionine supplementation, the lowest feed intake was observed in animals fed with DL2 diet. We also observed methionine effect on weight gain. The differences between the averages can be seen through of the contrasts (Table 4). Animals fed MD diet had lower weight gain than animal on DL1 (P=0.0499) and DL2 (P=0.0182) diets. There was no difference between DL1 and DL2 diets on weight gain (P = 0.6172).

Table 4- Daily weight gain (DWG), daily feed intake (DFI), and plasma creatinine of broilers from the grower period

		DWG, Kg		DFI, Kg		Creatinine, mg/dL	
		Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	0.084	0.004	0.154	0.007	0.38	0.03
	DL1	0.090	0.005	0.141	0.005	0.38	0.06
	DL2	0.085	0.011	0.139	0.009	0.36	0.00
Stress	MD	-0.339	0.262	0.074	0.012	0.32	0.03
	DL1	-0.155	0.048	0.067	0.011	0.38	0.03
	DL2	-0.103	0.029	0.060	0.006	0.36	0.05
Main effects							
Environment	Comfort	0.087	0.027	0.141[a]	0.012	0.37	0.03
	Stress	-0.199	0.027	0.067[b]	0.011	0.35	0.04
Diet	MD	-0.127	0.033	0.137[a]	0.044	0.35	0.04
	DL1	-0.032	0.033	0.104[a]	0.041	0.38	0.05
	DL2	-0.009	0.033	0.094[b]	0.037	0.36	0.04
Probabilities							
Environment		<.0001		<.0001		NS	
Diet		0.0457		0.0016		NS	
Interaction		NS		NS		NS	
Contrast ($P>X^2$)							
MD – DL1		0.0499					
MD – DL2		0.0182					
DL1 – DL2		0.6172					
Comfort – Environment		<.0001					

[a, b] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, methionine supplementation in excess.

The effects of supplementation and high temperature on proportional weights of the liver, heart, abdominal fat, breast and legs of animals from the starter and grower period are shown in Table 5 and 6, respectively.

In the starter period, heat stress decreased the proportional weight breast (P=0.0363), and animals on DL1 diet presented higher mean of this variable than animals on MD diet (P=0.0189). The treatments did not influence the proportional weights of the liver, heart, abdominal fat and legs.

Table 5- Proportional weight of organs and cuts of broilers from the starter period

		Proportional weight (%)									
		Breast		Legs		Liver		Abdominal fat		Heart	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	22.96	0.44	21.21	0.66	2.49	0.32	1.09	0.26	0.52	0.13
	DL1	25.23	1.09	19.29	1.02	2.50	0.08	1.23	0.41	0.39	0.10
	DL2	24.22	1.00	19.90	0.77	2.59	0.45	1.01	0.21	0.47	0.04
Stress	MD	21.05	1.57	20.50	0.77	2.14	0.18	1.07	0.30	0.40	0.02
	DL1	23.75	0.97	20.66	1.08	2.07	0.30	1.13	0.14	0.44	0.09
	DL2	23.10	2.45	21.63	1.29	2.58	0.33	1.00	0.19	0.58	0.17
Main effects											
Environment	Comfort	24.14^{a}	1.25	20.13	1.12	2.53	0.29	1.11	0.29	0.46	0.11
	Stress	22.63^{b}	2.00	20.93	1.06	2.27	0.34	1.07	0.20	0.48	0.12
Diet	MD	21.87^{b}	1.53	20.90	0.74	2.34	0.31	1.08	0.26	0.47	0.11
	DL1	24.39^{a}	1.22	19.88	1.20	2.32	0.29	1.18	0.30	0.41	0.09
	DL2	23.58^{ab}	1.92	20.64	1.30	2.59	0.37	1.00	0.19	0.52	0.12
Probabilities											
Environment		0.0363		NS		NS		NS		NS	
Diet		0.0189		NS		NS		NS		NS	
Interaction		NS		NS		NS		NS		NS	

$^{a, b}$ Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, methionine supplementation in excess.

In the grower period, animals that received the MD diet exhibited higher levels of abdominal fat than animals on DL2 diet (2.00 vs 1.52 %; P=0.0413). Regarding the environment, the percentage of abdominal fat was higher in animals that remained at the thermal comfort temperature (1.87 *vs* 1.58 %; P=0.0362).

Table 6- Proportional weight of organs and cuts of broilers from the grower period

		Proportional weight (%)									
		Breast		Legs		Liver		Abdominal fat		Heart	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	28.87	0.85	21.88	1.54	1.52	0.06	2.13	0.45	0.33	0.04
	DL1	30.25	0.79	21.89	1.90	1.40	0.19	1.71	0.33	0.36	0.03
	DL2	30.46	2.38	20.25	1.14	1.48	0.25	1.74	0.12	0.36	0.08
Stress	MD	27.30	2.26	23.30	0.07	1.40	0.32	1.76	0.12	0.30	0.07
	DL1	28.75	1.92	21.95	2.34	1.35	0.05	1.73	0.08	0.40	0.01
	DL2	30.39	2.62	21.26	0.86	1.50	0.09	1.08	0.16	035	0.03
Main effects											
Environment	Comfort	29.83	1.62	21.29	1.58	1.47	0.17	1.87^a	0.36	0.34	0.05
	Stress	28.82	2.24	22.18	1.46	1.42	0.17	1.58^b	0.35	0.35	0.05
Diet	MD	28.35	1.46	22.36	1.40	1.48	0.16	2.00^a	0.40	0.32	0.05
	DL1	29.66	1.39	21.92	1.79	1.38	0.14	1.72^{ab}	0.24	0.37	0.03
	DL2	30.44	2.19	20.59	1.09	1.48	0.20	1.52^b	0.35	0.35	0.07
Probabilities											
Environment		NS		NS		NS		0.0362		NS	
Diet		NS		NS		NS		0.0413		NS	
Interaction		NS		NS		NS		NS		NS	

[a, b] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, methionine supplementation in excess.

Gene expression

The results of IGF-I, GHR, PI3KR1, CTSL2, and atrogin-1 gene expression of broilers of 1-21 days of age are shown in table 7. In this phase, no effect of the interaction between methionine supplementation and temperature was observed on any gene expression.

HS animals had lower CTSL2 gene expression than animals that remained in comfort (0.015 *vs* 0.036 AU; P<.0001).

We observed methionine supplementation effect on IGF-I, GHR, CTSL2, and atrogin-1 gene expression. Animals on DL1 diet presented higher IGF-I expression than animals on MD diet (P=0.0144). The highest GHR expression, and the lowest atrogin-1 expression, respectively, were observed in animals fed with DL2 diet (P=0.0011). Animals on MD diet presented the highest CTSL2 expression (P=0.0004).

Table 7- IGF-I, GHR, PI3KR1, CTSL2, and atrogin 1gene expression of broilers from the starter period

		IGFI-I		GHR		PI3KR1		CTSL2		Atrogin-1[2]	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	0.90	0.12	1.89	0.37	0.036	0.01	0.019	0.007	1.56	0.59
	DL1	1.14	0.26	3.33	1.08	0.059	0.03	0.012	0.004	1.21	0.41
	DL2	0.90	0.47	4.95	2.02	0.051	0.02	0.014	0.008	0.63	0.28
Stress	MD	0.47	0.25	1.84	1.15	0.050	0.01	0.050	0.014	1.61	0.74
	DL1	1.06	0.45	2.53	1.26	0.054	0.02	0.027	0.007	1.37	0.43
	DL2	0.88	0.22	4.70	3.24	0.039	0.01	0.031	0.004	0.92	0.24
Main effects											
Environment	Comfort	0.98	0.32	3.39	1.79	0.049	0.02	0.015[b]	0.007	1.14	0.57
	Stress	0.80	0.40	3.02	2.34	0.048	0.03	0.036[a]	0.013	1.30	0.56
Diet	MD	0.68[b]	0.29	1.87[b]	0.82	0.043	0.03	0.035[a]	0.019	1.59[a]	0.64
	DL1	1.10[a]	0.35	2.93[b]	1.19	0.057	0.02	0.019[b]	0.010	1.29[a]	0.41
	DL2	0.89[ab]	0.35	4.82[a]	2.57	0.045	0.02	0.023[b]	0.010	0.78[b]	0.29
Probabilities											
Environment		NS		NS		NS		<0.0001		NS	
Diet		0.0144		0.0011		NS		0.0004		0.0012	
Interaction		NS		NS		NS		NS		NS	

[a, b] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, methionine supplementation in excess.
[2]Expressed as arbitrary unity (AU).

Methionine supplementation and heat stress had no effects on IGF-I and PI3KR1 gene expression of animals from grower phase (Table 8).

It was possible to observe an interaction of the factors on GHR (P=0.0252), and CTSL2 (P=0.0011) gene expression.

The highest GHR expression was observed in animals that remained in thermal comfort on DL2 diet, and the lowest expression, in HS animals fed with MD diet.

Animals subjected to heat stress and fed with MD diet presented the highest CTSL2 gene expression. Animals kept at thermal comfort on DL1 and DL2 diets presented the lowest CTSL2 expression.

Only methionine supplementation had effect on atrogin-1 gene expression (P<.0001); with higher methionine content in diet it was observed lower atrogin-1 gene expression.

