UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

EFEITO DA SUPLEMENTAÇÃO DE METIONINA NA FORMA LIVRE E DIPEPTÍDEO SOBRE A EFICIÊNCIA PRODUTIVA E REGULAÇÃO DA EXPRESSÃO GÊNICA EM FRANGOS DE CORTE SOB ESTRESSE TÉRMICO

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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
Março - 2019

Dados Internacionais de Catalogação na Publicação (CIP)
(Biblioteca Central - UEM, Maringá, PR, Brasil)

Sousa, Fabiana Cristina Belchior de

S725e Efeito da suplementação de metionina na forma livre e dipeptídeo sobre a eficiência produtiva e regulação da expressão gênica em frangos de corte sob estresse térmico / Fabiana Cristina Belchior de Sousa. -- Maringá, 2019.

xv, 88 f. : il. color.

Orientadora: Profª. Drª. Eliane Gasparino. Coorientadora: Prof.ª Dr.ª Ana Paula Del Vesco. Tese (doutorado) - Universidade Estadual de

Maringá, Centro de Ciências Agrárias, Programa de Pós-Graduação em Zootecnia, 2019.

1. Frango de corte - Estresse térmico. 2. Frango de corte - Metionina. 3. Estresse oxidativo. 4. Epigenética. I. Gasparino, Eliane, orient. II. Del Vesco, Ana Paula, coorient. III. Universidade Estadual de Maringá. Centro de Ciências Agrárias. Programa de Pós-Graduação em Zootecnia. IV. Título.

CDD 23.ed. 636.513

Síntique Raquel Eleuterio – CRB 9/1641

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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

APROVADA em 13 de março de 2019.

Profª Drª Sandra Maria Simonelli

Profª Drª Fernanda Losi Alves de Almeida

Profª Drª Simara Márcia Marcato

Profª Drª Adriana Gonela

Profª Drª Eliane Gasparino
Orientadora

Meu coração tem medo de sofrer – disse o rapaz ao Alquimista, numa noite em que olhavam o céu sem lua.

Diga a ele que o medo de sofrer é pior do que o próprio sofrimento. E que nenhum coração jamais sofreu quando foi em busca de seus sonhos, porque cada momento de busca é um momento de encontro com Deus e com a Eternidade.

"Cada momento de busca é um momento de encontro" disse o rapaz ao seu coração. "Enquanto procurei meu tesouro, todos os dias foram dias luminosos, porque eu sabia que cada hora fazia parte do sonho de encontrar. Enquanto procurei esse meu tesouro, descobri no caminho coisas que jamais teria sonhado encontrar, se não tivesse tido a coragem de tentar coisas impossíveis..."

O Alquimista – Paulo Coelho

A Deu,s por permitir que eu chegasse até aqui.

Aos meus pais, Adão Belchior de Sousa e Valdenia Alves da Silva. Meu pai, homem trabalhador, honesto que nunca mediu esforços para dar a educação aos seus filhos. Minha mãe, mulher forte, batalhadora, que tem os filhos sempre em primeiro lugar. Aquela que sempre tem uma palavra de conforto em tantos momentos de ansiedade e desânimo. Aos meus irmãos, Fabio Belchior, Rayssa Belchior e Ravena Maria Belchior, com os quais, sei que posso contar em qualquer momento.

Aos meus sobrinhos, Diogo Marcos e Enzo Gabriel

Ao meu noivo, Marcos André Arrais de Sousa

Dedico

AGRADECIMENTOS

A Deus pela vida, pela proteção, que esteve comigo em todos os momentos, dando-me todos os dias coragem, sabedoria e forças ao longo dessa jornada.

Aos meus pais, por serem incansáveis, pelo esforço em enfrentar todas as dificuldades, pela compreensão e por sempre apoiar os meus estudos.

Aos meus irmãos Fabio, Rayssa e Ravena pela amizade, incentivo mesmo estando distante, sempre acreditaram em mim.

Ao meu noivo Marcos Arrais, pelo amor, paciência e comprenssão.

À minha orientadora professora Dr.ª Eliane Gasparino, por toda ajuda, ensinamentos pela atenção, compreensão, incentivos, conselhos e pela simplicidade. Sempre tem uma ideia e rápida solução. Serei eternamente grata pelo apoio, e oportunidade.

À coorientadora professora Dr.ª Ana Paula Del Vesco, pela colaboração fundamental no desenvolvimento desta tese, pelos ensinamentos, paciência, dedicação e gentileza. Muito Obrigada!

Ao grupo de pesquisa da professora Dr.ª Ana Paula Del Vesco, em em especial a Thaís Pacheco, Vittor Zancanela, pela colaboração funtamental. Meu Muito Obrigada!

À minha amiga Katiene Régia, por me ajudar a superar os momentos de desânimo, e comemorar comigo os momentos de alegria, mesmo distante.

Aos meus amigos Jhonny e Jaiton, pela amizada, gentileza. Sempre dipostos a ajudar.

A Letícia Toledo e Thaune Costa, pelo convívio e pela amizade que tornaram esses anos mais leves e divertidos.

À Angélica Khatlab, por toda a ajuda, amizade e apoio desde o primeiro dia que cheguei em Maringá. Em especial, quero agradecer também aos seus pais que sempre foram tão gentis e carinhos comigo.

As amigas que me acolheram tão bem em Maringá, Camila, Ana e Rayane.

Aos companheiros do programa de Pós-Graduação em Zootecnia.

À Universidade Estadual de Maringá e ao Programa de Pós Graduação em Zootecnia, por ter tornado possível a realização deste projeto.

A Capes, pelo apoio financeiro concedido mediante da bolsa de estudo.

Enfim, a todos os familiares, amigos e colegas que direto ou indiretamente contribuíram com essa realização.

BIOGRAFIA

Fabiana Cristina Belchiorde Sousa, filha de Adão Belchior de Sousa e Valdenia Alves da Silva, nasceu em Manoel Emidio, Estado do Piauí, no dia 07 de novembro de 1987.

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Em março de 2014, iniciou no mestrado no Programa de Pós-Graduação em Zootecnia da Universidade Federal do Piauí, área de concentração Produção Animal- Melhoramento Genético Animal, sob orientação da Professora Dr.ª Katiene Régia Silva Sousa. Em fevereiro de 2016, submeteu-se à banca examinadora para defesa da Dissertação de mestrado.

Em março de 2016, ingressou no Programa de Pó- Graduação em Zootecnia da Universidade Estadual de Maringá, em nível de doutorado, área de concentração Produção Animal- Melhoramento Genético Animal, sob orientação da Professora Dr.ª Eliane Gasparino.

Em março de 2019, submeteu-se à banca examinadora para defesada tese apresentada, como parte das exigências para obtenção do título de doutora em Zootecnia.

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IV. FREE AND DIPEPTIDE FORMS OF METHIONINE SUPPLEMENTATION REDUCE HEAT STRESS EFFECTS BY GENE REGULATION AND EXPRESSION

RESUMO

O desempenho das aves esta relacionado com muitos fatores, que devem ocorrer de maneira totalmente integrada e coordenada visando a máxima eficiência produtiva. A busca por animais cada vez mais eficientes deixa claro a necessidade de conhecer melhor como os fatores nutricionais da dieta, e os fatores externos, como a temperatura ambiental estão envolvidos no desempenho das aves, em função das modificações ocorridas devido a mudanças fisiológicas em nível celular e/ou molecular. Sendo assim, este trabalho teve como objetivos avaliar os efeitos do estresse térmico agudo e crônico e da suplementação de duas fontes de metionina, sobre a eficiência produtiva e a regulação da expressão gênica em frangos de corte. Para isso, no primeiro experimento os frangos de corte de 21-42 dias de idade foram avaliados em três períodos experimentais: 24 horas de avaliação (21 a 22 dias de idade); 10 dias de avaliação (22 a 32 dias de idade); e 20 dias de avaliação (21-42 dias de idade), os frangos foram criados em temperatura de conforto térmico (21ºC) e exposto ao estresse térmico contínuo de 30ºC. Em ambos os grupos, os animais foram alimentados com dieta sem suplementação de metionina (SM); com suplementação de metionina como aminoácido livre (DL-M); e com suplementação de metionina na forma de dipeptídeo (DL-MM). Para o segundo experimento, os frangos de corte de 22-42 dias de idade foram divididos em duas temperaturas ambientais: um grupo foi criado no conforto térmico de 21ºC e o outro grupo em temperatura elevada de 30ºC dos 22-42 dias. Para ambos os grupos, os frangos foram alimentados com dieta sem suplementação de metionina (SM); com suplementação de metionina como aminoácido livre (DL-M); e com suplementação de metionina na forma de dipeptídeo (DL-MM). Nestes experimentos, foram avaliados, o desempenho, a qualidade da carne, parâmetros sanguíneos, a relação heterófilo/linfócito, a expressão e a metilação do DNA na região promotora dos genes relacionados a capacidade antioxidante: glutationa peroxidase (*GPx*), glutationa sintetase (*GSS*), e alguns marcadores biológicos do estresse oxidativo. No primeiro trabalho, foi observado que o estresse térmico reduziu o consumo de ração e o ganho de peso dos animais. No entanto, a suplementação com metionina melhorou o ganho de peso. Aos 32 dias de idade, os frangos criados em condições de estresse térmico apresentaram menor teor de HDL e maior de LDL que os que foram criados em conforto

térmico e aves alimentadas com dieta SM apresentaram maior conteúdo de TGI. Aos 42 dias de idade não foi observado diferença entre os frangos criados no estresse térmico e alimentados com dieta DL-MM dos mantidos em conforto térmico. Para a carne do peito aos 42 dias de idade, os frangos de corte criados em condições de estresse térmico e alimentados com dieta SM apresentaram menor valor de pH final. Os frangos criados em ambiente de conforto térmico e alimentados com dieta DL-M e DL-MM tiveram menor perda por cocção que os animais alimentados com dieta SM. Sob estresse térmico, frangos de corte alimentados com DL-M tiveram a menor perda por cocção. A maior perda por descongelamento foi observada em aves alimentadas com dieta SM e a menor em animais alimentados com dieta DL-MM. Para a carne da perna, o estresse térmico reduziu o valor de pH final e elevou o valor do componente L*. Os frangos de corte alimentados com dietas SM e DL-MM apresentaram, respectivamente, os menores e maiores valores de pH final. Aves alimentadas com dieta SM apresentaram maior valor de descongelamento e perda por cocção. No segundo experimento em que se avaliaram os efeitos da suplementação de duas fontes de metionina e do estresse térmico crônico, observou-se que aves criadas sob estresse térmico crônico apresentaram significativamente maior relação Heterófilo/Linfócito. Para aves criadas sob condições de estresse térmico, frangos de corte alimentados com dieta DL-M apresentaram tendência de menor relação H/L que as aves alimentadas com dietas SM e DL-MM. Maior concentração de proteínas carboniladas e menor concentração de glutationa (GSH) foram significativamente observadas em aves criadas em estresse térmico crônico em relação as aves do conforto térmico. Ao comparar as aves criadas sob estresse térmico crônico, aves alimentadas com dieta DL-M apresentaram menor concentração de TBARS e proteínas carboniladas que aves recebendo dieta SM. Maior expressão dos genes *GPx* e *GSS* foi observada em frangos de corte criados em ambiente de estresse térmico crônico. As aves que receberam a dieta SM tiveram expressão da *GPx* aumentada, e maior expressão da *GSS* em comparação com os frangos de corte que receberam a dieta SM em temperatura de conforto térmico. Em condições de estresse crônico, aves alimentadas com dieta DL-MM apresentaram os maiores valores de metilação na região promotora dos genes *GPx* e *GSS*. Foi observado efeito negativo entre a metilação do DNA e os níveis de expressão gênica. Os frangos no estresse, apresentaram menores níveis de metilação e maiores níveis de expressão que aves criados em ambiente de conforto recebendo ambas as dietas. O estresse calórico reduziu o consumo de ração. Entanto, a suplementação com metionina melhorou o ganho de peso. Assim, nosso resultados sugerem que o estresse calórico agudo e crônico pode afetar o desempenho e alterar o metabolismo em frangos, e que a suplementação de metionina, independente da forma, pode ajudar a atenuar os efeitos do estresse mediante ação dos genes relacionados ao mecanismo antioxidante da glutationa.

Palavras-chaves: antioxidante, desempenho, estresse oxidativo, epigenética

ABSTRACT

The performance of the birds is a function of many factors, which must occur in a fully integrated and coordinated aiming to maximize productive efficiency.The search for increasingly efficient animals makes clear the need to know better how the nutritional factors of the diet, and external factors, as the ambient temperature are involved in the performance of the birds, due to the changes occurred due to physiological changes at the cellular and / or molecular leve. The objective of this study was to evaluate the effects of acute and chronic thermal stress and the supplementation of two sources of methionine on the production efficiency and the regulation of gene expression in broilers. For this, in the first experiment the broilers of 21-42 days of age were evaluated in three experimental periods: 24 hours of evaluation (21 to 22 days of age); 10 days of evaluation (22 to 32 days of age); and 20 days of evaluation (21-42 days of age), broiler chickens were reared at thermal comfort temperature (21ºC) and exposed to continuous thermal stress at 30ºC. In both groups, the animals were fed a diet without methionine supplementation (SM); with methionine supplementation as free amino acid (DL-M); and with methionine supplementation as dipeptide (DL-MM). For the second experiment, 22-42-day-old broiler chickens were divided into two environmental temperatures: one group was created in thermal comfort at 21ºC and the other at a high temperature of 30ºC from 22-42 days. For both groups, the broilers were fed a diet without methionine supplementation (SM); with methionine supplementation as free amino acid (DL-M); and with methionine supplementation in dipeptide form (DL-MM). Performance, meat quality, blood parameters, heterophile / lymphocyte ratio, DNA expression and methylation in the promoter region of genes related to antioxidant capacity: glutathione peroxidase (GPx), glutathione synthetase (GSS), and some biological markers of oxidative stress. In the first study, it was observed that the thermal stress reduced the feed consumption and the weight gain of the animals. However, methionine supplementation improved weight gain. At 32 days of age, broilers raised under heat stress had lower HDL and higher LDL levels than those raised in thermal comfort, and birds fed SM diet presented higher TGI contents. At 42 days of age no difference was observed between broilers raised in the thermal stress

and fed with DL-MM diet of those kept in thermal comfort. For broiler meat at 42 days of age, broiler chickens raised under thermal stress conditions and fed SM diet had lower final pH values. Broilers raised in a thermal comfort environment and fed with DL-M and DL-MM diet had loss of cooking than animals fed SM diet. Under thermal stress, broilers fed DL-M had the lowest cooking loss. The highest loss by thawing was observed in birds fed SM diet and the lowest in animals fed DL-MM diet. For the leg meat, the thermal stress reduced the final pH value and raised the value of the L *. Broilers fed SM and DL-MM diets presented, respectively, the lowest and highest final pH values. Birds fed SM diet showed higher thawing value and cooking loss. In the second experiment in which we evaluated the effects of supplementation of two sources of methionine and chronic thermal stress, we observed that birds raised under chronic thermal stress had a significantly higher Heterophile / Lymphocyte ratio. For birds raised under thermal stress conditions, broilers fed DL-M diet showed a tendency of lower Heterophile / Lymphocyte ratio than birds fed SM and DL-MM diets. Higher concentration of carbonylated proteins and lower concentration of glutathione (GSH) were significantly observed in birds raised in chronic thermal stress in relation to birds of thermal comfort. When comparing birds raised under chronic thermal stress, birds fed DL-M diet presented lower concentration of TBARS and carbonylated proteins than birds receiving SM diet. Higher expression of the GPx and GSS genes was observed in broilers raised in a chronic thermal stress environment. Birds that received the SM diet had increased GPx expression and higher GSS expression compared to broiler chickens that received the SM diet at thermal comfort temperature. Under conditions of chronic stress, birds fed DL-MM diet had the highest values of methylation in the GPx and GSS promoter region. Negative effect was observed between DNA methylation and levels of gene expression. The chickens under stress had lower levels of methylation and higher levels of expression than birds raised in a comfort environment receiving both diets. Caloric stress reduced feed intake. However, methionine supplementation improved weight gain. Thus, our results suggest that acute and chronic caloric stress may affect performance and alter metabolism in broilers, and that methionine supplementation, regardless of form, may help to attenuate the effects of stress through the action of genes related to the antioxidant mechanism of glutathione.