Table 8- IGF-I, GHR, PI3KR1, CTSL2, and atrogin 1gene expression of broilers from grower period

		IGFI-I		GHR		PI3KR1		CTSL2		Atrogin 1[2]	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Comfort	MD[1]	0.22	0.03	2.10[bc]	0.41	0.017	0.004	0.024[cd]	0.009	1.83	0.7
	DL1	0.28	0.06	3.69[b]	1.20	0.014	0.008	0.015[d]	0.005	1.42	0.4
	DL2	0.24	0.11	5.49[a]	2.24	0.014	0.004	0.013[d]	0.003	0.74	0.3
Stress	MD	0.14	0.12	2.04[c]	1.28	0.012	0.001	0.074[a]	0.017	2.34	0.8
	DL1	0.26	0.11	2.80[bc]	1.39	0.013	0.004	0.034[bc]	0.009	1.61	0.5
	DL2	0.22	0.05	2.26[bc]	1.16	0.02	0.011	0.038[b]	0.006	1.09	0.3
Main effects											
Environment	Comfort	0.25	0.07	3.76	1.99	0.015	0.005	0.017	0.007	1.33	0.6
	Stress	0.21	0.10	2.37	1.25	0.015	0.010	0.049	0.021	1.68	0.7
Diet	MD	0.18	0.09	2.06	0.91	0.015	0.009	0.049	0.029	2.08[a]	0.7
	DL1	0.27	0.08	3.25	1.33	0.013	0.006	0.024	0.012	1.51[b]	0.4
	DL2	0.23	0.08	3.88	2.39	0.017	0.009	0.026	0.014	0.92[c]	0.3
Probabilities											
Environment		NS		0.0053		NS		<0.0001		NS	
Diet		NS		0.0113		NS		<0.0001		<0.0001	
Interaction		NS		0.0252		NS		0.0011		NS	

[a, b, c,d] Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).
[1]MD, methionine deficient; DL1, recommended level of methionine supplementation; DL2, methionine supplementation in excess.
[2]Expressed as arbitray unity (AU).

DISCUSSION

Quail, broiler, layer, and breeder production are quite affected by the high temperatures found in some tropical countries during the summer season. These temperatures cause damage to bird performance and the parts yield, which can be explained by the physiological changes in the bird's body (Geraert et al., 1996; Yunianto et al., 1997). When birds are subjected to heat stress, environmental and postural mechanisms such as feed intake reduction and increase in water intake are used primarily in an attempt to reduce metabolic heat production and increase heat dissipation (Mujahid et al., 2005).

In animals at 42 days, a huge decrease in the weight of broilers under the HS environment was observed, even though the period of evaluation was only 24 hours. We cannot completely explain what happened, but references in the literature show that broilers under high temperatures increase their plasma corticosterone levels, which stimulates a huge increase in the body's protein breakdown (Yunianto et al., 1997). This leads to a reduction in the relative weights of many organs such as the intestine, liver,

gizzard, lungs, proventriculus, and heart (Oliveira Neto et al., 2000), as well as a reduction in the absorptive area of the intestine (Mitchell and Carlisle, 1992), a reduction in parts yield (Geraert et al., 1996), and an increase in water consumption (Oliveira Neto et al., 2000). All of these aforementioned physiological changes may indicate that broilers lost protein from their bodies. Our study agrees with the data presented by other studies, since we observed that animals kept under heat stress not only demonstrated a lower feed intake and a greater water intake, but they also presented a greater plasma creatinine concentration.

The plasma concentration of creatinine is normally associated with renal functions; however, some studies show that high levels of creatinine are present in the blood when there is a greater breaking or turnover of protein, and that renal deficiency is possibly associated with the high turnover rates (Biolo et al., 1998).

In agreement with our expectations, we observed that methionine supplementation increased the body weight gain and reduced the feed intake. The supplementation with the highest methionine level (DL2 *vs*. DL1) did not demonstrate the best results regarding body weight gain, but DL2 was associated with the lowest fat deposition in birds between 22 to 42 days. Animals exposed to heat stress presented a lower fat content than animals kept at thermal comfort.

Likewise a lower fat content that was found in our study, some studies demonstrated that the decreased protein synthesis capacity and rate, lower growth rate, lower efficiency of protein deposition, as well as lower RNA levels have been also observed in animals exposed to heat stress (Temim et al., 2000). Chickens exposed to high temperatures presented lower circulating IGF-I concentrations, along with lower T3 and T4 levels, higher corticosterone levels, higher TBARS levels, and greater antioxidant activity (Willemsen et al., 2010). IGF-I activity is important not just in promoting protein synthesis, but also in decreasing the protein degradation rate in the ubiquitin-proteosome complex (Sacheck et al., 2004).

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.

The binding between IGF-I and its receptor results in autophosphorylation and conformational changes, which produce a signaling cascade involving many proteins. Among these is the insulin receptor substrate (IRS), which is also phosphorylated by the IGF-I receptor, the phosphatidylinositol 3-kinase (PI3K), the phosphoinositide-dependent kinase 1 (PDK1), and the PKB/Akt. After these two last proteins are recruited, a phosphorylation / activation of PKB/Akt by the PDK1 occurs. The Akt activation is important because this protein has a positive effect on the protein synthesis. Akt activation stimulates the mTOR, which controls a large number of components related to the initiation and elongation (Wang and Proud, 2006). However, Akt activation does have a negative effect on protein degradation, since it acts directly on FoxO family complexes (Schiaffino and Mannucari, 2001). The Akt phosphorylates (inhibits) the transcription factors FoxO family. FoxO family is necessary to activate the MURF-1 and antrogin 1 enzymes (Nakashima et al., 2006) that act on the degradation pathway of ubiquitin proteasome.

The degradation pathway of ubiquitin proteasome consists of enzymatic actions that result in the release of amino acids after the breakdown of proteins linked to ubiquitin. Three enzymatic compounds are necessary for this action: E1, Ub-activating enzyme; E2, Ub-carrier; and E3, Ub-protein ligases. The E3 are considered to be the key enzymes, since they are responsible for recognizing the protein that will be the substratum of the degradation and for transferring the ubiquitin (Lecker et al., 2006). Among the E3 are the MURF-1 and the atrogin 1 enzymes; because of their role in the degradation process, the coding gene of theses enzymes are called atrogenes, and their expression is high in stressful situations (Gomes-Marcondes and Tisdale, 2002) or in the deprivation of energy or amino acids (Lecker et al., 2006; Tesseraud et al., 2007).

In our study, we observed that higher amounts of methionine in the diet were associated with a greater GHR and IGF-I gene expression, and despite we did not observe variation in the PI3KR1 gene expression, the supplementation with the higher methionine level yielded the lower atrogin 1 gene expression. Perhaps, besides stimulating protein synthesis through a positive effect on IGF-I gene expression, methionine supplementation may also stimulate proteins, other than PI3KR1, upstream to atrogin 1, thereby signaling a lower degradation. Given the complexity and the numerous factors involved in the sensitive relationship between protein synthesis and degradation, various studies have been performed to evaluate the environmental and dietary nutrient effects (Morand, 1997; Willemsen et al., 2011) on the expression of

genes involved in this metabolism; some of these studies demonstrate, besides having a greater effect on expression and IGF-I circulation (Stubbs et al., 2002; Scanes, 2009; Del Vesco et al., 2013), the direct action of amino acids on the protein complex of mTOR. These studies suggest that the metabolic pathway by which amino acids act is distinct from that of the hormones, and occurs not through PI3K, but by Vps34 (Nobukuni et al., 2005; Yoon and Chen, 2013).

Protein degradation can still occur through enzymes present in lysosomes, and regardless of the form in which the substrate is brought into the interior of the lysosomes, everything will be denatured by the low lysosomal pH and degraded by the same lysosomal proteases. Among them are cathepsins B, D, K, and the lysosomal cysteine protease, cathepsin L (Wu, 2013). As for the ubiquitin-proteasome pathway, studies also show the effects of nutrient deficiency and stress on this degradation pathway. The lack of amino acids induces autophagy, not only by the lack of stimulation to the action of mTOR, but also due to the formation of complexes essential for the formation of the autophagosome (Jewell and Guan, 2013; Jewell et al., 2013). Moreover, in our study, we found that a deficiency in methionine led to increased expression of the gene CTSL2.

The expression of cathepsin L2 was also greater in animals under heat stress than in animals under thermal comfort. Just as the lack of amino acids and other nutrients affects proteolysis, the stress is also responsible for initiating actions that promote proteolysis. This is due to the fact that apart from the effects of an increased production of ROS on metabolism, stress is also associated with a lower expression of IGF-I, an increased expression of components of the ubiquitin-proteasome pathway (Gomes-Marcondes and Tisdale, 2002), activation of FoxO signaling (Furukawa-Hibi et al., 2002), and induction of autophagy (Lee et al., 2012), as was observed in our study.

These results allow us to suggest that the acute heat stress could signal greater protein degradation, as a greater expression of the cathepsin L2 gene occurred in stressed animals. Furthermore, methionine supplementation stimulates protein deposition, thereby not only ensuring a greater expression of genes related to synthesis, but also a lower expression of genes related to degradation.

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V. EFFECTS OF METHIONINE SUPPLEMENTATION ON THE REDOX STATE OF ACUTE HEAT STRESS-EXPOSED QUAILS

(Journal of Animal Science)

ABSTRACT- The aims of the present study were to evaluate the possible effects of heat stress on H_2O_2 production and to evaluate whether methionine supplementation could mitigate the deleterious effects on cell metabolism and the redox state induced by oxidative stress. Meat quails (*Coturnix coturnix coturnix*) were fed a diet that either met the nutritional demands for methionine (**MS**) or did not meet this demand (methionine deficient diet - **MD**) for seven days. The animals were either kept at a thermal comfort temperature (25˚C) or exposed to heat stress (**HS**) (38˚C for 24 hours, starting on the sixth day). Heat stress induced decreased food intake (P=0.0140), decreased daily weight gain (P<0.0001) and increased water intake (P=0.0211). A higher rate of hydrogen peroxide (**H_2O_2**) production was observed in HS animals (0.0802 vs. 0.0692 nmol of ROS produced/minute/milligram of protein; P=0.0042) and in animals fed with the MD diet (0.0808 vs. 0.0686 nmol of ROS produced/minute/milligram of protein; P= 0.0020). We observed effects of the interaction between diet and the environment on the activities of glutathione peroxidase (**GP-x**) and catalase (P=0.0392, P<0.0001, respectively). Heat stress induced higher levels of GP-x activity in animals on the MS diet and higher catalase activity in animals on the MD diet. Glutathione (**GSH**) levels were higher in animals on the MS diet (P= 0.0273) and in animals that were kept in thermal comfort (P=0.0018). The thiobarbituric acid reactive substances (**TBARS**) level was higher in HS animals fed with the MD diet (P=0.0386). Significant effects of the interaction between supplementation and environment were observed on uric acid concentration levels, which were higher in HS animals fed the MS diet (P=0.008), and on creatine kinase (**CK**) activity levels, which were lower in HS animals fed the MD diet (1620.33 U/L, P=0.0442). Our results suggest that under HS conditions, in which H_2O_2 production is increased, methionine supplementation was able to mitigate ROS-induced damage, possibly by increasing the activities of antioxidant elements such as GSH, GPx activity and uric acid concentration, which were present in higher levels in animals that were subjected to heat stress and fed the MS-diet.