Key words: antioxidant, performance, oxidative stress, epigenetic

I. INTRODUÇÃO

A avicultura industrial brasileira é um sistema de produção animal desenvolvido e avançado. Nas últimas décadas, esse segmento tem ganhado espaço na condição de produção privilegiada por contribuir com a obtenção de produtos finais de melhor qualidade e com menor tempo de produção. Esse desenvolvimento aconteceu por meio da evolução nos avanços tecnológicos, melhoria no manejo, controle sanitário, instalações, equipamentos, e pelo fato do Brasil apresentar alto nível de produção de milho e soja, que são os principais ingredientes que compõem as rações dos frangos de corte (Oliveira e Nääs, 2012; Sousa e Osaki, 2005).

Na alimentação avícola, o milho e o farelo de soja são os ingredientes que participam na formulação em maior quantidade na ração, no entanto, a composição de aminoácidos desses produtos não suprem totalmente as exigências nutricionais das aves, necessitando da inclusão de aminoácidos sintéticos para que as exigências dos animais sejam atendidas. A metionina é classificada como primeiro aminoácido limitante pois desempenha papel importante para o crescimento, e é utilizada para deposição de penas, além disso, participa da síntese proteica (Bunchasak, 2009).

Além dos fatores citados acima a avicultura deve muito da sua evolução ao melhoramento genético, uma vez que os frangos são obtidos de linhagens selecionadas para o crescimento rápido, melhor eficiência alimentar e maior rendimento de cortes nobres, entre outras características (Havenstein et al., 2003; Patricio et. al., 2012). Destaca-se ainda no campo da pesquisa envolvendo a genética, a epigenética, que nos últimos anos, com o esforço para compreender a influência da dieta nos processos de epigenéticos revelou que o metabolismo pode influenciar a expressão gênica (Hitchler e Domann, 2007) e os nutrientes da dieta podem modificar o padrão de metilação do DNA

(Zhang et al., 2017). Essa relação dinâmica entre nutrição e expressão gênica fornece uma perspectiva promissora sobre o crescimento e a saúde dos animais (Anderson et al., 2012; Reik, 2007).

Assim, o desenvolvimento do animal depende de mecanismos fisiológicos complexos, que agem de forma integrada. A manutenção adequada da homeostase é de fundamental importância para o organismo das aves. No entanto, sabe-se que os agentes estressores físicos, fisiológicos, nutricionais ou ambientais estão diretamente envolvidos com o desenvolvimento do animal (Altan et al., 2003). Entre esses agentes, a alta temperatura ambiental é o fator físico que mais interfere negativamente no crescimento e no desempenho produtividade dos animais, isso porque mudanças fisiológicas, que ocorrem no metabolismo das aves sob condições adversas, são responsáveis por causar redução na eficiência produtiva e rendimento de partes (Yahav, 2000; El-Kholv et al., 2017).

Além disso, o estresse térmico tem sido associado as alterações metabólicas que envolvem o estresse oxidativo, visto que, a maior produção de espécies reativas de oxigênio (ROS) e a menor atividade da cadeia respiratória mitocondrial são respostas fisiológicas ao estresse induzido por altas temperaturas (Lin et al., 2006; Yang et al., 2010). Segundo Yang et al. (2010) o estresse térmico também pode alterar a atividade de algumas enzimas envolvidas no sistema de defesa antioxidante.

Dessa forma, pode-se perceber que o desempenho das aves é função de muitos fatores, que devem ocorrer de maneira totalmente integrada e coordenada para máxima eficiência produtiva. Assim, 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 pelas mudanças fisiológicas em nível celular ou molecular (Del Vesco et al., 2014).

Apesar das diversas pesquisas que avaliam como as variáveis relacionadas ao ambiente e a nutrição exercem sobre a expressão de diversos genes que regem diversas rotas metabólicas, o mecanismo que atua no controle da expressão gênica, ainda não está totalmente conhecido. Sendo assim, este trabalho buscou avaliar os possíveis efeitos do estresse térmico agudo e crônico e da suplementação de metionina, sobre o desempenho e a regulação da expressão gênica em frangos de corte.

1.0 **Desempenho: Nutrição e Estresse térmico**

1.1 Metionina

Animais monogástricos são alimentados com base no conceito de proteína ideal. Desta maneira, as dietas são formuladas para atender às exigências dos animais e assim tornar mais eficiente os processos de mantença e crescimento (Emmert e Baker, 1997), reduzindo a excreção indevida de nitrogênio. Dessa forma, o conceito de proteína ideal propõe a redução do teor proteico das rações mantendo a capacidade de fornecimento dos aminoácidos essenciais dentro dos níveis recomendados (Oliveira Neto e De Oliveira, 2009).

A metionina é um aminoácido sulfurado essencial e o primeiro limitante nas dietas para frangos de corte à base de milho e farelo de soja (Kim et al., 2006). A metionina possui várias funções no organismo animal, incluindo a participação na síntese proteica (Li et al., 2007) e atua como doador de grupo metil que são necessários para várias reações metabólicas tais como, a síntese de colina e betaína (Corzo et al., 2006). A metionina também influencia no metabolismo lipídico, estimulando o catabolismo oxidativo dos ácidos graxos mediante seu papel na síntese de carnitina (Nukreaw et al., 2011), participa da síntese da glutationa, composto importante para a defesa do organismo contra o estresse oxidativo (Bunchasak, 2009).

Outra característica importante é que, além de ser utilizada como fonte de enxofre que pode ser doado para síntese de outros componentes químicos, a metionina tem grande participação na síntese da cisteína que é utilizada para a síntese da proteína corporal, para deposição de penas e pele, explicando a alta exigência desses aminoácidos pelas aves (Willke, 2014). Além disso, a metionina é precursora da S-adenosilmetionina (SAM), composto que desempenha papel principal como doador de grupo metil para a metilação de DNA (Dunlevy et al., 2006).

Em função da exigência em metionina, a suplementação de fontes industriais na dieta de aves é importante para se alcançar desempenho adequado. As principais fontes sintéticas de metionina utilizadas na alimentação de frangos de corte pode ser encontrada de diferentes formas, tais como DL-metionina (DL-Met), disponível comercialmente na forma de pó ou sua forma líquida como sal de sódio (DL-metionina-NA), metionina hidroxi-análoga (MHA), também comercializada na forma de pó, como sal de cálcio (MHA-Ca) ou na forma líquida como ácido livre (MHA-FA) (Leite et al., 2009).

Os análogos diferenciam-se da DL-metionina por possuírem uma molécula de hidroxila (OH), no lugar do grupamento amino (NH3+), localizado no carbono alfa da molécula (Oliveira Neto, 2014). As formas DL-Met em pó e o análogo MHA-FA são as fontes de suplementação mais usadas na produção de aves (Bunchasak, 2009). Além da utilização dessas fontes mais comuns na produção de aves, a metionina também pode ser encontrada na forma de dipeptídeo. O uso de metionina na forma de dipeptídeo é recente, e vem sendo administrado em organismos aquáticos, pela sua baixa solubilidade em água (Niu et al., 2018).

As proteínas fornecidas na dieta para os animais são hidrolisadas no trato digestório, produzindo aminoácidos livres, dipeptídeos, tripeptídeos, e outros polipeptídeos (Wu, 2013). Os peptídeos vêm atraindo atenção como novas fontes de nutrientes, isso porque algumas pesquisas têm mostrado que a utilização de peptídeos na dieta para animais e humanos pode ser nutricionalmente superior a utilização de aminoácidos livres, em virtude da absorção de aminoácidos na forma de peptídeos ser mais rápida e eficiente que os aminoácidos livre; os sistemas de transporte dos peptídeos são diferentes dos aminoácidos livres e isso pode minimizar a competição por locais de transporte; além disso, os peptídeos podem ser mais resistentes que os aminoácidos livres em condições em que o organismo apresenta estado de jejum, carências vitamínicas ou doenças intestinais (Sanioto, 2016; Tauqir, 2016).

A utilização de di, tri e tetrapeptídeos tem sido recomendada para organismos aquáticos visando menor perda em decorrência do meio aquoso, uma vez que os dipeptídeos podem ter menor lixiviação em água (Mamauag et al., 2012; Xie et al., 2017). Recentemente, trabalhos têm mostrado que a a suplementação com dipeptídeos na dieta também pode contribuir para reduzir o efeito causado por diferentes desafios, podendo minimizar o efeito adverso no desempenho do crescimento dos peixes (Li et al., 2018) e otimizar o efeito hipertrófico promovido pelo exercício (Coqueiro et al., 2017) e reduzir resposta inflamatória, hipermetabólica, a autofagia e atrofia muscular em ratos (Zhao et al., 2018).

Além disso a utilização de dipeptídeo de metionina *in vitro* é mais eficiente na promoção da síntese proteica do leite que a metionina livre, e ainda podem funcionar como moléculas sinalizadoras ativando vias de sinalização em células epiteliais mamárias de bovinos (Yang et al., 2015; Wang et al., 2018). Embora os resultados acima relatadas sejam importantes, o conhecimento sobre a utilização de metionina na forma de dipeptídeos para frangos de corte ainda não é conhecido.

1.2 Estresse térmico

A evolução no desenvolvimento de pesquisas na avicultura nos últimos anos esteve diretamente ligada com melhorias na área da nutrição, sanidade e do melhoramento genético. Essas mudanças contribuíram para obtenção de aves com maior taxa de crescimento e deposição de tecido muscular em menor período de tempo. Embora muitos avanços tenham sido realizados e contribuído para melhorias nesse setor, ainda assim, problemas na produção são encontrados.

A alta temperatura ambiental é uma das principais preocupações da indústria avícola, especialmente nas regiões mais quentes, pois a exposição ao calor afeta a produção das aves e tem impacto significativo no bem-estar e na eficiência alcançada na produção (Rajkumar et al., 2011; El-Tarabany, 2016). Tem-se sugerido que os genótipos modernos de frangos de corte produzem mais calor corporal devido a sua maior atividade metabólica, que aumenta a carga de calor sobre esses animais (Deeb e Cahaner, 2001).

As aves são animais classificados como endotérmicos, uma vez que podem manter uma temperatura corporal relativamente constante (Bride et al., 2010). Entretanto, este processo só se mostra eficiente quando a temperatura ambiente se encontra dentro da zona de conforto térmico (Abreu e Abreu, 2011). Sob condição de temperatura de conforto, as aves são capazes de manter a homeostase da temperatura corporal interna (Furlan e Macari, 2002), e assim podem expressar o máximo desempenho do seu potencial genético (Miragliotta, 2005). Nessa faixa de termoneutralidade não há gasto de energia para termorregulação, assim toda energia produzida pelo organismo é direcionada para fins produtivos (Macari et al., 2004).

Por serem animais endotérmicos, as aves dispõem de um centro termorregulador localizado no hipotálamo, que é constituído por neurônios que respondem ao calor e são acionados quando a temperatura corporal aumenta, desencadeando em reações comportamentais e mecanismos fisiológicos de termorregulação, responsáveis por manter e controlar a homeotermia pelas trocas de calor com o ambiente (Borges et al., 2003).

Quando a temperatura ambiental se eleva acima da zona de conforto térmico, o animal é submetido a condição de estresse, de modo que seu organismo reage de maneira compensatória na tentativa de aumentar a dissipação de calor e manter o equilíbrio térmico do corpo (Furlan e Macari, 2002). No entanto, quando estes mecanismos não são eficientes, o animal na tentativa de diminuir a produção de calor metabólico e aumentar

a dissipação de calor, realiza ajustes fisiológicos rápidos como, vasodilatação e aumento da frequência respiratória (Macari et al., 2004; Lavor et al., 2008). Frequência respiratória aumentada causa alcalose respiratória em função do desequilíbrio ácido-básico e aumenta ainda mais o estresse (Borges et al., 2003).

Todo processo que as aves realizam para a regulação da temperatura corpórea promove modificações fisiológicas que comprometem as respostas inflamatórias e imunológicas e afeta negativamente a utilização da energia, resultando na redução do desempenho (Ohtsu et al., 2015; Hu et al., 2011; Liu et al., 2014).

O estresse térmico pode ser classificado como agudo ou crônico. Ambos os tipos podem levar ao aumento da temperatura corporal, entretanto, esse aumento depende da intensidade e da duração de exposição ao calor (Zhang et al., 2012). A exposição ao estresse térmico agudo provoca choque térmico, resultando no aumento rápido da temperatura corporal (Amand et al., 2004). No entanto, quando expostos ao estresse térmico crônico por um longo período, após ser elevada, a temperatura corporal declinará para um estado estacionário correspondente ao estado de aclimatação (Cooper e Washburn, 1998; Collin et al., 2002; Renaudeau et al., 2010).