Key Words: antioxidant system, H_2O_2, methionine, oxidative stress

INTRODUCTION

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 (Moustafa et al., 2004; Lee and Wei, 2005). 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 (**HS**) with oxidative stress (Mujahid et al., 2009; Mujahid et al., 2005), and the effects of HS are possibly due to an acceleration in the rate of ROS formation and/or an increase in ROS reactivity (Bai et al., 2003). Despite extensive studies on the subject, the mechanism through which high temperatures influence the cell redox state is not yet fully clear. The decreased expression of uncoupling proteins (**UCP**) that occurs in birds exposed to heat stress (Del Vesco and Gasparino, 2012) might be one such mechanism involved in ROS production because proteins such as UCP and adenine nucleotide translocases (**ANT**) could depolarize mitochondrial internal membranes, thus exerting (along with the other remaining factors) physiological control on oxidative phosphorylation (Sack, 2006).

The defenses of organisms against reactive oxygen species may be mediated by non-enzymatic or enzymatic antioxidants, mainly represented by the enzymes superoxide dismutase and catalase, and the glutathione defense system (Abrashev et al., 2008).

Glutathione (**GSH**) is involved in various biological actions, including protection against toxic compounds, the reduction of disulfide bonds in proteins, serving as a precursor for DNA synthesis, serving as a cysteine pool and, above all, protection against free radicals (Morand et al., 1997). GSH is biosynthesized in the majority of tissues from three precursor amino acids, one of which, cysteine, can be synthesized from a methionine precursor via the homocysteine transsulfuration pathway (Shoveller et al., 2005).

Estimates have suggested that approximately 50% of glutathione is generated from homocysteine and that under oxidative stress conditions, the production rate increases through stimulation of the transsulfuration pathway via the increased expression and activity of cistathionine ß-synthase and the inhibition of methionine synthase, which are the enzymes responsible for cysteine and methionine synthesis, respectively (Mosharov et al., 2000; Persa et al., 2004). In addition to playing an

important role in glutathione synthesis (reviewed by Stipanuk, 2004), some studies have shown the direct protective effects of methionine on oxidative stress (Stadtman et al., 2002; Moskovitz et al., 2001; Levine et al., 2000).

The present study was performed to test the hypothesis that heat stress induces oxidative stress and that under stress conditions, methionine supplementation may contribute to antioxidative systems, thus reducing oxidative stress damage. Thus, the present study aimed: to evaluate the effects of heat stress and methionine supplementation on meat quails performance, and to evaluate the role of heat stress in H_2O_2 production and whether methionine supplementation could mitigate the deleterious effects of oxidative stress on the metabolism and redox state in birds.

MATERIALS AND METHODS

The procedures in this experiment were approved by the Committee on Animal Care of the Universidade Estadual de Maringá - Brazil.

Experimental Design and Animals

Sixty-four male meat quails (*Coturnix coturnix coturnix*) were raised in floor pens with wood shavings as litter under thermal comfort temperature until 35 days of age and were fed a balanced diet to meet their nutritional demands (Rostagno et al., 2011). After 35 days, the animals were divided into four groups (n=16 per group) and transferred to floor pens in temperature-controlled rooms (20 m^2) (room 1 and 2 - two groups per room), where they remained for seven days while adapting to the thermal comfort temperature: 25 ± 0.9°C with 60 ± 1.2% relative humidity. The temperature chosen for thermal comfort followed the recommendations of Pinto et al. (2003). In each room, one group received feed that was calculated to meet the nutritional demand for methionine (**MS**), according to Rostagno et al. 2011; and the other group received feed without methionine supplementation (methionine deficient diet - **MD**) (Table 1). Animals in room 1 were kept at thermal comfort during the seven days of evaluation, while animals in room 2 were kept at thermal comfort for six days. At the end of the sixth day, these animals were subjected to heat stress of 38ºC for 24 hours. All animals were sacrificed by cervical dislocation at the end of the seventh day. Water and feed intake were measured during the full thermal comfort period and the 24 hours of stress period.

Table 1- Diet components and calculated and analysed contents of the experimental diets (as-fed basis)

	Experimental Diets[1]	
Ingredients	MS	MD
Corn 7.8% CP	58.25	58.40
Soy bean meal 46.7% CP	35.40	35.40
Soy oil	2.40	2.50
Salt	0.40	0.40
Calcareous 38%	1.34	1.34
Dicalcium phosphate 20%	1.33	1.33
DL- Methionine 99%	0.27	-
L- Lysine HCl 78%	0.15	0.15
L-Treonine 78%	0.06	0.06
Premix[2]	0.40	0.4
Total	100.00	100.00
Composition analysis (%)		
CP	21.16	21.02
Lysine digestible	1.15	1.15
Met+Cis digestible	0.84	0.57
Treonine digestible	0.78	0.78
Triptophane digestible	0.23	0.23
Valine digestible	0.90	0.90
Isoleucine digestible	0.83	0.83
Arginine digestible	1.33	1.33
Composition Calculated (%)		
Ca	0.90	0.90
P	0.41	0.41
Na	0.18	0.18
AME (kcal/kg)[3]	2999.06	3000.08

[1]MD, methionine deficient; MS, methionine supplementation.
[2]Supplied by kilogram of diet: retinyl-acetate, 3.44 mg; cholecalciferol, 50 µg; DL-α-tocopherol, 15 mg; thiamine, 1.63 mg; riboflavin, 4.9 mg; pyridoxine, 3.26 mg; cyanocobalamin, 12 µg; D-pantothenic acid – 9.8 mg; D-biotin – 0.1 mg; menadione, 2.4 mg; folic acid, 0.82 mg; niacinamide, 35 mg; selenium – 0.2 mg; iron, 35 mg; Copper, 8 mg; Manganese, 60 mg; Zn, 50 mg; I, 1 mg; choline: 650 mg; salinomycin: 60 mg; avilamycin: 5 mg; Butyl hydroxy toluene, 80 mg.
[3]AME= apparent metabolizable energy.

Body weight and sample collection

To calculate the weight gain of the quails under thermal comfort and stress conditions, the specimens were weighed individually at the beginning and the end of the thermal comfort and stress periods, respectively.

The livers, legs, breasts and abdominal fat of six specimens from each treatment group (comfort MS, comfort MD, stress MS, stress MD) were weighed to obtain the proportional organ weights, which were calculated as (organ weight/bird weight) x 100.

Additionally, blood was collected from these animals for uric acid and creatinine content, and plasma creatine kinase (**CK**) activity analysis (n=6). Blood was collected from the jugular veins into heparin tubes and was kept on ice. After centrifugation (3.024 × g, 10 min, 4°C), plasma was collected and stored at -20ºC until analyzed.

Uric acid, creatinine and creatine kinase activity analyses were performed according to colorimetric methods with the following kits: uric acid MS 80022230171, creatinine-PP MS80022230066 and CK-NAC-PP MS 80022230088, respectively, following the manufacturer's recommendations (Gold Analisa, Belo Horizonte, Minas Gerais, Brazil). One unit (U) of creatine kinase activity was defined as the amount of enzyme needed to convert 1 mmol of creatine into CrP (creatine phosphate)/min at 37°C, pH 9.0.

Six quail livers from each treatment group were collected for thiobarbituric acid reactive substances (**TBARS**), GSH, catalase activity, and glutathione peroxidase activity analysis. All collected samples used in biochemical analyses were frozen in liquid nitrogen and stored at -80ºC until analyzed.

The production of ROS was evaluated in mitochondria isolated from the livers of four quails from each treatment group.

ROS Production

Four quails from each treatment group were sacrificed to analyze ROS production. The livers were collected for mitochondrial isolation and subsequent analysis of mitochondrial ROS content. For mitochondrial isolation, the livers were placed in a cooled Becker container with isolation medium (0.2 M mannitol, 0.075 M sucrose, 2.0 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, 100 µM phenylmethylsulphonylfluoride (**PMSF**) and 50 mg fatty acid-free bovine serum albumin) (all the products were obtained from Sigma-Aldrich, St. Louis, Missouri, USA) and sliced with scissors. The livers and isolation medium were transferred into a Dounce homogenizer. The homogenates were filtered and subjected to two sequential centrifugations at 536 x g and 7080 x g for 10 min at - 4ºC each. The precipitates were washed twice by centrifugation at 6392 x g for 10 min at - 4ºC. Mitochondria were homogenized in the same medium at a protein concentration of 80-100 mg/mL (Bracht et al., 2003).

The protein content of the sub-cellular fractions was determined according to the method described by Lowry et al. (1951) with bovine albumin as a standard and a spectrophotometer set for 700 nm wavelength.

The mitochondrial ROS production level was estimated by measuring the linear increase in fluorescence. H_2O_2 induces dichlorofluorescein diacetate (**DCFH-DA**) oxidation that generates a fluorescent compound (2'-7' dichlorofluorescein) in the presence of horseradish peroxidase (**HRP**) (Zaccagnino et al., 2009).