Como exposto acima, o efeito do estresse térmico depende da intensidade do estressor. Quinteiro-Filho et al. (2010 e 2012) relataram que os parâmetros de desempenho foram afetados em frangos de corte submetidos ao estresse térmico agudo e crônico de 31°C. Os mesmos autores relataram que ambas as temperaturas diminuíram significativamente o ganho de peso e consumo de ração, no entanto, o índice de conversão alimentar e a mortalidade apresentaram diferença quanto ao tipo de exposição ao calor, foi observado que os frangos de corte no estresse agudo diminuíram a conversão alimentar (270%) e aumentaram a taxa de mortalidade (26%), entretanto, as aves no estresse crônico não apresentaram alterações nos parâmetros analisados (2,51% e 0%). É provável que em algum momento as aves se adaptem ao estresse térmico, o que reduziria a perda de desempenho (Gonzalez-Esquerra e Leeson, 2005).

Segundo Zhang et al. (2012), aves mantidas em temperatura elevada constante (34°C) e/ou cíclica (36°C) apresentam redução no ganho de peso em 8,1% e 18,2%, respectivamente. Sohail et al. (2012) relataram que os frangos de corte aos 42 dias de idade expostos ao estresse crônico tiveram uma redução de 16,4% no consumo de ração e 32,6% no peso corporal. Em um outro estudo os autores observaram que o aumento na temperatura ambiente de 32,2°C levou a queda no consumo de ração a cerca de 5,5% por ave/dia, com redução de 3,3% na eficiência alimentar sob condição de estresse térmico

agudo (Habibian et al., 2016). Com a redução no consumo e perda de peso, os pesos absolutos de coxa, sobrecoxa e peito também são prejudicados, diminuindo o rendimento desse cortes nobres (Oliveira et al., 2006).

Ambientes com temperaturas elevadas também são responsáveis por várias alterações fisiológicas e metabólicas, como a redução no nível hormonal de tri-iodotironina (T3) (Geraert et al., 1996; Mashaly et al., 2004; Willemsen et al., 2011), inibição do funcionamento normal do sistema imune (Mashaly et al., 2004), modificações na atividade do sistema neuroendócrino das aves, resultando na ativação do eixo hipotálamo-hipófise-adrenal (HPA) elevando as concentrações plasmáticas de corticosterona (Star et al., 2008; Quinteiro-Filho et al., 2010; Quinteiro-Filho et al., 2012).

Além das alterações mostradas, o estresse térmico também induz mudanças na composição sanguínea. Xie et al. (2015) constataram elevação nas concentrações plasmáticas dos metabolitos triglicerídeo e colesterol total, e aumento na atividade da lactato desidrogenase em frangos de corte submetidos ao estresse térmico agudo ou crônico. Tem sido relatado que o estresse térmico induz a síntese de ácidos graxos importantes na produção de triglicerídeos (Flees et al., 2017; Jastrebski et al., 2017), e induz o metabolismo aeróbico das substâncias energéticas, resultando no aumento da glicólise e da deposição de gordura (Lu et al., 2017).

Além disso, o estresse térmico aumenta a lipase lipoproteica do tecido adiposo, o que sugere que o tecido adiposo de animais hipertérmicos tem uma capacidade aumentada para captar e armazenar triglicerídeos intestinais e hepáticos (Baumgard e Rhoads et al., 2013). Os mesmos autores relatam que o déficit energético do animal estressado pelo calor, é resultado da falta de mobilização de gordura do tecido adiposo, juntamente com uma resposta reduzida aos estímulos lipolíticos. O estresse térmico aumenta também a deposição de lipídeos em função da maior taxa de catabolismo de aminoácidos (Geraert et al., 1996).

O estresse térmico também está relacionado aos prejuízos na qualidade da carne (Petracci et al., 2010). As alterações nos parâmetros de qualidade são atribuídas a alteração da composição bioquímica, que provoca mudanças nas propriedades de qualidade, como cor, pH, perda de água por gotejamento e por cocção (Dransfield et al., 1999; Alnahhas et al., 2014; Almasi et al., 2015).

Segundo Spurio (2015), o aumento rápido na temperatura ambiental já pode ser considerado um ponto crítico para manutenção da qualidade da carne, e pode afetar a qualidade da carne do peito, causando variações na cor, pH e capacidade de retenção de

água (Dadgar et al., 2010). O estresse agudo ou de curta duração, leva a uma acidificação mais rápida e queda do pH, assim pode causar danos indesejáveis na qualidade da carne de frango com alterações na cor, capacidade de retenção de água, estabilidade de oxidação, resultando em carne pálida e exsudativa (Foaud et al., 2016). Por outro lado, o estresse crônico ou de longa duração, afeta negativamente o metabolismo da gordura, o crescimento muscular e reduz a qualidade da carne e o perfil químico devido ao desequilíbrio eletrolítico e a ativação da peroxidação lipídica (Babinszky et al., 2011; Sokołowicz et al., 2016; Kim et al., 2017).

A superprodução de espécies reativias de oxigênio (ROS) sob estresse por calor pode causar danos oxidativos em vários órgãos, incluindo tecido muscular esquelético, o que pode levar ao declínio da qualidade da carne de frango (Wang et al., 2009; Azad et al., 2010). A oxidação lipídica e proteica induzida pelo estresse térmico agudo, juntamente com a diminuição do pH final, poderia reduzir as funcionalidades proteicas do músculo do peito de frango (Wang et al., 2009).

Os principais defeitos de qualidade na indústria da carne são a carne pálida, flácida e exsudativa (PSE) e a carne escura, dura e seca (DFD), que reduzem a aceitação pelos consumidores, a vida útil do produto, o rendimento da carne, e como consequência prejuízo a indústria avícola (Adzitey e Nurul, 2011). Na indústria avícola mundial a carne PSE tem se tornado um problema econômico, sendo o Brasil responsável por uma perda de US$ 30 milhões causada pela redução do peso da carcaça entre 1 a 1,5% (Droval et al., 2012).

Além disso, temperaturas ambientais acima da zona termoneutra nas aves têm sido associadas ao estresse oxidativo (Lin et al., 2006; Yang et al., 2010). Foi relatado que o estresse térmico aumenta a produção de ROS e malodialdeído (MDA) e diminui a atividade da superóxido dismutase (SOD) e da catalase (CAT) (Yu et al., 2013). Níveis elevados de produtos de peroxidação lipídica foram reconhecidos como índice de estresse oxidativo (Akbarian et al., 2016). No entanto a elevação do MDA não ocorre na mesma extensão no estresse térmico agudo ou crônico. Por exemplo, frangos expostos ao estresse agudo (40ºC por 5h) apresentaram maior elevação dos níveis de MDA (Mujahid et al., 2009; Wang et al., 2009) que as aves do estresse térmico crônico (32°C por 8 h/14 dias) (Azad et al., 2010).

Tem sido indicado que dependendo da gravidade e duração do estresse térmico, o sistema antioxidante e as enzimas associadas se comportam de maneira diferente. Tradicionalmente, após o estresse agudo, a atividade das enzimas antioxidantes

(CAT, GSH-Px e SOD) aumentam para proteger as células contra a formação de superóxido em excesso (Akbarian et al., 2016). No entanto, resultados inconsistentes foram relatados por Sahin et al. (2010). Os mesmo autores observaram redução na atividade hepática das enzimas SOD, GSH-Px e CAT em codornas em condições de estresse térmico crônico.

Outra diferença metabólica de resposta entre o estresse agudo e crônico é quanto a regulação da proteína desacopladora (avUCP). O estresse agudo regula negativamente a síntese da avUCP; como consequência estimula a capacidade de oxidação metabólica nas mitocôndrias, levando a níveis mais elevados de superóxido mitocondrial e maior produção de ROS (Mujahid et al.,2009). O estresse crônico, enquanto isso, leva à redução da capacidade metabólica oxidativa mitocondrial, e à regulação positiva da proteína desacopladora das aves (Azad et al., 2010). Dessa forma, o estresse térmico afeta os mecanismos biológicos de defesa, induz distúrbios metabólicos, levando a baixa produtividade dos frangos (Ohtsu et al., 2015; Xie et al., 2014).

2.0 **Metabolismo: Estado oxidativo e regulação gênica**

2.1 Estado oxidativo

Substâncias oxidantes são geradas em processos endógenos, assim como, podem ser adquiridas de fontes exógenas (Surai, 2007; Winterbourn, 2008). A maioria dos radicais livres que danificam os sistemas biológicos são radicais livres de oxigênio, e estes são geralmente conhecidos como espécies reativas de oxigênio (ROS) (Rahman, 2007). Os ROS são moléculas de oxigênio com elétrons instáveis que podem causar degeneração e morte celular. Essas moléculas são resultantes do metabolismo do oxigênio, pois durante a produção de energia pelo organismo, nem todo o oxigênio utilizado como aceptor de elétrons pelas mitocôndrias é totalmente reduzido a água (Ray et al., 2012).

A produção de ROS ocorre durante o metabolismo celular normal, sendo assim formados fisiologicamente nos sistemas biológicos celulares. Em baixas concentrações, essas moléculas atuam de forma benéfica no organismo, como na produção de energia ou como sinalizadores; no entanto, quando em excesso, produzem modificações adversas nos componentes celulares, como lipídios, proteínas e DNA (Barreiros et al., 2006; Celi, 2010; Oliveira e Schoffen, 2010).

Os três principais ROS que são de significado fisiológicos são o superóxido ($O2^{-\cdot}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\cdot}OH$) (Sharma et al., 2012). O superóxido é o primeiro produto da redução do oxigênio molecular, é produzido em diversos processos biológicos, entre eles, na cadeia transportadora de elétrons das mitocôndrias, a partir da adição de um elétron a uma molécula de oxigênio. O peróxido de hidrogênio é gerado por meio da reação de dismutação catalisada pela enzima superóxido dismutase (SOD), em que, o superóxido é convertido a peróxido de hidrogênio (Birben et al., 2012). O peróxido de hidrogênio ainda pode ser convertido em água, pela ação das enzimas catalase e glutationa peroxidase (Droge, 2002).

Apesar dos radicais livres serem importantes para alguns processos fisiológicos (Surai, 2007), quando o sistema antioxidante de defesa não consegue neutralizar o excesso de substâncias oxidantes, inicia-se o estado de estresse oxidativo devido ao desequilíbrio entre a produção e a eliminação dos ROS (Gessner et al., 2016; Hussain et al., 2016).

Tem sido relatado que o estresse por calor é um dos fatores ambientais responsáveis pelo estresse oxidativo em aves (Ghazi Harsini et al., 2012; Mujahid et al., 2007). De acordo com alguns autores, a influência do estresse térmico na indução do estresse oxidativo pode estar relacionada as mudanças nas taxas de reações bioquímicas e fisiológicas, em função do aumento na produção de ROS (Mujahid et al., 2007; El-Kholy et al., 2017, El-Kholy et al., 2018).

Os mecanismos pelos quais o estresse térmico induz ao estresse oxidativo ainda não estão bem compreendido. Segundo Kikusato e Toyomizu (2013), aves submetidas a altas temperaturas podem apresentar elevação no potencial de membrana mitocondrial, podendo ter efeito substancial na geração de ROS. Além disso, o estresse por calor pode causar disfunção mitocondrial em função do desacoplamento dos complexos I, II, e III da cadeia transportadora de elétrons (Huang et al., 2011; Mujahid et al., 2009; Kikusato et al., 2010). Assim, o rompimento do metabolismo mitocondrial e da função respiratória pode levar ao vazamento de elétrons, culminando no aumento da produção de ROS (Volodina et al., 2017).

O excesso de ROS podem ser eliminadas do organismo por meio de substâncias antioxidantes. O organismo dispõe de diversos mecanismos de defesa enzimático e não enzimático. Entre as substâncias não enzimáticas com ação antioxidante pode-se destacar as vitaminas lipossolúveis, vitaminas hidrossolúveis, carotenoides, flavonoides e oligoelementos (Zinco, cobre, selênio, magnésio); entre os antioxidantes enzimáticos, as

enzimas superóxido dismutase, catalase e pelo sistema de defesa da glutationa (Birben et al., 2012).

A glutationa é um tripeptídeo formado por três aminoácidos (glutamato, cisteína e glicina), e existe no organismo em duas formas, a de glutationa reduzida (GSH) e glutationa oxidada (GSSG). A glutationa atua em muitos processos biológicos importantes, incluindo a síntese de proteínas, reservatório de cisteína, e a principal, na defesa celular contra os radicais livres (Masella et al., 2005).

O sistema da glutationa é formado pelas enzimas glutationa oxidase (GO), glutationa peroxidase (GPx) e pela glutationa redutase (GR). Assim, a ação de defesa antioxidante depende da atividade em conjunto de todo o sistema. Dessa forma, para desempenhar sua função na redução das espécies reativas de oxigênio a glutationa é oxidada a glutationa de dissulfureto (GSSG) pela ação das enzimas GO e GPx. A glutationa pode ser regenerada a partir de GSSG pela enzima glutationa redutase (GR) na presença de sua coenzima ($NADPH + H^+$), permitindo que a mesma molécula seja usada mais de uma vez para eliminar os ROS (Droge et al., 2002). Esse sistema também catalisa a dismutação do peróxido de hidrogênio em água, por ação da enzima glutationa peroxidase (Barreiros et al., 2006).

Os danos fisiológicos e metabólicos causados pelo estresse térmico no organismo dos animais também podem ser minimizados por meio dos nutrientes. Entre os vários nutrientes conhecidos que podem agir como antioxidantes, está o aminoácido essencial metionina (Levine et al., 1996). A metionina pode atuar como antioxidante direto na proteção sobre o estresse oxidativo (Stadtman et al., 2002). Os resíduos de metionina são suscetíveis à oxidação pelos radicais livres (Swennem et al., 2011).

A metionina ao reagir com o ROS, é oxidada de maneira reversível, formando metionina sulfóxido. A maioria das células possuem redutases específicas, como a enzima metionina sulfóxido redutase (Msr), essa enzima faz a catalização de redução da metionina sulfóxido de volta à metionina (Weissbach et al., 2005), em uma reação dependente da enzima tiorredoxina (TRx). Assim, a redução dos resíduos de metionina em proteínas podem reagir novamente com os ROS. A enzima tiorredoxina é oxidada na reação, e então é reduzida novamente por meio da reação catalisada pela enzima tiorredoxina redutase (TRxR), à custa de NADPH. Dessa forma, a partir desse complexo ciclo enzimático de oxidação e redução dos resíduos de metionina, faz com que aja a eliminação das espécies reativas de nitrogênio e oxigênio (revisado por Luo e Levine, 2009).

Estudos mostram que a suplementação com fontes de metionina nas dietas de frangos de corte apresentam efeitos benéficos como melhora no desempenho, capacidade antioxidante, melhora a qualidade da carne do peito e nas respostas imunes das aves (Lai et al., 2018; Zhang et al., 2018; Yaqoob et al., 2018). A metionina também é conhecida como capaz de atenuar o efeito do estresse térmico (Willemsen et al., 2011).

Além disso, sob condições de estresse térmico agudo, a alimentação com DL-metionina para frangos de corte contribui para minimizar os efeitos negativos do estresse por meio do aumento dos níveis de expressão dos genes relacionados à atividade antioxidante, incluindo a cistationina β-sintase, GSH sintetase e GPx (Del Vesco et al., 2015).

2.2 Epigenética e Regulação gênica

Como descrito acima, o ambiente pode atuar no metabolismo das aves mediante alterações na expressão gênica. Novos estudos têm sido conduzidos para melhor descrever como alterações ambientais são capazes de interferir no desenvolvimento e desempenho dos animais por meio da epigenética.

A palavra epigenética foi introduzida por Conrad Hal Waddington em 1942, derivada da palavra *epigenesis.* Esse termo foi citado para descrever os mecanismos causais que dão origem a fenótipos a partir de genótipos nos processos de desenvolvimento e diferenciação. Desde então, o termo epigenética tem apresentado vários conceitos e, o mais recente introduz uma definição molecular referindo-se as alterações químicas ao nível da transcrição de genes associadas à estrutura da cromatina que são herdadas durante a divisão celular e ao longo das gerações, não sendo portanto atribuídas a alterações na sequência do DNA (Triantaplyllopoulos et al., 2016).

A regulação epigenética apresenta importante papel para o desenvolvimento normal do organismo e são fundamentais para estabelecer a regulação correta da expressão dos genes. Durante o desenvolvimento animal, modificações químicas ocorrem nos cromossomos que não alteram a sequência de nucleotídeos, tais modificações são chamadas de marcas epigenéticas, as quais estão associadas com alterações na expressão dos genes, nos processos de transcrição e tradução, assim como, estão envolvidas no desligamento ou ativação de um gene em certos tecidos, em que a sua expressão se faz ou não necessária (Goddard e Whitelaw, 2014; Ibeagha-Awemu e Zhao, 2015).

Com a descoberta das modificações epigenéticas, muitos processos passaram a ser mais compreendidos. Recentemente, foi proposto que os mecanismos epigenéticos também podem mediar os efeitos nutricionais da dieta sobre a fisiologia do animais modelos e a expressão gênica sem alterar a sequência do DNA e afetar o desenvolvimento do embrião, crescimento e a saúde dos animais (Goddard e Whitelaw, 2014; Feil e Franga et al., 2012; Gluckman, 2011).

As modificações epigenéticas modificam o acesso à informação genética, sendo estas reversíveis e variando de um tipo celular para outro, de modo que os fatores ambientais podem afetar o epigenoma, e assim, produzir efeitos sobre a expressão gênica alterando as marcas epigenéticas (Goddard e Whitelaw, 2014; Jirtle e Skinner, 2007).

Embora a grande maioria dos fatores ambientais não possa alterar a sequência de DNA, os processos epigenéticos podem ser alterados em resposta aos fatores ambientais da nutrição à temperatura, uma vez que os doadores de grupos metil são obtidos pela dieta e são principalmente a metionina, folato, colina e vitamina B12 (Skinner, 2014).

Existem dois mecanismos principais de alterações epigenéticas: a metilação do DNA e a modificação de histonas. A metilação do DNA é talvez a modificação epigenética mais amplamente estudada, que desempenha um papel importante na regulação da expressão gênica e da arquitetura da cromatina (Donkena et al., 2010), uma vez que constitui uma das modificações mais estáveis com impacto importante nos processos biológicos, incluindo a regulação adequada da expressão gênica, silenciamento de genes no cromossomo X inativado, imprinting e elementos repetitivos, assim como é essencial para o desenvolvimento embrionário (Baylin e Herman, 2000; Kohli e Zhang, 2013).

A metilação do DNA é uma modificação química na qual ocorre a adição de um grupo metil ($-CH_3$) ao carbono 5’ de uma citosina na sequência do DNA que geralmente precede uma guanina (no contexto 5’CG3’), resultando em uma 5-metilcitocina, essa reação é catalisada por uma classe de enzimas conhecidas como DNA metiltransferases (Dnmts) que utilizam a *S*-adenosilmetionina como doador do grupo metil (Figura 1)(Miranda e Jones, 2007).

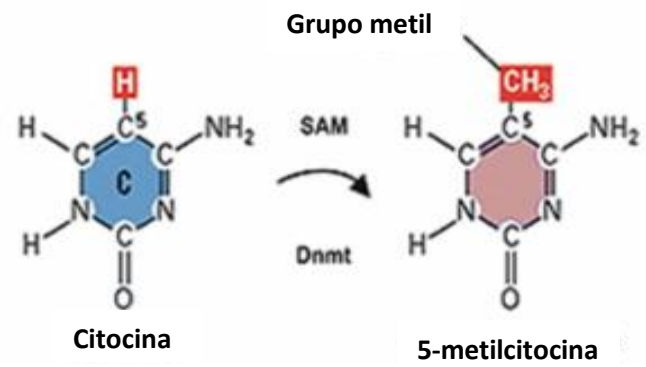

Figura 1. Metilação do DNA mediada por DNA metiltransferases (Dnmts). (Fonte: Adaptada de Gronbaek et al., 2007).

As DNA metiltransferases (Dnmts) são divididas em duas classes baseadas em suas diferenças na especificidade do substrato CpG. A enzima Dnmt I é conhecida como metilase de manutenção envolvida na metilação de fitas hemimetiladas (apenas uma fita metilada) do DNA, que funcionam durante a replicação do DNA, copiando padrões de metilação da cadeia parental para a cadeia recém-sintetizada com papel principal de passar o controle epigenético da expressão gênica (Williams et al., 2011). A segunda classe de Dnmts é relacionada a metilação do DNA de novo. A metilação de novo é catalisada pelas enzimas Dnmt3a e Dnmt3b, que atuam em sítios sem nenhum tipo de indicação de metilação, ou seja não têm preferência de ligação ao estado hemimetilado (Figura 2) (Jurkowska et al., 2011).

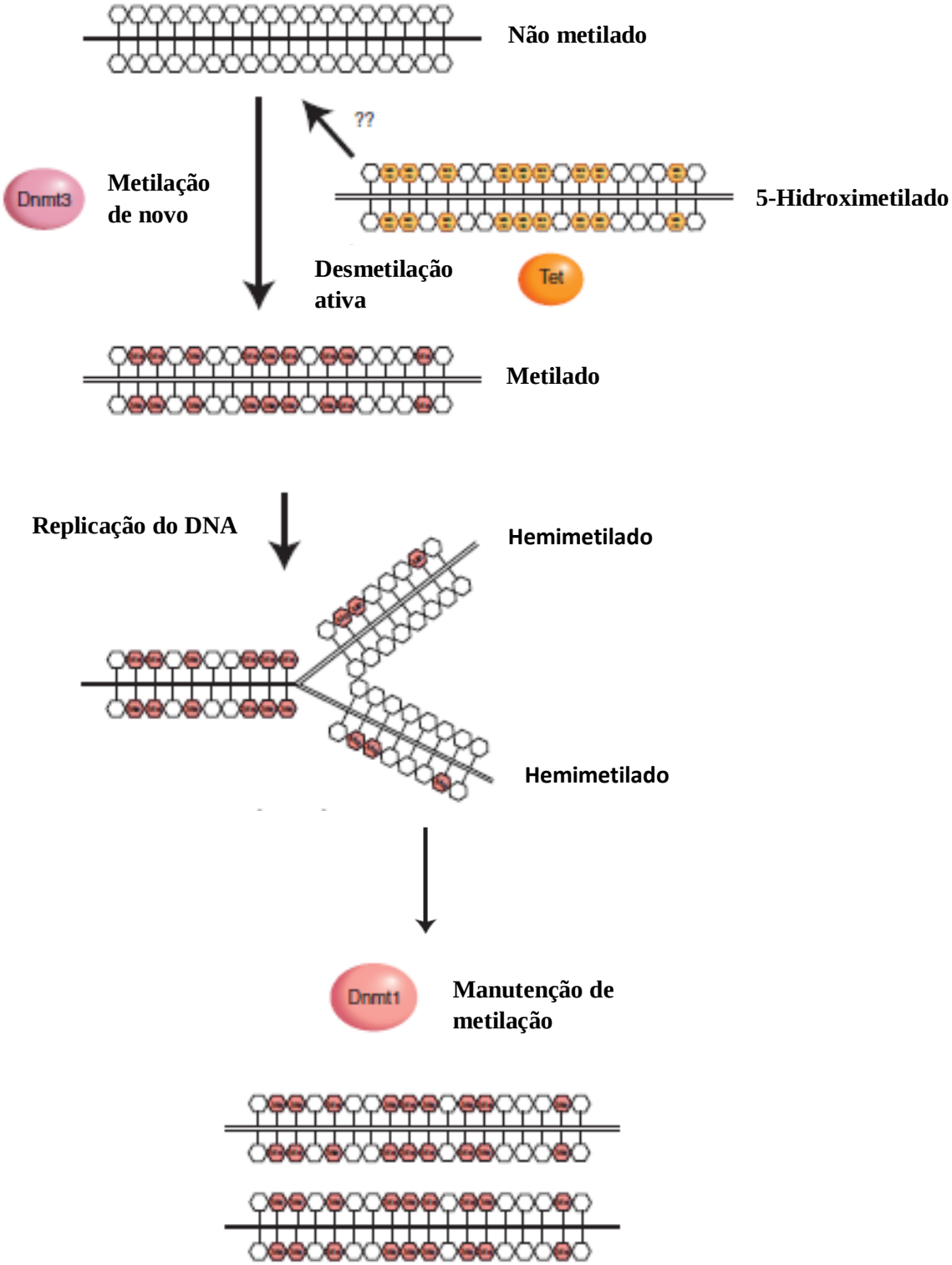

Figura 2. Metilação de novo e metilação de manutenção do DNA. (Fonte: Adaptada de Lin e Zhang, 2014).

A metilação do DNA ocorre principalmente nos locais de dinucleotídeos CpG, com exceção de algumas plantas, fungos e invertebrados, e foi encontrado em outras bases

C (Zemach et al., 2010). O genoma dos vertebrados apresenta baixa frequência dos nucleotídeos CG, no entanto, existe regiões específicas em que há grande concentração destes nucleotídeos, que são chamadas de ilhas CpG (Laird, 2003). Essas regiões correspondem a sequências intercaladas do DNA que diferem do padrão genômico por serem ricas em CG, e predominantemente CpG não metiladas (Deaton et al., 2011). De fato, a maior parte do genoma é deficiente em CpG e cerca de 70-80% dos dinucleotídeos CpG esparsos encontram-se metilados (Lister et al., 2009). Acredita-se que a metilação de CpG suprime a transcrição em locais indevidos (Jones et al., 2012), visto que a metilação geralmente está relacionada, ao silenciamento gênico (Toraña et al., 2016).

As ilhas CpG são frequentemente encontradas em regiões promotoras e no primeiro éxon de muitos genes, assim estão relacionadas diretamente com o controle da transcrição. Para que a expressão gênica ocorra, o promotor do gene deve estar prontamente acessível aos fatores de transcrição para reconhecer uma sequência específica de DNA, e pode ser alterado pelo padrão de metilação de tais ilhas (Bird, 2002; Deaton e Bird, 2011). O genoma é constante, mas os efeitos da regulação gênica na metilação do DNA podem variar entre posições de metilação em genes, tecidos e estágios de desenvolvimento (Ibeaghaawemu e Zhao, 2015).

A metilação do DNA pode alterar a expressão gênica por meio de dois mecanismos principais, direta ou indiretamente; Primeiro, a metilação do DNA pode interferir diretamente na ligação dos fatores de transcrição, pois existem sítios CpG metilados na região do promotor que impede a ligação dos fatores de transcrição a seus respectivos domínios específicos e, portanto, suprime a regulação da expressão gênica (Jaenisch e Bird, 2003). Em segundo lugar, o mecanismo de repressão à transcrição é mediado por proteínas que possuem afinidade pelo radical metil dos sítios CpG metilados. Uma família de proteínas, conhecidas como proteínas do domínio de ligação metil-CpG (ou MBDs), são atraídas e se ligam aos dinucleotídeos CpG metilados, como, as proteínas MeCP1 e MeCP2 (do inglês, methyl-CpG binding protein). Tais proteínas ao se ligarem ao DNA metilado podem competir com fatores de transcrição pelos seus respectivos domínios ou, ainda, promover um rearranjo na estrutura do DNA, originando cromatina de alta densidade, a qual se torna incompatível com o processo de transcrição (Li e Zhang, 2014).

No centro do processo de metilação do DNA está a transferência de um grupo metil da *S*-adenosilmetionina (SAM) para a citosina de um dinucleotídeo CpG (adjacente a uma única fita de DNA), imediatamente após a replicação do DNA (Jeltsch, 2006;

Jurkowska et al., 2011). De fato, a SAM é um doador de metil universal, sendo o substrato para enzimas Dnmts controlar o estado metilado do DNA, RNA e proteínas, como as histonas (Matzke e Mosher, 2014; Kouzarides, 2002). É importante ressaltar que a fonte de grupamentos metil proveniente da *S*-adenosilmetionina (SAM) é formado pela rota metabólica da metionina durante o processo de síntese da homocisteína (Stipanuk, 2004).

3.0 **Estresse térmico, metionina e metilação do DNA**

Modificações epigenéticas do DNA representam um mecanismo importante pelo qual a atividade da expressão gênica pode ser modificada em resposta aos estímulos ambientais, como da dieta nutricional e estresse térmico (Zhang, 2018; Vinoth et al., 2017). Uma série de estudos em modelos vegetais foram realizados para saber o papel da metilação do DNA durante o estresse térmico mediante controle epigenético da expressão gênica (Boyko e Kovalchuk, 2008; Pecinka et al., 2010; Gao et al., 2014). No entanto, há poucos estudos sobre essas alterações em aves (Luo et al., 2011; Vinoth et al., 2017).