Intact mitochondria (10 µL containing ≈ 0.8 mg protein) were added to 2 mL of 250 mM Mannitol, 10 mM HEPES, pH 7.2 buffer with 1.36 µM DCFH-DA and succinate (10 mM) + rothenone (10 µM) or succinate (10 mM) + rothenone + antimycin (15 µM). The reactions were started by the addition of 0.4 µM HRP, and the fluorescence levels were measured every minute for 10 minutes (Siqueira et al., 2005) (all the products were obtained from Sigma-Aldrich, St. Louis, Missouri, USA). The assays were performed in a spectrofluorometer with stirring. The results are expressed as the nmoles of produced ROS/minute/milligram of protein.

Enzymatic analysis

Six quails from each treatment group were used for glutathione peroxidase (**GP-x**) activity analysis. Immediately after slaughter, the livers were collected from the animals, pressed and stored in liquid nitrogen until analyzed. For enzymatic activity analysis, the samples were weighed and crushed in liquid nitrogen with a mortar and pestle. The crushed samples were transferred to cooled test tubes, to which 10% of the sample weight of iced phosphate buffer (0.1 M K_2HPO_4 and KH_2PO_4, pH 7.4) was added. The samples were homogenized with a Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged at 6,392 × g for 10 min at -4°C. and enzymatic activities were determined in the supernatants. The protein contents were determined according to Lowry et al. (1951) with bovine serum albumin as the standard.

Glutathione peroxidase activity was determined with hydrogen peroxide (H_2O_2) according to the method described by Paglia and Valentine (1967). The activity of the enzyme was determined by the amount of nicotinamide adenine dinucleotide phosphate (**NADPH**) oxidized that was detected using the spectrophotometer at a wavelength of 340 nm to measure the fluorescent signal emitted by the consumed NADPH. The fluorescent signal was measured every ten seconds for 50 seconds. The consumed NADPH was measured based on the decay in absorbance over the evaluated time

period.

For the reactions, 350 µL of 171 mM potassium phosphate buffer, 250 µL of 6 mM GSH, 300 µL of 0.9 mM NADPH, 10 µL of 2U/mL glutathione reductase (all the products were obtained from Sigma-Aldrich, St. Louis, Missouri, USA), 40 µL of supernatant and 520 µL of deionized H_2O were combined. The solution was homogenized, and 30 µL of 6 mM H_2O_2 were subsequently added. The fluorescence values were recorded every 10 seconds for 50 seconds. The activity was expressed as the nmoles of oxidized NADPH/mg of protein/minute.

Catalase activity was determined by the rate of hydrogen peroxide reduction into water and oxygen, according to the method proposed by Beers and Sizer (1952). The results were expressed as U/mg of protein.

Lipid peroxidation and GSH determination

Lipid peroxidation was measured in the liver homogenates by evaluating the thiobarbituric acid reactive substances (TBARS) with malondialdehyde (**MDA**; 1,1,3-3-tetrametoxipropane), according to Iguchi et al.(1993). The results were expressed as nmoles of TBARS/mg of protein.

For GSH analysis, the liver fractions were homogenized with perchloric acid and centrifuged at 15,000 x g for 1 min at 4º C. 5,5′-dithiobis-2-nitrobenzoate (**DNTB**) was added to the supernatants and the reactions were performed according to the method described by Teare et al. (1993). The results are expressed as µg/mg of protein.

Protein concentrations were determined according to the method described by Lowry et al. (1951) with bovine serum albumin as the standard.

Statistical analysis

The results are expressed as averages and standard deviations. The UNIVARIATE procedure was applied to evaluate data normality. The experiment was conducted in a completely randomized factorial design, with two environments (thermal comfort and heat stress) and two methionine supplementation levels (MS and MD). The averages were compared using Tukey’s test ($P<0.05$) (SAS Inst. Inc., Cary, NC).

RESULTS

Water and feed consumption, DWG and proportional organ weight

Figure 1 shows the water and feed intakes that were recorded for the two diets and two environments being studied. Methionine supplementation was not found to affect either factor; however, HS significantly increased water intake (P=0.0211) and decreased feed intake (P=0.0140).

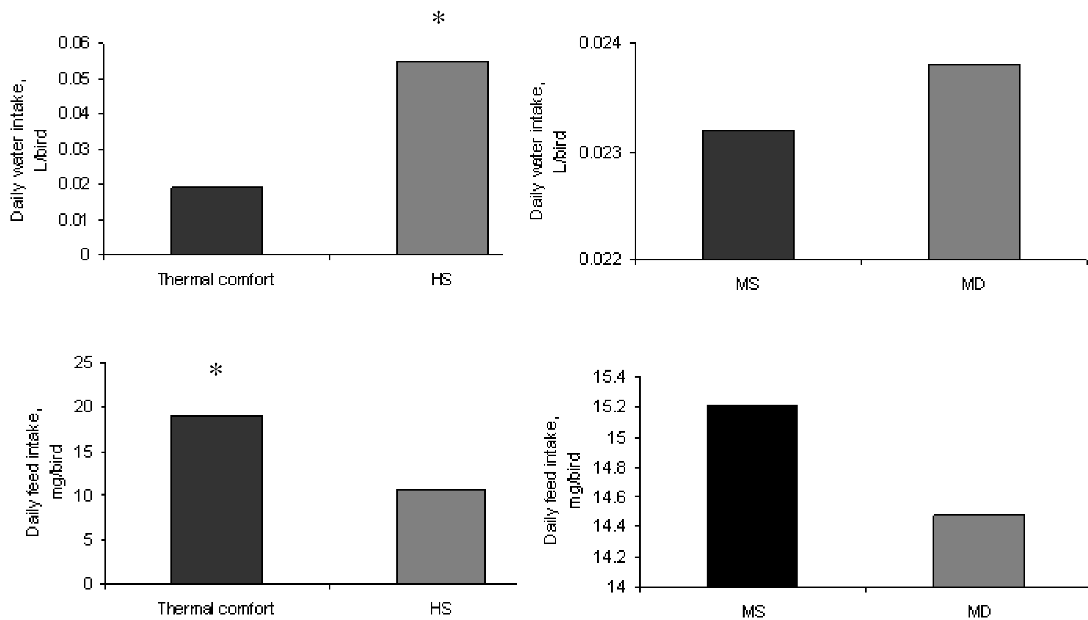

Figure 1 - Daily water and feed intake of quails in thermal comfort or heat stress (HS) and fed with methionine deficient (MD) or methionine supplementation (MS) diet. * P < 0.05 for thermal comfort *vs* HS group.

The effects of supplementation and high temperature on the daily weight gain (DWG) and the proportional weights of the liver, abdominal fat, breast and legs are shown in Table 2.

Table 2 – Daily weight gain (DWG) and proportional weight of organs and muscles

Diet-Environment	Diet X Environment	DWG (g)		Proportional weight (%)							
				Liver		Abdominal Fat		Breast		Legs	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MS[1]	Comfort	1.61	0.079	1.10	0.19	2.21	0.50	25.92	1.79	14.96	0.44
	Stress	0.31	0.036	0.91	0.39	1.01	0.51	25.79	0.97	14.96	0.53
MD[2]	Comfort	1.65	0.094	1.18	0.40	1.60	0.40	24.84	1.83	15.31	0.21
	Stress	-0.23	0.023	0.88	0.35	0.58	0.20	24.69	1.44	14.94	0.39
Main effects											
Diet	MS	1.17	0.41	1.01	0.30	1.61[a]	0.79	25.85	1.33	14.96	0.45
	MD	1.00	0.36	1.03	0.39	1.10[b]	0.62	24.77	1.53	15.12	0.35
Environment	Comfort	1.63[a]	0.41	1.14	0.29	1.91[a]	0.52	25.38	1.77	15.13	0.37
	Stress	0.04[b]	0.004	0.90	0.34	0.80[b]	0.42	25.24	1.28	14.95	0.43
Probabilities											
Diet		NS		NS		0.0319		NS		NS	
Environment		<0.0001		NS		0.0002		NS		NS	
Interaction		NS		NS		NS		NS		NS	

[a, b] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MS, methionine supplementation.
[2]MD, methionine deficient; MS, methionine supplementation.

Heat stress significantly decreased the DWG (1.63 vs. 0.04 g; P<0.0001), while methionine supplementation had no effect on this variable.

No effect of the interaction between diet and temperature was observed on abdominal fat. Animals that received the MS diet exhibited higher levels of abdominal fat (1.61 vs. 1.10%; P=0.0319). Regarding the environment, the percentage of abdominal fat was higher in animals that remained at the thermal comfort temperature (1.91 vs. 0.80%; P=0.0002). The treatments (comfort MS, comfort MD, stress MS, stress MD) did not influence the proportional weights of the liver, breast and legs.

ROS Production, oxidative stress markers and enzymatic activity in the liver

Hydrogen peroxide (H_2O_2) production was influenced by both of the study variables (Figure 2). Increased H_2O_2 production was observed in the HS animals (0.0802 vs. 0.0692 nmoles of ROS produced/minute/milligram of protein; P=0.0042). Regarding diet, ROS production was increased in the MD diet animals (0.0808 vs. 0.0686 nmoles of ROS produced/minute/milligram of protein; P=0.0020).