A metilação do DNA é susceptível a influência e alterações ambientais, dessa forma, o estresse térmico pode causar aumento ou diminuição nos níveis de metilação do DNA em todo o genoma ou em loci específicos. Em um estudo de expressão gênica global, foi observado que a expressão dos genes envolvidos com desenvolvimento e o crescimento do músculo esquelético de suínos exibiram hipometilação após estresse térmico constante (Hao et al., 2016). Em um outro estudo com frangos, os autores observaram que a exposição aos ROS regulou negativamente a expressão da catalase via hipermetilação de uma ilha CpG no promotor da catalase (Quan et al., 2011). A metilação do promotor atua como um marcador epigenético repressivo que regula negativamente a expressão gênica (Li et al., 2012; Su et al., 2014). Isso é consistente com o estudo de Gan et al. (2013) que observaram a relação inversa entre a expressão do mRNA e a metilação na região promotora da HSP70 no músculo da perna de galinha quando submetida a condição estressante.

O estresse térmico é frequentemente considerado um dos mecanismos prejudiciais por induzir o estresse oxidativo (Li et al., 2006; Azad et al., 2010), sendo a influência do estresse térmico conhecida por aumentar a formação de ROS (Mujahid et al., 2007). A elevação na temperaturas ambiental não está somente relacionada com maior produção de ROS, pode causar também alterações nas atividades de enzimas antioxidantes. Segundo Pamok et al. (2009) animais expostos as altas temperaturas, em estresse térmico

crônico, apresentam maior atividade da enzima GPx, e essa reposta pode ser uma tentativa do organismo de combater o ROS, que também tem sua produção aumentada nessa condição.

A enzima GPx trabalha em conjunto com a glutationa (GSH). A biossíntese da GSH ocorre na maioria dos tecidos a partir de três aminoácidos precursores. Entre esses está a cisteína que durante o metabolismo pode ser sintetizado a partir da metionina por meio da rota de transsulfuração da homocisteína (Shoveller et al., 2005). Trabalhos na literatura têm relatado que o estresse oxidativo e a metilação do DNA estão metabolicamente ligados pela relação entre o metabolismo de um carbono e a via de transulfuração (Zhang et al., 2018; Niedzwiecki et al., 2013; García-Giménez et al., 2017).

A metionina tem um papel importante nos processos epigenéticos, servindo como doadora de grupo metil para a metilação da citosina em ilhas CpG (Tehlivets et al., 2013). A S-adenosilmetionina (SAM) é formada pela rota metabólica da metionina durante o processo de síntese da homocisteína (Stipanuk, 2004). Esse aminoácido está envolvido no metabolismo da homocisteína por meio de duas rotas biológicas, remetilação e transsulfuração. As reações envolvem vários passos, iniciando com a formação da S-adenosilmetionina pela metionina, catalisada pela metionina adenosiltransferase (MATs), posteriormente, as metiltranferase (MTs) catalisam a formação da S-adenosil homocisteína (SAH), convertendo em homocisteína mediante a hidrolise da SAH (Finkelstein e Martin, 2000). Uma vez que a SAH é convertida em homocisteína, esta também pode ser reciclada no ciclo da metionina por meio da rota da remetilação sendo reconvertida em metionina novamente pela ação das enzimas metionina sintetase (MS) e betaína:homocisteína metiltransferase (BHMT), usando como doador de grupo metil o metiltetrahidrofolato ou a betaína, respectivamente. Além disso, a homocisteína também pode ser incorporada na via da transsulfuração para conversão em cisteína por duas etapas enzimáticas: Primeiro, a homocisteína é convertida em cistationina pela ação da cistationina B-sintase (CBS). Na segunda, a cistationina é metabolizada, com ação da cistationina B-liase, ocorrendo a síntese da cisteína (Stipanuk, 2004). Após a formação de cisteína, este aminoácido é incorporado na via da biossíntese da GSH (Frau et al., 2013).

A γ- glutamilcisteína sintetase é a primeira enzima limitante da velocidade na síntese da GSH, ligando o ácido glutâmico à cisteína, resultando em γ-L-glutamil-L-cisteína. Na segunda etapa, a enzima glutationa sintetase (GSS) liga o dipeptídeo com a

glicina para obter GSH. Para prevenção de síntese excessiva de glutationa e acúmulo intermediário da γ-glutamilcisteína, a γ-glutamilcisteína sintetase, pode sofrer um feedback negativo a partir da GSH (Lu, 2014).

A redução da captação de cisteína e qualquer alteração nas principais enzimas relacionadas ao metabolismo da GSH pode desregular seus níveis normais, afetando assim, o perfil de metilação (Garcia-Gimenez e Pallardo et al., 2014). Sob condições de estresse térmico crônico as aves podem apresentar alta produção de homocisteína e, baixa de vitamina B12 e ácido fólico, indicando conversão de metionina em cisteína para suportar o reabastecimento de GSH, como resultado alteração na metilação do DNA pode ser observado pela desregulação da S-adenosilmetionina (Fuso et al., 2005).

Há uma estimativa de que a cerca de 50% da produção da glutationa é de origem da homocisteína, cisteína proveniente da homocisteína pela rota de transsulfuração, e que sob condições de estresse oxidativo, na qual é requisitada maior produção de glutationa, a cistationina ß-sintase aumenta sua atividade, e assim, ocorre maior expressão desta enzima (Mosharov et al., 2000).

Quando essas mudanças na expressão gênica são acompanhadas a demandas crescentes de glutationa, o ciclo da metionina é afetado levando a alteração na metilação do DNA (Anthony e Domann, 2011). Isso ocorre porque sob condições de estresse oxidativo crônico os níveis de GSH são reduzidos, assim a atividade da enzima cistationina β-sintase (CBS) aumenta para direcionar o fluxo de homocisteína por meio da via de transulfuração para formar GSH (Mosharov et al., 2000). Ao favorecer a entrada de homocisteína na via de transulfuração, menos homocisteína é direcionado para a regeneração da metionina, assim o aumento da produção de GSH influencia os processos epigenéticos, incluindo a metilação do DNA e, limita a disponibilidade de S-adenosilmetionina, o cofator utilizado durante o controle epigenético da expressão gênica (Hitchler e Domann, 2007).

Assim, um alto nível de ROS esgota a glutationa endógena, e o excesso de homocisteína, que não é reciclado, impede a regeneração da metionina, afetando a formação de *S*-adenosilmetionina para metilação e também a formação de cisteína, precursora da glutationa (Menezo et al., 2016). Dessa forma, a redução da glutationa (GSH) pode levar à hipometilação do DNA global, possivelmente pela redução da *S*-adenosilmetionina (García-Gímenes e Pallardó, 2014).

Nos últimos anos, um esforço para compreender a influência da dieta nos processos epigenéticos revelou que o metabolismo pode influenciar a expressão gênica

(Hitchler e Domann, 2007). Os nutrientes da dieta, como por exemplo, a metionina podem modificar o padrão de metilação do DNA (Zhang et al., 2017). Como um aminoácido essencial, a metionina interage com outros nutrientes envolvidos no metabolismo (Bunchasak, 2009) e desempenha um papel único nos processos epigenéticos ao servir como penúltimo doador de metil para as reações de metilação em mamíferos (Waterland, 2006).

Existem três maneiras pela qual a dieta com metionina influencia os padrões de metilação do DNA; Primeiro, o fornecimento de substrato necessário para a metilação adequada do DNA; segundo, a atividade enzimática da Dnmt pode ser modulada pelo fornecimento de cofatores (Exemplo; colina, betaina); terceiro, a atividade das enzimas que regulam o ciclo de um carbono pode ser alterada (Zhang et al., 2015). Como doadora de grupo metil, a S-adenosilmetionina (SAM) é sintetizada no ciclo da metionina, assim a disponibilidade reduzida de metionina pode alterar os níveis metabólicos de SAM e SAH resultando em alterações na expressão de genes relacionados ao crescimento. Por outro lado, dieta suplementada com metionina aumenta a metilação do DNA (Zhang, 2017).

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II. OBJETIVOS GERAIS

Estudar o desempenho, a expressão gênica e a metilação do DNA em frangos de corte alimentados com suplementação de duas fontes de metionina e submetidas a diferentes temperaturas ambientais.

Objetivos Específicos:

Avaliar os efeitos do estresse térmico agudo e crônico e da suplementação de metionina na forma de aminoácido livre ou como dipeptídeo sobre o desempenho, qualidade da carne, e sobre os níveis sanguíneos de triglicerídeos, colesterol total, e suas respectivas frações LDL e HDL em frangos de corte.

Estudar o efeito do estresse térmico crônico e da suplementação de metionina sobre a metilação do DNA na região promotora dos genes: glutationa peroxidase (*GPx*) e glutationa sintetase (*GSS*);

Determinar se há correlação entre o padrão de metilação do DNA e os níveis de expressão de mRNA dos genes glutationa peroxidase (*GPx*) e glutationa sintetase (*GSS*);

Analisar o efeito do estresse térmico crônico e da suplementação de metionina como aminoácido livre ou como dipeptídeo sobre o desempenho, a atividade das enzimas antioxidantes superóxido dismutase, catalase, e sobre os marcadores biológicos do estresse oxidativo: níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), glutationa (GSH), proteína carbonilada (PC) em frangos de corte.

III. METHIONINE AS FREE AMINO ACID AND DIPEPTIDE ON PRODUCTIVE EFFICIENCY AND MEAT QUALITY OF BROILERS UNDER ACUTE AND CHRONIC HEAT STRESS

(Animal Feed Science and Technology)

Abstract

This study was carried under the hypothesis that the supplementation of methionine as free amino acid and dipeptide could reduce the effect of acute and chronic heat stress on the productive efficiency and meat quality of broiler chickens. For this, broilers were evaluated at three experimental periods: 24 hours of evaluation (21-22 days of age); 10 days of evaluation (22-32 days of age); and 20 days of evaluation (21-42 days of age). Broilers were divided into two groups: one group was raised in comfortable temperature, and the other group was raised in continuous heat stress (HS) of 30ºC. From both groups, animals received diet without methionine supplementation (MD diet); with supplementation of methionine as free amino acid (DL-Methionine; DL-M); and with methionine as dipeptide (DL-methionyl-DL-methionine; DL-MM). HS reduced weight gain (WG) after 10 and 20 days of evaluation ($P<0.05$). At 42 days of age, birds fed MD diet presented higher relative weight of abdominal fat ($P = 0.0188$) and lower relative weight of breast ($P <0.0001$) than chickens fed DL-M and DL-MM. At 32 days of age, broilers under HS had lower HDL and higher LDL content than birds that remained in comfort. At 42 days of age, there was no difference between broilers under HS fed DL-MM and broilers raised in comfort. Meat quality of breast and legs was evaluated at 42 days of age. In the breast meat, broilers under HS fed MD diet had the lowest value of ultimate pH. Broilers under thermoneutral temperature fed with DL-M or DL-MM diets had lower cooking loss than broilers fed MD diet. Under HS, broilers fed DL-M had the lowest cooking loss. The highest and lowest thawing losses were observed in broilers fed MD and DL-MM diet, respectively. HS caused lower value of ultimate pH ($P = 0.0045$) and higher value of component L* ($P = 0.0482$) in the meat of legs. Broilers fed MD and DL-MM diets had, respectively, the lowest and highest ultimate pH values (6.26 vs 6.37; $P = 0.0437$). Chickens fed MD diet showed the highest value of thawing and cooking losses. Our results suggest that acute and chronic heat stress could impact the broilers performance in different ways. We also demonstrated that methionine supplementation contributes to reduce the effects of stress. There were no notable differences between the supplementation of methionine as free amino acid or dipeptide.

Keywords: amino acids, cooking loss, high temperatures, peptides, thawing loss

Abbreviations: HS, heat stress; MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid; DL-MM, diet with supplementation of methionine as dipeptide; HDL, high density lipoproteins; LDL, low density lipoproteins

1. Introduction

Genetic selection used in the last decades has resulted in broiler chickens more susceptible to environmental factors such as high temperature (Deeb and Cahaner, 2001). On the other hand, temperatures at or above 30°C are common in tropical countries or in countries with heat waves during the summer and cause economic losses of millions of dollars each year (Mignon-Grasteu et al., 2015). Physiological changes that occur in the metabolism of birds under adverse conditions are responsible for reducing production efficiency and yield of parts (Yahav, 2000; El-Kholv et al., 2017). For example the physiological imbalance caused by high temperatures that directly influences the muscle glycogen reserves reflecting on meat quality (Aksit et al., 2006; Gregory, 2010; Tang et al., 2013).

Heat stress (HS) can be expressed as acute or chronic (Akbarian et al., 2016). Acute stress refers to sudden and short periods of high temperatures, while chronic stress refers to prolonged periods of high temperatures (Gonzalez-Esquerra and Leeson, 2006). Both types of heat stress have been related to metabolic changes involving oxidative stress, since higher production of reactive oxygen species (ROS) and lower mitochondrial respiratory chain activity are physiological responses to stress induced by high temperatures (Lin et al., 2006; Yang et al., 2010). Oxidative stress induced by heat stress has been related to lower productive efficiency (Del Vesco et al., 2013).

Mechanisms of defense against ROS can be mediated by non-enzymatic dietary antioxidants such as copper, zinc, selenium, magnesium, some plant derivatives, and by enzymatic antioxidants, represented mainly by the enzymes superoxide dismutase, catalase and by the glutathione defense system (Kuss, 2005). Methionine is one of the amino acids that are part of the metabolic pathway of glutathione (GSH) biosynthesis. Studies have shown that under stress conditions higher amount of endogenous methionine is directed towards the production of glutathione through cysteine production (Romestaing et al., 2008).

Methionine used in poultry diets is usually commercialized as DL-methionine (DL-Met) powder or its liquid form as sodium salt (DL-methionine-Na), or as methionine hydroxy-analogue (MHA) powder as calcium salt (MHA-Ca) or in its liquid form as free acid (MHA-FA) (Leite et al., 2009). In addition to these formula most commonly used in poultry production, methionine can also be found as dipeptides. However, regarding diets used for animal production, the use of methionine as dipeptide is directed to the production of aquatic organisms, since it presents lower leaching in the water (Niu et al., 2018).

Besides the role of dipeptides in diets of aquatic organisms described above, some researches also points out that the use of peptides in the diet may be nutritionally superior to the use of free amino acids. The absorption of amino acids as peptides can be faster and advantageous than as free amino acids; competition for transporters is lower when using peptides; and these can be more resistant than the free amino acids under fasting state (Sanioto, 2016; Tauqir, 2016). Thus, our study was conducted to test the hypothesis that supplementation of methionine as free amino acid (DL-methionine) or as methionine dipeptide (methionyl-methionine dipeptide) could reduce the damage caused by acute and chronic heat stress. For this, we evaluated the effects of HS and supplementation of two sources of methionine on the productive efficiency of broilers in three experimental periods: 24 hours of evaluation (21-22 days of age); 10 days of evaluation (22 to 32 days) and 20 days of evaluation (21 to 42 days of age) and the meat quality of breast and legs of 42 days old broilers. Here we showed for the first time the effect of supplementation of methionine as dipeptide for broilers under acute and chronic heat stress.