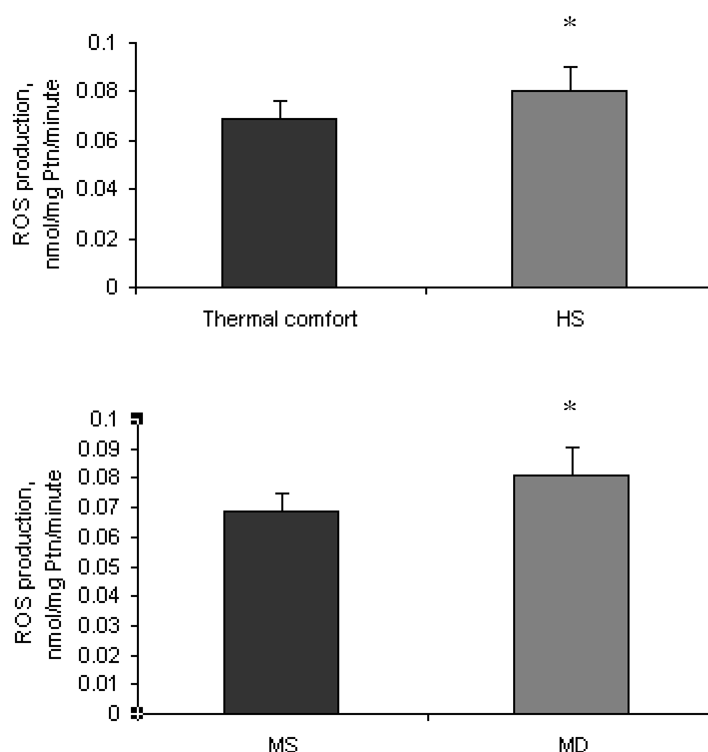

Figure 2 – Production of mitochondrial reactive oxygen species (ROS) in the liver of the quails exposed to thermal comfort or heat stress (HS), and fed with methionine supplementation (MS) or methionine deficient (MD) diet. The results are expressed in nanomoles (nm) of ROS produced per minute per milligram of protein. The results are shown as the average, and the standard deviation is represented by the vertical bars. *P<0.05 for thermal comfort *vs* HS group, and for MS *vs* MD group.

Total GSH concentrations, TBARS levels, catalase and glutathione peroxidase activities in the liver are summarized in table 3.

The total GSH concentration was influenced by both methionine supplementation and heat stress. Higher GSH levels were observed in animals that received the MS diet (11.92 vs. 8.87 µg/mg of protein; P= 0.0273) and in animals kept in thermal comfort (11.41 vs. 9.39 µg/mg of protein; P=0.0018).

Lipid peroxidation was evaluated by analyzing thiobarbituric acid-reactive substances (TBARS) in the liver. The HS quails had increased TBARS levels in comparison to birds kept in thermal comfort (11.39 vs. 7.36 nmoles TBARS/mg of

protein; P<0.0001). It was also possible to observe an interaction of the factors on TBARS levels (P=0.0386), with the highest values found in animals that were subjected to heat stress and the MD diet (13.14 nmoles TBARS/mg of protein, P=0.0386).

Table 3 – Analysis of oxidative stress markers and antioxidant enzyme activities in the liver

Diet-Environment	Diet x Environment	GSH[1]		TBARS[2]		GPx Activity[3]		Cat Activity[4]	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
MS[5]	Comfort	13.70	3.23	7.57^{b}	2.15	172.46^{bc}	8.08	29.91^{c}	4.80
	Stress	10.15	0.37	9.63^{b}	1.78	245.72^{a}	37.63	89.16^{b}	12.11
MD[6]	Comfort	9.12	1.22	6.15^{b}	1.12	162.54^{c}	18.04	31.66^{c}	3.33
	Stress	8.63	2.28	13.14^{a}	3.01	195.23^{b}	14.91	203.92^{a}	34.56
Main Effects									
Diet	MS	11.92^{a}	2.86	9.10	1.62	209.09	36.23	59.54	7.82
	MD	8.87^{b}	1.75	10.14	2.67	178.88	23.24	117.79	23.08
Environment	Comfort	11.41^{a}	3.33	7.36	3.85	167.49	14.28	30.79	4.04
	Stress	9.39^{b}	1.74	11.39	2.42	220.47	37.95	146.54	21.06
Probabilities									
	Diet	0.0273		NS		0.0037		<0.0001	
	Environment	0.0018		<0.0001		<0.0001		<0.0001	
	Interaction	NS[7]		0.0386		0.0392		<0.0001	

[a, b, c] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]GSH= glutathione.Expressed as ug/mg of protein.
[2]TBARS= Thiobarbituric acid-reactive substances. Expressed as nmol TBARS/ mg of protein.
[3]GP-x= glutathione peroxidase. Expressed as nmol of oxidized NADPH /mg of protein/minute.
[4]Cat=CatalaseExpressed as U/mg of protein.
[5]MS= methionine supplementation.
[6]MD= methionine deficient.
[7]NS= not significant.

Glutathione peroxidase activity in the quail livers were influenced by the interaction between temperature and diet (P=0.0392). The enzymatic activity levels were higher in HS animals fed with MS diet (245.72 nmoles of oxidized NADPH/mg of protein/minute).

Catalase activity was also influenced by an interaction between the two factors (P<0.0001). The highest levels of catalase activity were found in birds subjected to heat stress and the MD diet (203.92 U/mg of protein).

We also analyzed the uric acid and creatinine plasma concentrations, as well as the creatine kinase activity (Table 4). Uric acid concentrations were influenced by the interaction between diet and environment and the highest concentration was observed in HS birds fed with MS diet (P=0.008).

Table 4- Plasma analysis of uric acid, creatinine, and creatine kinase (CK) activity levels

Diet-Environment	Diet x Environment	Uric acid, mg/Dl		Creatinine, mg/dL		CK Activity,U/L	
		Mean	SD	Mean	SD	Mean	SD
MS[1]	Comfort	6.13^b	1.41	0.20^c	0.0002	2707.83^a	138.75
	Stress	10.52^a	2.26	0.23^b	0.0052	2377.00^a	176.99
MD[2]	Comfort	6.43^b	1.93	0.20^c	0.0003	2068.33^a	150.40
	Stress	5.42^b	0.70	0.30^a	0.002	1620.33^b	203.68
Main Effects							
Diet	MS	8.32	2.91	0.20	0.0001	2388.10	176.11
	MD	5.93	1.48	0.27	0.049	1989.70	131.44
Environment	Comfort	6.28	1.62	0.22	0.04	2542.04	183.18
	Stress	7.97	3.10	0.25	0.05	1844.30	173.05
Probabilities							
	Diet	0.0023		< 0.0001		NS[3]	
	Environment	0.0237		0.0049		0.0347	
	Interaction	0.0008		0.0049		0.0442	

[a, b, c] Mean values within a column with unlike superscript letters were significantly different (P<0.05).
[1]MS= methionine supplementation.
[2]MD= methionine deficient.
[3]NS= not significant.

Similarly, creatinine concentration (P=0.0049) and creatine kinase activity levels (P=0.0442) were also influenced by interactions between the factors. The highest creatinine levels (0.30 mg/dL) and lowest creatine kinase activity values (1620.33 U/L) were observed in animals that were on the MD diet and exposed to heat stress.

DISCUSSION

When birds are subjected to heat stress, environmental and postural mechanisms such as feed intake reduction are used primarily in an attempt to reduce metabolic heat production and increase heat dissipation (Mujahid et al., 2005). In our study, HS led to a significant decrease in feed intake, concomitant with an increase in water consumption. Daily weight gain (DWG) was decreased in birds exposed to heat stress. Contrary to our expectations, methionine supplementation, which has been linked directly to better performance in birds (Kauomar et al., 2011), had no effect on DWG. This result can be attributed to the age of the birds used in the present study, as the birds were not at the stage of greater muscle deposition. The age of the birds may have also influenced fat deposition in animals that were fed the MS diet. According to Nikolova et al. (2007), abdominal fat deposition in broiler chickens increased from 0.73% at 35 days old to 1.06% at 49 days old.

The exposure of birds to high temperatures has been associated constantly with decreased production efficiency. Additionally, heat-induced acute (Lin et al., 2006) and chronic stress (Yang et al., 2010) have been linked to metabolic changes that are related to oxidative stress. According to the authors, high temperature-induced stress caused increased ROS production and decreased activity of the mitochondrial respiratory chain.

Reactive oxygen species are produced mainly as a function of proton leakage during phosphorylative oxidation; however, the mechanism of ROS production in heat stressed birds is not yet fully known (Tan et al., 2010). Possible mechanisms of ROS production under heat stress conditions might be linked to protein oxidation and decreased activity in respiratory chain complexes or to other forms of mitochondrial damage. Such damage could be associated with body temperature (due to increased ROS production), followed by decreased body weight gain in birds that exhibit higher body temperatures (Mujahid et al., 2005). Because chemical and biochemical reaction rates increase with temperature, it is likely that an increase in body temperature would lead to ROS generation via the accelerated metabolic reactions in the cells and tissues (Lin et al., 2006).

Contrary to our expectations, hydrogen peroxide production was influenced not only by ambient temperature but also by methionine supplementation. A higher rate of H_2O_2 production was observed in HS quails and in those fed a diet without methionine supplementation.

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 and 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 day old quails, the expression of UCP-encoding mRNA is decreased in comparison to seven day old birds, and the expression was also decreased in meat quails that were exposed to acute heat stress.

Cellular mechanisms such as UCP, which reduce ROS production, cannot fully eliminate this process that occurs constantly during energy generation, in which 2 to 4% of the total amount of O_2 used as an electron acceptor is not fully reduced to water (Bottje et al., 2006). Therefore, to maintain the redox state and prevent oxidative stress, cells need to rely on mechanisms that are capable of eliminating the free radicals being produced, such as antioxidant enzymes.

In the present study, quails were subjected to heat stress to evaluate whether this factor could induce increased ROS production. Methionine supplementation in the diet was studied mainly to evaluate the possible role of this amino acid in antioxidant mechanisms, which might thus confer protection against cell damage from oxidative stress.

Antioxidant enzymes such as catalase and glutathione peroxidase are essential in the elimination of ROS. Both are able to protect cells mainly against H_2O_2. Studies have shown that under conditions where ROS production is elevated, such as in animals subjected to heat stress, the activities of these enzymes also increase (Tan et al., 2010; Yang et al., 2010).

The results of our study are in agreement with data available in the literature. Catalase activity is increased in quails exposed to heat stress in comparison to birds kept exclusively at thermal comfort. Catalase activity was highest in the HS birds subjected to a diet without methionine supplementation. This result could be related to a cellular compensation mechanism to quench increased H_2O_2 production because other defense mechanisms, such as glutathione peroxidase activity, were decreased in birds that received the methionine-deficient diet.

In addition to GSH, the glutathione system is comprised of the enzymes glutathione oxidase, glutathione peroxidase (GP-x) and glutathione reductase; therefore,

the antioxidant activity is dependent on the activity of the entire system. In our study, the greatest glutathione peroxidase activity was found in HS birds and fed a methionine-supplemented diet. This result can be explained by the cellular need for increased GP-x activity while under stress conditions (Yang et al., 2010) and by the larger amount of GSH available while under methionine supplementation that enable the glutathione antioxidant system to operate adequately.

Glutathione is a tripeptide synthetized from glutamate, glycine and cysteine. Cysteine can be synthetized from homocysteine from the precursor methionine (Shoveller et al., 2005); as such, when dietary methionine is available in an adequate amount, larger quantities can be directed towards cysteine synthesis via the transsulfuration pathway.

Some studies have shown that under stress conditions, the majority of metabolically available homocysteine is directed towards glutathione synthesis. This might be explained either by the increased activity of cystathionine ß-synthase, which is the enzyme responsible for cysteine production (Persa et al., 2004), or by the decreased activity of methionine synthase, which is the enzyme responsible for the synthesis of methionine from a homocysteine substrate; decreased activity of methionine synthase results from the oxidation of the cobalamine domains, which temporarily inactivates the enzyme (Muratore, 2010).

We observed higher amounts of GSH in animals that were fed the MS diet; this result could be explained by the increased amount of available methionine that led to an increased capacity to synthesize total GSH. Likewise, we also found lower GSH amounts in HS animals; we believe that this result is due to a greater requirement for glutathione to target the increased ROS production under stress conditions. Previously, Mager and Kruijff (1995) observed that cells exposed to high temperatures presented with a decreased maintenance of glutathione levels.

In addition to taking part in glutathione synthesis, methionine can act as a direct antioxidant. In cells, several ROS can react with methionine residues to generate methionine sulfoxide. This product can be catalyzed back to methionine by the enzyme methionine sulfoxide reductase, in a thioredoxin-dependent reaction. Therefore, methionine residues can act as direct antioxidants and protect the proteins where these residues are located and several other molecules from ROS (reviewed by Luo and Levine, 2009).

Uric acid, like methionine, has been reported in the literature as one of many elements that exhibits antioxidant activity. At the physiological pH range, it is commonly found as urate, a powerful ROS scavenger released into the bloodstream by deleterious reactions such as hemoglobin auto-oxidation or peroxide production by macrophages. Urate can inactivate an oxidant before they can react with biological molecules such as DNA, proteins and lipid membranes (Sautin and Johnson, 2008).

Birds possess specific mechanisms that contribute to increased urate concentrations in the blood, such as the absence of the enzyme uricase and the ability to encapsulate uric acid with proteins. Studies have indicated a relationship between higher uric acid concentrations and the decreased presence of oxidative stress markers (Klandorf et al., 2001; Simoyi et al., 2002).

In our work, we observed increased uric acid concentrations in quails that were exposed to heat stress and a MS diet. This suggests that stress demands an increased concentration of this antioxidant in the plasma and that this increased level results from methionine supplementation because the presence of this amino acid supplement can increase feed intake and concomitantly glycine intake, which is a necessary element for uric acid synthesis. Methionine supplementation (Bunchasak et al., 2006) and increased feed intake have been linked to increased plasma uric acid concentration levels in broiler chickens (Machin et al., 2004).

Thiobarbituric acid-reactive substances in the liver were used as biological markers of the extent of lipid peroxidation damage caused by increased H_2O_2 production. Creatine kinase activity in quail plasma was also used as a marker, as increased concentrations of this enzyme in the plasma have been linked to decreased membrane integrity and were observed in birds exposed to heat stress (Willemsen et al., 2011). However, creatine kinase contains thiol groups that are easily oxidized by ROS, rendering it relatively unstable because the formation of internal disulfide bonds causes a loss of activity (Gunst et al., 1998).

We observed an interaction between the effects of ambient temperature and methionine supplementation on the levels of TBARS and creatine kinase activity; increased TBARS and decreased creatine kinase activity were found in HS animals that were fed a MD diet. Animals exposed to heat stress but fed the MS diet retained their TBARS levels and the enzymatic activities of these animals were significantly similar to those kept in thermal comfort.

In agreement with our study, Mujahid et al. (2009) observed increased TBARS levels in birds exposed to heat stress. The authors suggested that the negative effects caused by high temperatures could be attenuated with a dietary supplement of olive oil, which was linked to possible increases in UCP-encoding mRNA expression and decreases in ROS production.

Previous studies in the literature also link oxidative stress and decreased creatine kinase activity (Glaser et al., 2010; Aksenova et al., 2002), possibly via thiol group oxidation. The activity of the enzyme can be preserved by endogenous glutathione, which serves as a protective agent during half-life of the enzyme in circulation; the loss of activity under certain conditions cannot be recovered when the extracellular GSH concentration is decreased, even in the presence of thiol-reducing agents (Gunst et al., 1998).

In light of the known effects on bird performance that are caused by heat stress and methionine supplementation, we evaluated the creatinine concentrations in quail plasma to establish which condition induced greater muscle catabolism. We found that the plasma creatinine concentrations were highest in animals that were fed the MD diet and subjected to heat stress.

These results allow us to suggest that under heat stress conditions, in which the H_2O_2 production was highest, methionine supplementation could attenuate ROS-induced damage, possibly consequent to the increased antioxidant activity of GSH, GPx activity, and uric acid concentration, as these were present in larger amounts in animals that were exposed to heat stress and received methionine-supplemented feed.

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VI. ACUTE HEAT STRESS AND DIETARY METHIONINE EFFECTS ON IGF-I, GHR, AND UCP MRNA EXPRESSION IN LIVER AND MUSCLE OF QUAILS

(Genetics and Molecular Research)

ABSTRACT. This study evaluated the expression of insulin-like growth factor I (IGF-I), growth hormone receptor (GHR), and uncoupling protein (UCP) mRNA in muscle and liver of quails that were in thermal comfort or exposed to heat stress and that were fed diets with or without methionine supplementation. Meat quails were fed a diet that either met the nutritional demands for methionine (MS) or did not meet this demand (methionine-deficient diet, MD). The animals were either kept at a thermal comfort temperature (25˚C) or exposed to heat stress (HS, 38˚C for 24 hours starting on the 6th day). RNA was extracted from liver and breast muscle, and cDNA was synthesized and amplified using quantitative reverse transcription polymerase chain reaction. Animals that were fed the MS diet and remained at the thermal comfort temperature exhibited increased IGF-I mRNA expression in the liver (0.56 AU). The GHR mRNA expression in the liver and muscle was influenced by both of the study variables. Animals receiving the MS diet showed higher GHR expression, while increased expression was observed in animals at the thermal comfort temperature. The UCP mRNA expression in the muscle was influenced by both methionine supplementation and heat stress. Higher expression was observed in animals that received the MD diet (2.29 *vs* 3.77 AU) and in animals kept in thermal comfort. Our results suggest that heat stress negatively affects the expression of growth-related genes and that methionine supplementation is necessary to appropriately maintain the levels of IGF-I, GHR, and UCP transcripts for animal metabolism.
Key words: Growth hormone receptor; Insulin-like growth factor I; Heat stress; Methionine; Uncoupling protein

INTRODUCTION

The growth rate is related to the feed efficiency and the deposition of muscle mass. The efficiency of an animal to convert food into muscle is related to the efficiency of energy production. Studies show that birds with lower ATP production because of lower efficiency of ATP production from substrate in the mitochondria have poor feed efficiency or feed conversion (Bottje and Carstens, 2009). The efficiency of

energy production depends on not only perfect coordination between the respiratory chain complexes but also the availability of nutrients and an antioxidant system that protects the mitochondria from damage that is generated during the production of ATP.

The broiler performance is also affected by the environmental conditions to which the animals are exposed. High environmental temperatures cause low feed intake, poor nutrient utilization efficiency, low weight gain, low egg production, and poor feed efficiency (Aksit et al., 2006; Menten et al., 2006). This negative effect on performance is mainly a result of reduced T3 and T4 levels, changes in water and ion balances, the kinetics of important enzymes that change the ratio of anabolic and catabolic products, immune depression, and changes in growth hormone levels (Barbour et al., 2010).

In addition to environmental temperature (Gabillard et al., 2003; Gabillard et al., 2006), studies show that the expression of growth-related hormones, such as insulin-like growth factor I (IGF-I) and growth hormone receptor (GHR), may be influenced by other factors, such as nutrition (Katsumata et al., 2002; Gasparino et al., 2012). According to Kimball and Jefferson (2004), amino acids play a key role in regulating some cellular processes, such as the regulation of gene expression by mRNA modulation. Still, according these authors, the cells are able to recognize the availability of amino acids and generate changes in translational signaling pathways, which are also regulated by hormones and growth factors.

Because of its importance in the deposition of muscle tissue and feathers, and in biochemical processes (donor of methyl groups, -CH3), methionine is the first limiting amino acid for broilers. The requirement of methionine and cystine (Met + Cys) depends on the performance parameters that are evaluated, with a higher requirement of sulfur amino acids for feed conversion and breast yield than for weight gain (Schutte and Pack, 1995). The main function of methionine is to allow the animal growth (muscle mass). However, methionine has a complex metabolism that is involved in several functions in the animal organism, such as the methylation of RNA, DNA, proteins, and lipids, and the production of various cellular components, such as glutathione, which is the main antioxidant system in the cytosol of the cells, polyamines (spermine and spermidine), taurine, which facilitates the digestion of lipids and also acts as an antioxidant in the cell membrane (Stipanuk, 2004), and H_2S, which has an anti-inflammatory action (Bauchart-Thevret et al., 2009).