2. Material and methods

All the activities performed during the experiment were approved by the Institutional Animal Care and Use Committee of Universidade Federal de Sergipe (Protocol number 041/2017).

2.1. Animals and experimental design

One hundred and fifty male broilers (*Gallus gallus*) (Cobb 500) of 21 days with live weight of 900g were distributed in a completely randomized design in factorial scheme 3 (diets) x 2 (environments) with five replicates per treatment and five birds per experimental unit.

The chickens were divided into two groups regarding environmental temperature, in one group birds were raised in thermal comfort (according to the recommendation of the lineage for each age), while in the other group broilers were raised at a constant temperature of 30°C ± 1,5ºC from 21 to 42 days of age. Chickens from both temperature groups were fed with three diets related to methionine supplementation: diet without methionine supplementation (MD), supplementation with DL-methionine (free-form amino acid, DL-M), and with methionine dipeptide (DL-methionyl-DL-methionine; DL-MM) (Table 1). In all experimental period the birds had free access to water and feed. The rations were formulated to attend the recommendations of Rostagno et al. (2017), with the exception of methionine + cystine levels.

Table 1. Experimental diets (expressed as-fed basis).

	22-42 days of age		
Ingredients (%)	**MD**	**DL-M**	**DL-MM[1]**
Corn 7.8% CP	598.05	598.05	598.05
Soy oil	45.00	45.00	45.00
Soy bean meal 46% CP	324.00	324.00	324.00
Salt	4.30	4.30	4.30
Calcitic calcareous 38% Ca	9.30	9.30	9.30
Dicalcium phosphate 20%	10.70	10.70	10.70
DL-methionyl-methionine 97%	-	-	2.80
DL-Methionine 99%	-	2.70	-
L-Threonine 98,5%	0.20	0.20	0.20
L-Lysine HCl 78%	1.55	1.55	1.55
Premix[2]	4.00	4.00	4.00
Inert	2.90	0.20	0.10
Total	1000	1000	1000
Calculated composition (%)			
Crude protein	20	20	20
Digestible Lys	1.080	1.080	1.080
Digestible Met + Cist	0.543	0.810	0.809
Digestible Thr	0.700	0.700	0.700
Digestible Trp	0.218	0.218	0.218
Digestible Val	0.842	0.842	0.842
Digestible Ile	0.772	0.772	0.772
Digestible Arg	1.243	1.243	1.243
Ca	0.68	0.68	0.68
Available phosphorus	0.35	0.35	0.35
Na	0.19	0.19	0.19
Metabolizable energy (Kcal/Kg)	3.169	3.169	3.169

[1]MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid; DL-MM, diet with supplementation of methionine as dipeptide. [2]Vitamin and mineral mix (Guaranted levels/Kg of product): 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; iodine, 1mg; choline: 650 mg; salinomycin: 60 mg; avilamycin: 5 mg; Butyl hydroxy toluene, 80 mg.

2.2. Performance and relative weight

Weight gain (WG) and feed intake (FI) were calculated based on three experimental periods: 24 hours of evaluation (acute heat stress, 21-22 days of age); 10 days in experimentation (chronic heat stress, 22 to 32 days of age) and 20 days in experimentation (chronic stress, 21 to 42 days of age). The WG was calculated by the difference between the weight of the birds at the end and beginning of each experimental period. The feed intake was determined by the difference between the feed supplied during the experimental periods and the leftovers.

At 32 (10 days of evaluation) and 42 days of age (20 days of evaluation), six birds from each treatment were slaughtered for cervical dislocation to assess the relative weight of breast, legs and abdominal fat. The birds were selected by the mean weight (± 10%) of the treatment and subjected to a five-hour feed fast for complete elimination of the contents of the gastrointestinal tract. The relative weight was calculated as percent of body weight**.**

2.3. Plasma parameters

In order to evaluate the content of triglycerides (TRI), total cholesterol (TC) and its respective LDL and HDL fractions, the blood of six birds from each treatment was collected from the jugular vein and stored in tubes containing heparin at 22, 32 and 42 days of age. The blood was centrifuged at 3,000 x*g* for 10 min at 4°C; the plasma was collected and stored at -20°C until the time of analysis.

The content of TG, CT, LDL, and HDL were analyzed using commercial kits according to the methodology specified by the manufacturer (Labtest, Minas Gerais, Brazil): TRIGLYCERIDES LIQUIFORM 87-2/100, CHOLESTEROL LIQUIFORM 76-2/100, CHOLESTEROL LDL 146-1/40 and CHOLESTEROL HDL 13-1/50. The tests were performed by kinetic method in EPOCH microplate spectrophotometer (BioTek® Instruments, Vermont, USA) in triplicate.

2.4. Meat quality

For evaluation of the quality of breast and legs meat at 42 days of age, six birds per treatment were selected according to the mean weight of their respective treatment (± 10%). The breasts and legs of the birds were deboned and separated in left and right parts, considering the bird in the ventral direction. The water loss by thawing and cooking, ultimate pH and meat color were evaluated.

For the evaluation of water loss by thawing, samples from right side (breast and leg) were frozen at -22°C for 24 hours, weighed and stored in the refrigerator for 24 hours for thawing. After 24 hours of thawing, samples were taken from the refrigerator, rinsed with paper towel and weighed again. Thawing loss was calculated by difference between weight of frozen and thawed samples. Cooking losses was evaluated according to Bridi and Silva (2009).

The evaluation of ultimate pH was performed on the cranial part of breast muscle (*Pectoralis major*) and legs (*Gluteus maximus*) after 24 hours of cooling the samples at 4ºC in the refrigerator. The assays were performed using a HI 99163 digital pHmeter (Hanna Instruments) with insertion electrode, following the methodology described by Bridi and Silva (2009).

Color measurements were performed 24 hours post-mortem in the breast muscle and legs at three different reading points per sample. The color measurements were analyzed using the Konica Minolta's CR-400 colorimeter. The values of L * (luminosity), a* (red-green component) and b* (yellow-blue component) were expressed in the CIELAB color system.

2.5. Statistical analysis

The data were submitted to two-way ANOVA. For significant effects, means were compared by Tukey's test ($P < 0.05$) (SAS Version 9.0, SAS Inst. Inc., Cary, NC, USA). In the model we considered the effects of supplementation sources, environmental temperature, and diet-environment interaction. The results are expressed as mean and standard error.

3. Results

3.1. *Performance and relative weight*

The effects of diets and environments on feed intake (FI) and weight gain (WG) at 22, 32 and 42 days of age are presented in Table 2. After 24 hours of evaluation (22 days of age), diet and temperature effects were observed on FI, as birds from thermoneutral temperature presented higher FI than chickens from HS, and animals fed DL-M diet had higher feed intake than birds fed MD diet. No significant effects were observed on WG after 24 hours of experimentation.

Regarding the second period of evaluation (22 to 32 days of age), animals raised in thermal comfort presented higher FI ($P < 0.0001$) and higher WG ($P < 0.0001$) than

animals under HS for 10 days. Methionine supplementation, regardless of source (DL-M or DL-MM), resulted in greater weight gain (P <0.0001). At 42 days of age (20 days of experimentation), higher WG was observed in birds from thermoneutral group and in birds receiving diet with supplementation of any source of methionine.

At 32 days of age, effect of interaction between environment and diet was observed on the relative weight of the legs (P = 0.0136), as chickens fed MD diet under HS presented the highest relative weight of legs. There was no significant difference between diets under comfortable temperature. Both sources of methionine supplementation caused higher relative weight of breast. At 42 days of age, birds fed MD diet presented higher relative weight of abdominal fat (P = 0.0188) and lower relative weight of breast (P <0.0001) than chickens fed DL-M and DL-MM diets (Table 3).

Table 2. Performance of broiler chickens at 22, 32 and 42 days of age.

		22 days				32 days				42 days			
		FI(g)		WG(g)		FI(g)		WG(g)		FI(g)		WG(g)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Comfort	MD	124.11	3.53	86.78	3.76	1800.80	38.03	1015.81	28.59	1649.13	40.74	784.78	68.21
	DL-M	131.78	1.92	97.44	4.59	1784.82	19.98	1170.90	24.55	1620.27	41.16	918.74	42.51
	DL-MM	128.60	2.91	91.63	6.32	1720.78	21.11	1157.67	20.15	1575.40	39.66	912.27	42.80
HS	MD	109.00	3.46	75.74	5.09	1558.28	49.78	837.88	26.89	1438.20	24.85	65.40	53.05
	DL-M	122.80	1.74	93.67	4.89	1589.30	31.69	1023.20	20.28	1407.67	57.94	824.50	65.08
	DL-MM	112.81	4.19	80.93	3.50	1585.40	39.92	1038.10	26.89	1391.07	46.33	772.87	54.88
Main effect													
Environment	Comfort	128.18[a]	1.70	91.94	3.14	1766.51[a]	16.75	1118.21[a]	18.81	1614.93[a]	23.13	882.56[a]	27.48
	HS	114.24[b]	2.13	83.63	3.08	1577.66[b]	22.29	966.39[b]	21.86	1412.31[b]	24.70	749.59[b]	36.53
Diet	MD	116.56[b]	3.01	82.69	3.67	1666.07	52.39	922.16[b]	28.28	1543.66	41.74	701.42[b]	40.98
	DL-M	127.85[a]	1.72	95.79	3.28	1687.06	37.06	1097.05[a]	22.96	1513.96	48.76	866.39[a]	39.71
	DL-MM	121.12[ab]	3.06	86.56	3.83	1653.09	31.01	1097.88[a]	21.35	1483.23	42.08	842.57[a]	40.20
Probability													
Environment		<.0001		0.0521		<.0001		<.0001		<.0001		0.0079	
Diet		0.009		0.0555		0.5925		<.0001		0.3856		0.0295	
Interaction		0.5223		0.7973		0.3321		0.5042		0.9342		0.9062	

[a, b] Mean values within a column with different superscript letters are significantly different (P<0.05).
FI, Feed intake; WG, weigh gain. MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid; DL-MM, diet with supplementation of methionine as dipeptide.

Table 3. Relative weight of abdominal fat, breast and legs of broilers at 22 and 32 days of age.

		32 days						42 days					
		Abdominal fat		Breast		Legs		Abdominal fat		Breast		Legs	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Comfort	MD	1.59	0.09	23.01	0.35	17.97[b]	0.42	1.88	0.21	26.00	0.44	22.02	0.42
	DL-M	1.30	0.26	26.97	0.69	18.49[b]	0.40	1.65	0.14	29.83	0.46	20.77	0.27
	DL-MM	1.34	0.11	26.69	0.67	17.77[b]	0.35	1.54	0.17	30.83	0.58	19.88	0.33
HS	MD	1.48	0.13	23.20	0.68	20.12[a]	0.59	1.99	0.10	25.76	0.62	21.49	0.27
	DL-M	1.49	0.15	27.23	0.47	18.23[b]	0.16	1.44	0.14	31.33	0.43	21.32	0.52
	DL-MM	1.14	0.18	26.30	0.79	18.32[b]	0.29	1.49	0.15	30.19	0.63	21.03	0.31
Main effect													
Environment	Comfort	1.41	0.09	25.56	0.54	18.08	0.23	1.68	0.10	28.88	0.57	20.89	0.28
	HS	1.37	0.09	25.58	0.55	18.89	0.29	1.64	0.09	29.09	0.66	21.28	0.21
Diet	MD	1.53	0.08	23.10[b]	0.37	19.04	0.47	1.94[a]	0.11	25.87[b]	0.36	21.76[a]	0.25
	DL-M	1.39	0.14	27.10[a]	0.40	18.36	0.21	1.55[b]	0.10	30.57[a]	0.38	21.05[ab]	0.29
	DL-MM	1.24	0.11	26.49[a]	0.49	18.04	0.23	1.52[b]	0.11	30.51[a]	0.42	20.46[b]	0.28
Probability													
Environment		0.7438		0.9727		0.0161		0.7116		0.6326		0.2043	
Diet		0.2093		<.0001		0.0464		0.0188		<.0001		0.005	
Interaction		0.4905		0.8508		0.0136		0.6042		0.1218		0.0845	

[a, b] Mean values within a column with different superscript letters are significantly different (P<0.05).
MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid;
DL-MM, diet with supplementation of methionine as dipeptide.

3.2. *Plasma parameters*

Results of plasma parameters at 22, 32 and 42 days of age are presented in Tables 4, 5 and 6, respectively. At 22 days of age, interaction effects were observed on triglyceride content (TRI) and total cholesterol (TC). Higher TRI content was observed in chickens under HS fed MD diet. Regarding TC content, broilers raised in comfortable temperature fed DL-MM diet had the highest TC content. There was no effect of treatments on the other parameters evaluated at 22 days of age.

At 32 days of age, there was an interaction effect on TC content, as broilers raised in comfort receiving DL-M diet presented the highest TC value (106.38 mg/dL). Temperature effects were observed on HDL and LDL contents; broilers raised in HS condition had lower HDL and higher LDL content than birds that remained in comfort. The diet had a significant effect on triglyceride content as higher value was observed in birds fed DL-M diet.

At 42 days of age, interaction effects were observed on TRI, TC, HDL and LDL content. For broilers raised under HS, the highest value of TRI was observed in chickens fed MD diet. Regarding TC content, there was no significant difference between birds raised in HS, however, for birds raised in comfortable temperature, chickens fed DL-MM had the lowest content of TC. There was no significant difference between broilers raised under HS on LDL content, while under thermoneutral temperature, broilers fed MD diet had the highest LDL content. Regarding HDL results, there was no difference between broilers raised in HS fed DL-MM and broilers that remained in comfortable temperature.

Table 4. Plasma triglycerides (TRI), total cholesterol (TC), HDL and LDL content of broiler at 22 days of age.

		TRI (mg/dL)		CT (mg/dL)		HDL (mg/dL)		LDL (mg/dL)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Comfort	MD	126.873[b]	6.698	73.416[b]	2.667	27.000	3.785	26.183	3.559
	DL-M	122.000[b]	2.996	74.345[b]	3.687	26.166	0.600	27.403	5.372
	DL-MM	135.243[ab]	1.527	91.380[a]	0.859	30.833	1.301	35.546	2.889
HS	MD	144.926[a]	4.322	77.730[b]	1.882	20.000	1.527	33.036	3.086
	DL-M	124.036[b]	2.340	78.913[b]	2.178	26.166	1.201	29.956	1.106
	DL-MM	131.576[b]	3.138	79.230[b]	0.885	26.330	2.420	26.913	1.153
Main effect									
Main effect	Comfort	128.040	2.901	79.714	3.211	28.000	1.371	29.711	2.513
Environment	HS	133.513	3.487	78.624	0.898	24.333	1.409	29.968	1.337
Diet	MD	135.900	5.385	75.573	1.750	23.500	2.404	29.610	2.605
	DL-M	123.020	1.760	76.630	2.170	26.166	0.600	28.680	2.518
	DL-MM	133.410	1.763	85.305	2.772	28.833	1.520	31.230	2.379
Probability									
Environment		0.1094		0.5650		0.0521		0.9234	
Diet		0.0141		0.0018		0.0731		0.7304	
Interaction		0.0410		0.0041		0.2792		0.0825	

[a, b] Mean values within a column with different superscript letters are significantly different ($P<0.05$).
MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid;
DL-MM, diet with supplementation of methionine as dipeptide.

Table 5. Plasma triglycerides (TRI), total cholesterol (TC), HDL and LDL content of broiler at 32 days of age.

		TRI (mg/dL)		TC(mg/dL)		HDL (mg/dL)		LDL (mg/dL)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Comfort	MD	90.348	1.797	83.685[b]	4.152	33.511	9.067	35.043	1.881
	DL-M	99.138	2.251	106.388[a]	9.634	48.331	10.020	37.891	2.582
	DL-MM	90.956	3.154	89.096[b]	3.179	26.008	2.823	43.008	1.586
HS	MD	92.566	3.018	87.196[b]	4.274	23.106	3.855	44.723	0.865
	DL-M	95.600	1.809	83.173[b]	1.069	18.551	1.060	43.735	0.709
	DL-MM	92.543	1.810	84.830[b]	0.544	19.923	0.800	43.680	2.161
Main effect									
Environment	Comfort	93.481	1.653	93.056	4.158	35.950[a]	4.873	38.647[b]	1.373
	HS	93.570	1.287	85.068	1.446	20.527[b]	1.358	44.056[a]	0.771
Diet	MD	91.457[b]	1.707	85.440	2.889	28.309	4.952	39.883	1.761
	DL-M	97.369[a]	1.476	94.783	5.794	33.441	6.574	40.813	1.551
	DL-MM	91.750[ab]	1.750	86.963	1.666	22.965	1.672	43.344	1.282
Probability									
Environment		0.9638		0.0517		0.0031		0.0008	
Diet		0.0305		0.1335		0.2208		0.1446	
Interaction		0.4245		0.0277		0.1171		0.0512	

[a, b] Mean values within a column with different superscript letters are significantly different ($P<0.05$).
MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid;
DL-MM, diet with supplementation of methionine as dipeptide.

Table 6. Plasma triglycerides (TRI), total cholesterol (TC), HDL and LDL content of broiler at 42 days of age.

		TRI (mg/dL)		TC (mg/dL)		HDL (mg/dL)		LDL (mg/dL)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Comfort	MD	88.756^{bc}	0.986	91.830^{a}	2.396	44.486^{a}	2.835	28.680^{a}	1.638
	DL-M	94.666^{a}	0.796	89.020^{a}	1.422	45.085^{a}	1.451	25.100^{b}	0.965
	DL-MM	89.455^{b}	1.327	73.968^{b}	1.468	38.606^{bc}	2.487	19.911^{c}	0.680
HS	MD	89.743^{b}	0.959	74.951^{b}	1.319	40.060^{ab}	1.612	16.633^{c}	0.700
	DL-M	85.603^{c}	0.837	71.431^{b}	2.842	35.115^{c}	2.158	18.118^{c}	0.167
	DL-MM	85.803^{c}	1.549	76.215^{b}	2.536	42.420^{ab}	0.956	18.731^{c}	1.049
Main effect									
Environment	Comfort	90.959	0.859	84.939	2.142	42.726	1.450	24.230	1.665
	HS	87.050	0.779	74.199	1.354	39.198	1.160	17.973	0.615
Diet	MD	89.250	0.672	83.390	2.624	42.273	1.692	22.656	2.004
	DL-M	90.135	1.473	80.225	3.053	40.100	1.948	21.827	1.025
	DL-MM	87.629	1.117	75.091	1.437	40.513	1.394	18.821	0.597
Probability									
Environment		0.0002		0.0001		0.0408		0.0001	
Diet		0.0893		0.0016		0.5281		0.0042	
Interaction		0.0004		0.0001		0.0070		0.0001	

[a, b, c] Mean values within a column with different superscript letters are significantly different (P<0.05).
MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid;
DL-MM, diet with supplementation of methionine as dipeptide.

3.3. Meat quality

Results of meat quality of breast and legs of broilers chickens at 42 days of age are presented in Tables 7 and 8, respectively.

Breast results- Effect of interaction between temperature and diet was observed on ultimate pH value (P=0.0063) and cooking loss (P=0.0411). Broilers under HS fed MD diet had the lowest value of ultimate pH. Regarding cooking loss results, broilers raised in comfortable temperature fed with DL-M and DL-MM diets had lower cooking loss than broilers fed MD diet. Under HS, broilers fed DL-M had the lowest cooking loss. Temperature effect was observed on b* component (P=0.0202), as higher b* value was observed in broilers from thermoneutral group. Significative effect of diet was observed on L*, a*, and thawing loss, as broilers fed MD diet had the highest L* value and broilers fed DL-M and DL-MM diets had lower *a (redness) value. The highest thawing loss was observed in broilers fed MD diet and the lowest loss in broilers fed DL-MM diet (20.27 vs 16.26 g).

Legs results- It was observed that HS caused lower value of ultimate pH (P = 0.0045) and higher value of component L* (P = 0.0482), indicating a trend of pale meat. Regarding the diet, broilers fed MD and DL-MM diets had the lowest and highest ultimate pH values, respectively (6.26 vs 6.37; P = 0.0437). Significant difference was observed between the sources of methionine supplementation on the b* component: birds fed DL-M and DL-MM diet presented respectively the highest and lowest values of component b*. Chickens fed MD diet showed the highest values of thawing and cooking loss; there was no difference between broilers receiving DL-M and DL-MM diets.

Table 7. Breast's meat quality of broilers at 42 days of age.

		pH		L*		a*		b*		Thawing loss (g)		Cooking loss (g)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Comfort	MD	5.945^{c}	0.018	47.741	0.743	7.897	0.491	23.366	0.232	21.245	1.566	10.391^{a}	0.802
	DL-M	6.095^{a}	0.019	46.161	0.555	6.055	0.307	22.244	0.242	19.190	2.103	8.061^{b}	0.495
	DL-MM	6.038^{ab}	0.023	45.577	0.958	6.238	0.297	22.622	0.446	15.486	1.717	7.470^{b}	0.119
HS	MD	5.816^{d}	0.009	46.877	0.373	7.488	0.435	22.316	0.429	20.255	0.698	9.705^{a}	0.653
	DL-M	6.001^{bc}	0.026	44.922	0.685	6.500	0.474	22.333	0.250	20.393	1.896	7.995^{b}	0.359
	DL-MM	6.056^{ab}	0.028	45.888	0.559	6.111	0.368	21.627	0.275	17.051	1.783	9.706^{a}	0.736
Main effect													
Environment	Comfort	6.026	0.019	46.493	0.473	6.730	0.286	22.744^{a}	0.208	18.640	1.138	8.641	0.427
	HS	5.958	0.028	45.896	0.358	6.700	0.272	22.092^{b}	0.195	19.233	0.923	9.135	0.382
Diet	MD	5.880	0.022	47.309^{a}	0.418	7.693^{a}	0.319	22.841	0.282	20.750^{a}	0.827	10.048	0.504
	DL-M	6.048	0.021	45.541^{b}	0.460	6.278^{b}	0.278	22.288	0.167	19.971^{ab}	1.362	8.028	0.292
	DL-MM	6.047	0.018	45.733^{ab}	0.531	6.175^{b}	0.227	22.125	0.292	16.269^{b}	1.204	8.588	0.490
Probability													
Environment		0.0007		0.2846		0.9271		0.0202		0.6699		0.3028	
Diet		<.0001		0.0251		0.0009		0.0862		0.0308		0.0045	
Interaction		0.0063		0.4923		0.5650		0.1604		0.7172		0.0411	

$^{a, b,c,d}$ Mean values within a column with different superscript letters are significantly different (P<0.05).
MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid;
DL-MM, diet with supplementation of methionine as dipeptide.
Ultimate pH, pH at 24 hours*post mortem;* L*, luminosity; a*, red-green component; b*, yellow-blue component.

Table 8. Legs' meat quality of broilers at 42 days of age.

		pH		L*		a*		b*		Thawing loss (g)		Cooking loss (g)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Comfort	MD	6.356	0.053	44.377	0.348	8.683	0.380	20.505	0.212	5.470	0.310	8.540	0.671
	DL-M	6.368	0.029	42.747	0.537	7.738	0.483	20.625	0.341	3.616	0.234	6.685	0.518
	DL-MM	6.380	0.043	43.788	0.442	8.194	0.554	20.411	0.220	4.368	0.421	5.596	0.343
HS	MD	6.175	0.034	45.694	0.546	8.883	0.672	21.122	0.397	5.096	0.341	6.911	0.350
	DL-M	6.211	0.044	45.127	0.626	8.425	0.398	21.216	0.387	3.433	0.134	6.930	0.393
	DL-MM	6.378	0.058	43.430	1.153	9.105	1.045	19.786	0.343	4.006	0.170	5.898	0.239
Main effect													
Environment	Comfort	6.368^{a}	0.024	43.637^{b}	0.294	8.205	0.276	20.513	0.145	4.485	0.258	6.940	0.411
	HS	6.255^{b}	0.033	44.750^{a}	0.503	8.804	0.414	20.708	0.258	4.178	0.210	6.580	0.216
Diet	MD	6.265^{b}	0.041	45.036	0.367	8.783	0.370	20.813^{ab}	0.234	5.283^{a}	0.227	7.725^{a}	0.437
	DL-M	6.290^{ab}	0.035	43.937	0.533	8.081	0.316	20.920^{a}	0.262	3.525^{b}	0.132	6.807^{ab}	0.312
	DL-MM	6.379^{a}	0.035	43.609	0.591	8.650	0.581	20.098^{b}	0.216	4.187^{b}	0.224	5.747^{b}	0.205
Probability													
Environment		0.0045		0.0482		0.2542		0.4699		0.2012		0.3258	
Diet		0.0437		0.0950		0.5062		0.0345		<.0001		0.0005	
Interaction		0.1154		0.1310		0.8480		0.1099		0.9336		0.0604	

[a, b,c,d] Mean values within a column with different superscript letters are significantly different (P<0.05).
MD, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid;
DL-MM, diet with supplementation of methionine as dipeptide.
Ultimate pH, pH at 24 hours*post mortem;* L*, luminosity; a*, red-green component; b*, yellow-blue component.

4. Discussion

In our study, we observed that broilers subjected to heat stress had lower feed intake than broilers from thermoneutral group after 24 hours, 10 and 20 days of experimentation. Regarding the results of weight gain, after 24 hours of experimentation, birds submitted to HS showed similar weight gain to the birds that remained at comfortable temperature. However, after 10 or 20 days of experimentation, broilers from HS group had WG 13.6% (10 days) and 15% (20 days) lower than birds that remained in comfortable temperature, respectively. As observed in our study, the effects of heat stress appear to be dependent on the intensity and duration of stress. Continuous high temperature (Sahin et al., 2017) or intermittent high temperature for short (Ezzat et al., 2017) or extensive (Xu et al., 2018) periods are associated with metabolic alterations that result in lower productive efficiency and increased morbidity and mortality among other undesirable characteristics. To maintain body temperature within the zone of thermoneutrality, birds submitted to high temperature environments usually present higher water consumption, lower feed intake, and spend more time at rest. According to Mignon-Grasteau et al. (2015), temperatures about 30°C already have great negative effects on the performance of birds, and some characteristics, such as feed intake for example, may be altered at temperatures even below 30°C.

The effects of stress can also be determined through hormonal regulation and the interaction between endocrine, immune and nervous systems (Calefi et al., 2017). Heat stress is generally associated with activation of the hypothalamic-pituitary-adrenal axis (HPA) that results in elevation of plasma corticosterone (Quinteiro-Filho et al., 2010), which has as main function to prepare the body to defend itself from challenging situations by inhibiting the absorption of glucose by muscle cells and adipose tissue. According to Quinteiro-Filho et al. (2012), the activation of HPA axis and the increase in corticosterone levels is responsible for part of the negative effects observed in the performance and immune function of broilers under HS. The activity of thyroid and growth-related hormones is also influenced by stress and contributes to the observed changes in performance (Del Vesco et al., 2017; Roushdy et al., 2018).

Different nutritional strategies have been shown to be effective in minimizing the effects of heat stress, such as high-energy diets (Syafwan et al., 2011), and diets with supplementation of vitamins, minerals or specific amino acids (Zangeneh et al., 2018, Mohamed et al., 2017, Han et al., 2017). Our research group has shown that supplementation of DL-methionine in diets of broiler chickens under acute heat stress

can help to attenuate the negative effects of stress through the action of antioxidant components and somatotropic axis, for example (Del Vesco et al., 2015a, Del Vesco et al., 2015b). In the literature, other sources of methionine supplementation have also been related to improve bird performance (Park et al., 2018) and reported as capable of attenuating the effect of heat stress (Willemsen et al., 2011). However, until now, supplementation of methionine as free amino acid was always used in these studies. In this study, we evaluated for the first time the effect of supplementation of methionine as dipeptide on productive efficiency of broilers subjected to acute and chronic heat stress.

We observed that broilers raised in comfortable temperature had higher WG when they were fed with DL-M and DL-MM diets compared to the MD diet. The methionine supplementation was found even more important for broilers under HS, since broilers receiving diets with methionine supplementation had WG 18% and 21% higher than broilers fed MD diet at 10 and 20 days of experimentation, respectively. There was no significative difference between sources of methionine supplementation. Some researchers have shown that the supplementation of amino acids as dipeptides could be more efficient (Sanioto, 2016; Tauqir, 2016) and that the supplementation of dipeptides could contribute to reduce the effect caused by different challenges in mice or in vitro (Je et al., 2015; Chen et al., 2018). However, little is known regarding the effect of the supplementation of methionine as dipeptide for broilers. Silva et al. (2016) and Mencalha et al. (2016) have shown similar bio-efficacies for DL-M and DL-MM for weigh gain of starting and growing broilers, however, the role of dipeptides in diets of broilers needs to be further investigated.