This study was performed to test the hypothesis that heat stress can damage cellular metabolism, influencing the expression of genes related to growth and ATP

production, and that supplementing methionine could minimize the heat stress effect on the expression of such genes. Thus, this study aimed to evaluate the expression of IGF-I, GHR, and uncoupling protein (UCP) mRNA in muscle and liver of quails that were in thermal comfort or exposed to heat stress (38ºC for 24 h) and that were fed diets with or without methionine supplementation.

MATERIAL AND METHODS

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

The experiment was conducted on the Iguatemi Experimental Farm of the Universidade Estadual de Maringá-UEM. Sixty-four meat quails (*Coturnix coturnix coturnix*) were raised conventionally until 35 days of age and were fed a balanced diet to meet their nutritional demands. After 35 days, the animals were divided into 4 groups (N = 16 per group) and transferred into temperature-controlled rooms (room 1 and 2; 2 groups per room), where they remained for 7 days while adapting to the thermal comfort temperature: 25 ± 0.9°C with 60 ± 1.2% relative humidity. The temperature chosen for thermal comfort followed the recommendations of Pinto et al. (2003). In each room, 1 group received feed that was calculated to meet the nutritional demand for methionine (MS) according to Rostagno et al. (2011), and the other group received feed without methionine supplementation (methionine-deficient diet, MD) (Table 1). Animals in room 1 were kept at thermal comfort during the 7 days of evaluation, while animals in room 2 were kept at thermal comfort for 6 days. At the end of the 6th day, these animals were subjected to heat stress of 38ºC for 24 hours. All animals were sacrificed by cervical dislocation at the end of the 7th day. Water and feed intake were measured during the full thermal comfort period and the 24 h of stress, and it was calculated as the difference between the amount of feed offered and the feed residue at the end of the trial. The feed conversion ratio was calculated by dividing feed intake by weight gain, and it was corrected for mortality. To calculate the weight gain of the quails under thermal comfort and stress conditions, the specimens were weighed individually at the beginning and the end of the thermal comfort and stress periods, respectively.

Table 1- Experimental diets centesimal composition (expressed as-fed basis)

Ingredients	Experimental Diets MS	MD
Corn 7.8% CP	58.25	58.40
Soy bean meal 46.7% CP	35.40	35.40
Soy oil	2.40	2.50
Salt	0.40	0.40
Calcareous 38%	1.34	1.34
Dicalcium phosphate 20%	1.33	1.33
DL- Methionine 99%	0.27	-
L- Lysine HCl 78%	0.15	0.15
L-Treonine 78%	0.06	0.06
Premix*	0.40	0.4
Total	100.00	100.00
Composition analysis (%)		
CP	21.16	21.02
Lysine digestible	1.15	1.15
Met+Cis digestible	0.84	0.57
Treonine digestible	0.78	0.78
Triptophane digestible	0.23	0.23
Valine digestible	0.90	0.90
Isoleucine digestible	0.83	0.83
Arginine digestible	1.33	1.33
Composition Calculated (%)†		
Ca	0.90	0.90
P	0.41	0.41
Na	0.18	0.18
AME (kcal/kg)	2999.06	3000.08

MD, methionine deficient; MS, methionine supplementation.
*Supplied by kilogram of diet: retinyl-acetate, 3.44 mg; cholecalciferol, 50 µg; DL-α-tocopherol, 15 mg; thiamine, 1.63 mg; riboflavin, 4.9 mg; pyridoxine, 3.26 mg; cyanocobalamin, 12 µg; D-pantothenic acid – 9.8 mg; D-biotin – 0.1 mg; menadione, 2.4 mg; folic acid, 0.82 mg; niacinamide, 35 mg; selenium – 0.2 mg; iron, 35 mg; Copper, 8 mg; Manganese, 60 mg; Zn, 50 mg; I, 1 mg; choline: 650 mg; salinomycin: 60 mg; avilamycin: 5 mg; Butyl hydroxy toluene, 80 mg.

For gene expression analysis, samples of the liver and breast muscle (Pectoralis superficialis) were collected and stored in RNA Holders (BioAgency Biotecnologia, São Paulo, Brasil) at -20ºC until total RNA extraction.

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer instructions, at a ratio of 1 mL for every 100 mg tissue. All utilized materials were previously treated with an RNase inhibitor (RNase AWAYs, Invitrogen, Carlsbad, CA, USA). Tissue specimens with Trizol were ground using an electric Polytron homogenizer until their complete dissociation, after which,

200 μL chloroform was added, and the samples were manually homogenized for 1 min. Samples were then centrifuged for 15 minutes at 12,000 rpm and 4ºC, the liquid phase was collected and transferred to clean tube, and 500 μL isopropanol was added. Samples were 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 75% ethanol. The material was again centrifuged for 5 minutes at 12,000 rpm, and the supernatant was discarded. The pellet was dried for 15 minutes and then resuspended in RNAase-free ultrapure water.

The total RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. The integrity of the RNA was evaluated on a 1% agarose gel in the presence of 0.5 mg/mL ethidium bromide and visualized with ultraviolet light. RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove possible genomic DNA contamination according to the manufacturer recommendations.

Complementary DNA (cDNA) was prepared using the SuperScriptTM First-Strand Synthesis Super Mix kit (Invitrogen Corporation, Brazil), according to the manufacturer instructions. The cDNA mixture contained 6 mL total RNA, 1 mL oligo (dT: 50 μM oligo (dT)20) and 1 mL annealing standard. The reaction was incubated for 5 min at 65ºC and then placed on ice for 1 min. Subsequently, 10 μL 2X First-Strand Reaction Mix and 2 μL solution containing the enzyme reverse transcriptase SuperScript III and RNAase inhibitor were added. The solution was incubated for 50 min at 50ºC to allow cDNA to be synthesized. The reaction was then incubated for 5 min at 85ºC and immediately placed on ice. Samples were stored at -20ºC until analysis.

Real-time PCR used the fluorescent dye SYBR GREEN (SYBR GREEN PCR Master Mix, Applied Biosystems, Carlsbad, CA, USA). Real-time PCR analyses were performed in the apparatus StepOnePlus v.2.2 (Applied Biosystems, Carlsbad, CA, USA). All reactions were performed under the same analysis conditions and were normalized by the signal of a passive reference dye (ROX Reference Dye, Invitrogen, Carlsbad, CA, USA) to correct reading fluctuations caused by volume variations and evaporation during the reaction.

The UCP primers that were used in the reactions were designed according to Ojano-Dirain et al. (2007), and the GHR and IGF-I primers were designed at www.idtdna.com, using gene sequences from www.ncbi.nlm.nih.gov (access No. NM001001293.1 and FJ977570.1, respectively) (Table 2). We tested 2 housekeeping

genes, the ß-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes, and we used the GAPDH gene (access No. NM_204305) because it presented better efficiency in the reaction. All analyses were performed in a volume of 25 μL and in duplicate.

Table 2- Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Amplicom (bp)	Annealing Temperature (ºC)	Primers sequence (5'- 3')
GHR	145	60ºC	AACACAGATACCCAACAGCC
			AGAAGTCAGTGTTTGTCAGGG
IGF-I	140	60ºC	CACCTAAATCTGCACGCT
			CTTGTGGATGGCATGATCT
UCP	41	60ºC	GCAGCGGCAGATGAGCTT
			AGAGCTGCTTCACAGAGTCGTAGA
GAPDH	77	60ºC	GTTCTGTTCCCTTCTGTCTC
			GTTTCTATCAGCCTCTCCCA

The $2^{-\Delta CT}$ method was used to analyze the relative expression. The results are expressed as averages and standard deviations. The UNIVARIATE procedure was applied to evaluate data normality. The experiment was conducted in a completely randomized factorial design, with 2 environments (thermal comfort and heat stress) and 2 methionine supplementation hypotheses (MS and MD). The averages were compared using Tukey's test ($P < 0.05$) (SAS Inst. Inc., Cary, NC, USA).

RESULTS

Methionine supplementation did not affect water and feed intakes and daily weight gain (DWG); however, HS significantly increased water intake and decreased feed intake and DWG (1.63 *vs* 0.04) (Figure 1).

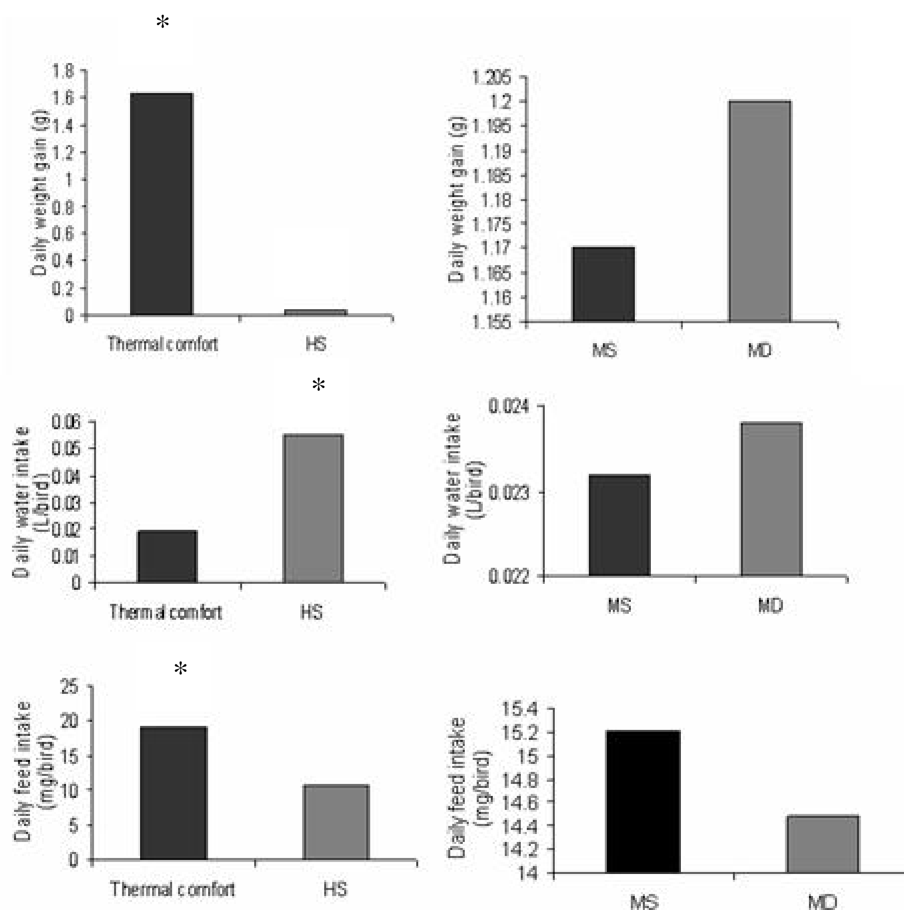

Figure 1 – Daily weight gain, daily water and feed intake of quails in thermal comfort or heat stress (HS) and fed with methionine deficient (MD) or methionine supplementation (MS) diet. * $P < 0.05$ for thermal comfort vs HS group.

The effect of the interaction between diet and temperature on the expression of IGF-I mRNA in the liver was observed. Animals fed the MS diet that remained at the thermal comfort temperature exhibited increased IGF-I expression (0.56 AU), and lower IGF-I mRNA expression was observed in animals that were fed the MD diet in comfort and heat stress (0.19 and 0.24 AU, respectively).

The expression of GHR mRNA in the liver was influenced by both of the study variables. Animals receiving the MS diet showed higher expression (309.93 AU) than those receiving the MD diet, and increased expression was observed in animals from the thermal comfort condition (309.72 AU).

The expression of UCP mRNA was influenced only by diet. Animals fed the MD diet showed increased expression of UCP mRNA in the liver (0.040 AU) (Table 3).

Table 3 - Expression of the IGF-I, GHR and UCP genes in the liver of quails

		IGF-I		GHR		UCP	
		Mean	SD	Mean	SD	Mean	SD
MS[1]	Comfort	0.56a	0.06	314.71	44.66	0.028	0.002
	Stress	0.47b	0.03	305.14	34.43	0.029	0.004
MD	Comfort	0.19c	0.02	304.73	41.99	0.036	0.004
	Stress	0.24c	0.03	217.89	32.01	0.045	0.008
Main Effects							
Diet	MS	0.51	0.06	309.93a	37.27	0.028b	0.003
	MD	0.21	0.04	261.31b	57.88	0.040a	0.008
Environment	Comfort	0.37	0.002	309.72a	40.47	0.037	0.010
	Stress	0.35	0.001	261.52b	55.88	0.032	0.005
Probabilities							
	Diet	<0.0001		0.0271		0.0004	
	Environment	0.3841		0.0281		0.0737	
	Interaction	0.0036		0.0686		0.1430	

[1]MD, methionine deficient; MS, methionine supplementation
[a, b, c] Mean values within a column with unlike superscript letters were significantly different (P<0.05).

In the muscle, no effect of the interaction between diet and temperature on the expression of the evaluated genes was observed. IGF-I expression was influenced only by diet. Animals fed the MS diet showed increased expression of IGF-I mRNA in the muscle (0.025 AU) (Table 4).

The highest GHR mRNA expression was observed in animals fed the MS diet (0.48 AU). Regarding environment, HS caused lower GHR mRNA expression than the thermal comfort condition.

The UCP mRNA expression was influenced by both methionine supplementation and heat stress. Higher expression was observed in animals that received the MD diet (3.77 AU) and in animals kept in thermal comfort (3.41 AU) (Table 4).

Table 4 - Expression of the IGF-I, GHR and UCP genes in the muscle of quails

		IGF-I		GHR		UCP	
		Mean	SD	Mean	SD	Mean	SD
MS[1]	Comfort	0.026	0.0035	0.57	0.09	2.76	0.19
	Stress	0.025	0.0016	0.39	0.04	1.81	0.12
MD	Comfort	0.014	0.0016	0.44	0.05	4.06	0.48
	Stress	0.013	0.0010	0.23	0.01	3.48	0.14
Main Effects							
Diet	MS	0.025a	0.003	0.48a	0.12	2.29b	0.53
	MD	0.014b	0.001	0.34b	0.12	3.77a	0.45
Environment	Comfort	0.020	0.007	0.51a	0.098	3.41a	0.77
	Stress	0.019	0.006	0.31b	0.087	2.65b	0.89
Probabilities							
	Diet	<0.0001		0.0003		<0.0001	
	Environment	0.3268		<0.0001		0.0001	
	Interaction	0.8497		0.6777		0.2106	

[1]MD, methionine deficient; MS, methionine supplementation
[a, b] Mean values within a column with unlike superscript letters were significantly different (P<0.05).

DISCUSSION

In animal production, performance (e.g., feed and reproduction efficiency) is expressed according to the animal's genetic background, the environment to which it is exposed, and the interaction between genetics and the environment. Thus, this study was conducted to test the hypothesis that methionine supplementation could mitigate the deleterious effects of heat stress on cell metabolism.

Animal growth is mainly due to protein deposition, which is given by a balance between protein synthesis and degradation. Reports suggest that these 2 distinct paths are products of the same biological pathways (Sacheck et al., 2004), and that hormone concentrations and diet are factors that can determine which of these paths will prevail.

Supplementing methionine in the diet may lead to protein deposition by stimulating body protein synthesis through the induction of greater amounts of IGF-I and GHR. Although how changes in amino acid supplementation can initiate changes in expression of genes such as GH and IGF-I is not yet fully clarified, it is suggested that

the mammalian target of rapamycin (mTOR) activity, an enzyme involved in regulating protein synthesis, may mediate this effect (Stubbs et al., 2002).

We observed that IGF-I and GHR mRNA expression was lower in both tissues that were evaluated in animals receiving the MD diet and in those exposed to heat stress. In the literature, it is possible to find other results about the influence of diet on somatotropic axis gene expression (Louveau and Le Dividich, 2002; Stubbs et al. 2002). According to Stubbs et al. (2002), the lower IGF-I mRNA expression in the liver of sheep fed with diets without methionine supplementation may be because of the ability of methionine to affect the release of IGF-I that induced by GH. Because the authors did not observe reduced release of IGF-I when the animals received a diet with a reduced total amino acid content, they suggested that restricted methionine can selectively block the transcriptional response to GH and affect the expression of IGF-I in the liver. Louveau and Le Dividich (2002) suggested that reduced IGF-I expression in animals suffering food restriction or a nutrient deprivation may be associated with reduced binding capacity of GH and/or lower levels of GHR in the liver. Therefore, adequate nutrition is indeed required for the maturation of the GH/IGF-I axis.

A result that should be highlighted is that we observed a significant interaction effect on the IGF-I mRNA expression in the liver, with the highest expression observed in animals that were maintained in thermal comfort and fed the MS diet. These beneficial effects of methionine supplementation are due in part to the fact that methionine participates in not only protein synthesis but also other important metabolic reactions to maintain adequate performance, such as acting as a donor of methyl groups (Simon, 1999), participating in glutathione synthesis as a sulfur donor (Wu and Davis, 2005), and participating in the synthesis of cysteine and S-adenosylmethionine, which is responsible for the biosynthesis of a variety of cellular compounds, such as creatine, carnitine, phospholipids, and proteins (Stipanuk, 2004).

Regarding heat stress, we can say that this factor not only influenced the expression of growth-related genes but also reduced the DWG and feed intake. Willemsen et al. (2011) observed lower IGF-I blood levels in broilers that were submitted to heat stress, which also presented lower T3 and T4 levels, higher corticosterone levels, higher thiobarbituric acid reactive substances concentrations, and higher antioxidant enzyme activity. The authors suggested that heat stress may trigger oxidative stress, thereby contributing to a lower metabolic rate. Besides reduced production efficiency and induced reactive oxygen species (ROS) production, exposure

to high temperatures is also associated with reduced activity of the mitochondrial respiratory chain (Yang et al., 2010).

The production of ROS in animals that are exposed to high temperatures has been correlated with the potential of the mitochondrial membrane and the expression of the UCP gene (Fink et al., 2005). UCPs are transporters in the inner mitochondrial membrane that divert ATP synthesis energy to heat production, which is catalyzed by protons leaking through the inner membrane (Ledesma et al., 2002). A greater membrane potential is related to greater ROS production, and greater UCP mRNA production is associated with the production of fewer free radicals and to less cellular damaged as a result of ROS. A greater membrane potential and less avUCP mRNA expression is observed in animals that are subjected to heat stress (Mujahid et al., 2009; Del Vesco and Gasparino, 2013).

Like in previous studies, we also observed that animals that were maintained in thermal comfort showed higher UCP mRNA expression in muscle and liver than animals that were subjected to heat stress. Additionally, animals fed the MD diet showed greater expression of UCP mRNA. Despite the beneficial effect of UCP in reducing damage to the DNA and cell proteins, as it reduces the production of free radicals a higher expression of UCP mRNA may worsen the feed vonversion ratio, as it reduces ATP production(Ojano-Dirain et al., 2007).

Our results allow us to suggest that heat stress of 38°C for 24 hours negatively affected the expression of genes related to growth, and that methionine supplementation is necessary to appropriately maintain the levels of IGF-I, GHR, and UCP transcripts for animal metabolism.

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CONCLUSÕES GERAIS

A análise dos nossos resultados de forma conjunta, leva à conclusão de que o estresse térmico induz o estresse oxidativo e maior proteólise; e que a suplementação de metionina pode atenuar os efeitos do estresse, contribuindo para maior expressão e atividade de elementos relacionados à atividade antioxidante, e pode estimular a deposição protéica, não apenas por garantir maior expressão de genes relacionados à síntese, mas também, menor expressão dos genes relacionados à degradação.