The results of weight gain are followed by the relative weight of breast. Broilers receiving methionine supplementation from both sources had higher relative weight of breast than broilers fed MD diet. For animals raised in HS, we observed that animals fed MD diet had relative weight of breast 15% and 18% lower than animals fed DL-M diet after 10 and 20 days of experimentation, respectively. This result can be explained in part by the action of methionine on the action of growth hormones and protein metabolism, as methionine acts by stimulating protein synthesis and reducing the catabolic rate (Stubbs et al., 2002; Métayer et al., 2008). After 10 days of experimentation, higher relative weight of legs was observed in broilers from HS group receiving MD diet. Higher relative weight of legs in birds raised under heat stress was also observed by Zhang et al. (2012). According to Temim et al. (2000), higher relative weight of legs is observed in HS group because birds submitted to stress present higher relative weight of *Sartorius* and

Gastrocnemius muscles when they have lower relative weight of the *Pectoralis major* muscle. Also according to the authors, heat stress seems to exert a higher effect on protein synthesis than on catabolism.

In our study we showed that broilers from HS group had lower feed intake followed by lower WG. This feature is related to the birds' attempt to reduce heat production, however, the decreased productive efficiency caused by HS does not occur only due to the reduction in feed consumption. HS also causes changes in hormones level, immune function, blood flow and blood metabolites that undergo changes in the attempt to decrease body temperature (Lara and Rostagno, 2013). It should be notice that the changes in plasma metabolites appear to be dependent on the duration of stress, since different alterations were observed in animals subjected to acute or chronic stress. These differences between acute and chronic were also observed by Xie et al. (2015). In our study, after 24 hours of experimentation, we observed a higher triglyceride content in birds subjected to HS receiving MD diet. After 10 days of experimentation, birds under HS had lower HDL and higher LDL content than birds that remained in comfortable temperature. After 20 days of evaluation, for birds from HS group, the highest level of triglycerides and LDL was observed in birds receiving MD diet. Broilers from HS group fed DL-MM diet had content of HDL similar to birds from comfort group. Comparing the sources of methionine supplementation, we can observe that birds fed DL-MM presented lower concentration of LDL than birds fed DL-M.

The results described above may be related to the increased level of corticosterone induced by heat stress, since corticosterone can cause significant changes in the metabolism of carbohydrates and lipids (Pan et al., 2018). Birds under HS condition had increased production of fatty acids used to produce triglycerides (Jastrebski et al., 2017). These authors have shown that the HS induces the glucose production through glycogenolysis and gluconeogenesis. The increase in lipid production is due to the action of the enzyme SREBP-1 (Sterol-regulatory element-binding protein-1) and of the growth hormone receptor (De Antônio et al., 2017). The increase in lipid deposition induced by stress is also due to the higher rate of amino acid catabolism. In this sense, methionine supplementation may have contributed to the positive results observed in the reduction of triglycerides and LDL content (Table 6) and in abdominal fat deposition (Table 3) of broilers from HS group. Saleh et al. (2017) also showed that methionine supplementation may contribute to reduce the level of cholesterol and to increase HDL levels in broilers.

Accompanied by performance and plasma metabolites results, chronic heat stress also impaired the meat quality of breast and legs, as HS reduced the ultimate pH and b* values and increased cooking loss and value of L*. Animals that are subjected to some kind of stress prior to slaughter may have postmortem glycogen depletion. Such depletion may result in increased value of ultimate pH, or in accelerated rate of glycolysis with higher lactate concentration. Increased lactate concentration results in a marked drop in pH after slaughter accompanied by denaturation of sarcoplasmic proteins (Listrat et al., 2016). All these changes result in reduced ability to retain water and increased value of the L* (El Rammouz et al., 2004). As denatured proteins have lower capacity of interaction of water the luminosity is increased due to higher dispersion and reflection of light (Listrat et al., 2016). Thus, as observed in our study, meat of broilers subjected to HS has higher values of L* and lower pH, b*, a* and water holding capacity (Zhang et al., 2012).

Similar results caused by chronic stress were observed in birds fed MD diet; lower ultimate pH and higher L* value accompanied by higher thawing and cooking losses. Similar results were also observed by Wen et al. (2017). It should also be noted that birds raised in HS receiving DL-M diet presented results of cooking loss similar to birds from thermoneutral group (Table 7). Since the water holding capacity may be related to the chemical characteristics of the meat, the result observed in this study may demonstrate that the lower protein deposition usually found in birds fed diets without supplementation of methionine (Corzo et al., 2006) may contribute to the lower water holding capacity in the meat of breast and legs of broilers fed MD diet.

5. Conclusion

In conclusion, heat stress impaired the performance, yield and meat quality of breast and legs of broiler chickens. Different effects were observed due to acute or chronic heat stress, with the worst performance and yield results observed due to chronic stress. Since there were no notable differences between the sources of supplementation, methionine as free amino acid or as dipeptide can be used to improve broilers performance under thermoneutral temperature and to mitigate the effects of heat stress.

Acknowledgment

This work was supported by the National Council for Scientific and Technological Development (CNPq) [407669/2016-7].

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IV. FREE AND DIPEPTIDE FORMS OF METHIONINE SUPPLEMENTATION REDUCE HEAT STRESS EFFECTS BY GENE REGULATION AND EXPRESSION

(Archives of Animal Nutrition)

Abstract

Our previous studies have shown that methionine supplementation could help to attenuate the effects of heat stress on the metabolism of broiler chickens. Here, we investigated for the first time the effect of methionine supplementation as the DL-methionyl-methionine (DL-MM) dipeptide in the diet of broilers subjected to high temperature (HT) during the growth phase (22-42 days of age). Broilers were divided into two groups: one group was raised in comfortable temperature, and the other group was raised under continuous heat stress (HT) of 30ºC. From both groups, animals were fed with diet without methionine supplementation (MS diet); with supplementation of methionine as free amino acid (DL-M); and with methionine as dipeptide (DL-MM). Broilers raised under HT had lower feed intake, weight gain, relative weight of liver, heart and spleen than broilers from confort group ($P<0.05$). There were no differences between methionine sources for performance and relative weight results. Birds raised under HT had significantly H/L ratio. For birds raised under HT, broilers fed DL-M diet had a trend of lower H/L ratio compared to birds fed SM and DL-MM diets. Higher concentration of carbonylated proteins and lower concentration of glutathione (GSH) were significantly observed in birds raised in HT compared to birds of comfort. Comparing birds raised under HT, birds fed DL-M diet had lower concentration of TBARS and carbonylated proteins than birds receiving SM diet ($P<0.05$). Higher expression of *GPx* and *GSS* was observed in broilers raised in HT environment compared to broilers from comfort goup ($P<0.05$). Under HT, broiler fed SM diet presented the highest expression of *GPx*. Broilers fed SM diet in HT environment had higher expression of *GSS* than broilers receiving SM diet in comfort temperature. We also investigated the association between our treatments and the DNA methylation in the promoter region of *GPx* and *GSS* genes. In HT conditions, birds fed DL-MM diet had the highest methylation values in the promoter region of the *GPx* and *GSS* genes. There was a negative association between DNA methylation and gene expression levels. Broilers from HT had lower methylation and higher expression levels than broilers raised in comfort enviroment receiving any diet. Our results showed that methionine supplementation as free amino acid or dipeptide may help to attenuate the effects of stress through the action of genes related to the antioxidant mechanism of glutathione. The

Methionine effects could be found at gene regulation, gene expression and at post-translational levels.

Keywords: broiler, heat stress, methionine, methionine dipeptide,oxidative stress

1. Introduction

Heat stress (HS) has been identified as one of the main causes of pathophysiological changes that culminate in decreased welfare and productive efficiency of chickens (Varasteh et al. 2015). The damages are due to changes in the intestinal environment that impair the absorption of nutrients and facilitate infection by pathogens (Quinteiro-Filho et al. 2012), reduction of immune function (Kamel et al. 2017), and also due to the higher production of reactive species of oxygen (ROS) (Lin et al. 2006; Yang et al. 2010).

Besides increased ROS production, HS causes changes in the activity of antioxidant enzymes accompanied by increased lipid oxidation and depletion in the concentration of antioxidant components (Willemsen et al. 2011; Akbarian et al. 2016). Reactive oxygen species cause damage to proteins, nucleic acids and lipids, and are responsible for economic losses observed from the production in the farm to the final product quality (Estévez 2015). In order to minimize the effect of heat stress on oxidative stress, the effect of supplementation of different nutrients such as vitamins (Attia et al. 2016; Zeferino et al. 2016), minerals (Habibian et al. 2015) and amino acids (Wadden et al. 2011) has been extensively investigated in order to potentiate the action of antioxidant components and to avoid the damage caused by oxidative stress.

Amino acids are precursors of different products. They act on different pathways and have different functions (Tesseraud et al. 2011). Methionine, as well as important for protein synthesis, has antioxidant function as a precursor of cysteine and glutathione through the transulfurization pathway (Swennen et al. 2011). Methionine also have direct action against ROS through the action of the enzyme methionine sulfoxide reductase (Luo and Levine 2009). As the observed by other authors (Ebrahimzadeh et al. 2013, Saleh et al. 2018), our research group has demonstrated the positive effect of methionine supplementation as DL-methionine (Del Vesco et al. 2015) and as methionine hydroxy analog (Gasparino et al. 2018) on the activity of different antioxidant components of birds subjected to acute heat stress. Through these results we can note that the effect of methionine supplementation depends on different factors, such as production phase, intensity and duration of stress, and source of supplementation, for example.

In addition to the free form, methionine can also be found as dipeptides conjugated to different amino acids or to another molecule of methionine. Study have shown that dipeptides can avoide cell damage (Zhang et al. 2013). For broilers, dipeptides may increase levels of antioxidant enzymes improving antioxidative capacity (Cong et al. 2016). In this study we investigated for the first time the effect of methionine supplementation as the DL-methionyl-methionine (DL-MM) dipeptide in the diet of broilers subjected to high temperature throughout the growth phase (22-42 days of age). We also investigated the association between our treatments and the DNA methylation in the promoter region of genes related to antioxidant capacity. Although several studies show the importance of DNA methylation in gene regulation, as well as the importance of methionine to methylation metabolism, the effect of environment and methionine supplementation on methylation of specific regions is still controversial (reviewed by Zhang 2018 and Tesseraud et al. 2009). Thus, this study was conducted to evaluate the effect of methionine supplementation as a free amino acid (DL-methionine, DL-M) and as dipeptide (DL-MM) on the antioxidant metabolism of broiler chickens raised under heat stress conditions.

2. Matherial and Methods

The activities carried out during this experiment were approved by the animal production research ethics committee of the Federal University of Sergipe (protocol nº041 / 2017).

2. 1. Animals and experimental design

One-hundred and fifty male broilers (*Gallus gallus*) (Coob 500) of 22 days of age were distributed in two environmental temperatures in factorial scheme 3 (diets) x 2 (environments): one group (75 birds) was raised at a temperature of thermal comfort (following recommendations of line for each age) and the other group (75 birds) was raised at high temperature (HT, 30°C ± 1.5°C) from 22 to 42 days of age. In each temperature group, the birds were fed three diets (N=50): diet without methionine supplementation (SM), with supplementation of DL-methionine (DL-M) or with methionine supplementation as DL-methionyl-methionine dipeptide (DL -MM). The experimental diets were formulated according to the recommendations contained in the Brazilian tables for birds and swine (Rostagno et al. 2011) in order to meet the nutritional requirements of the birds (Table 1).

Table 1. Experimental diets (expressed as-fed basis).

	22-42 days of age		
Ingredients (%)	**SM**	**DL-M**	**DL-MM[1]**
Corn 7.8% CP	598.05	598.05	598.05
Soy oil	45.00	45.00	45.00
Soy bean meal 46% CP	324.00	324.00	324.00
Salt	4.30	4.30	4.30
Calcitic calcareous 38% Ca	9.30	9.30	9.30
Dicalcium phosphate 20%	10.70	10.70	10.70
DL-methionyl-methionine 97%	-	-	2.80
DL-Methionine 99%	-	2.70	-
L-Threonine 98,5%	0.20	0.20	0.20
L-Lysine HCl 78%	1.55	1.55	1.55
Premix[2]	4.00	4.00	4.00
Inert	2.90	0.20	0.10
Total	1000	1000	1000
Calculated composition (%)			
Crude protein	20	20	20
Digestible Lys	1.080	1.080	1.080
Digestible Met + Cist	0.543	0.810	0.809
Digestible Thr	0.700	0.700	0.700
Digestible Trp	0.218	0.218	0.218
Digestible Val	0.842	0.842	0.842
Digestible Ile	0.772	0.772	0.772
Digestible Arg	1.243	1.243	1.243
Ca	0.68	0.68	0.68
Available phosphorus	0.35	0.35	0.35
Na	0.19	0.19	0.19
Metabolizable energy (Kcal/Kg)	3,169	3,169	3,169

[1]SM, diet without methionine supplementation; DL-M, diet with supplementation of methionine as free amino acid; DL-MM, diet with supplementation of methionine as dipeptide. [2]Vitamin and mineral mix (Guaranted levels/Kg of product): 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; iodine, 1mg; choline: 650 mg; salinomycin: 60 mg; avilamycin: 5 mg; Butyl hydroxy toluene, 80 mg.

2.2. Performance, relative weight and blood parameters

Weight gain (WG) and feed intake (FI) were calculated for the experimental period (22-42 days of age). At 42 days of age, all birds were slaughtered by cervical dislocation and the liver, heart, spleen and bursa of Fabricius (n=6) were extracted and weighed to obtain the relative weight which was calculated as: organ weight / weight of the fasting bird x 100.

The blood of five birds from each treatment (n = 5) was collected in a collection tube containing heparin. A blood sample of 10 µl was collected and deposited on a slide for clean microscopy, and with the aid of another (distending) slide, a slight backward movement was made until the drop of blood spreads to the edges of the slide and, at one stroke, the distributor was slid to the end of the blade. The slides were air dried and then stained. The dyes used were: triarylmethane (for ten seconds), xanthenes (for five seconds) and thiazines (for ten seconds), respectively. After staining the microscopes (40x objective), 100 leukocytes were counted for the differential count of the heterophiles and basophils, monocytes and lymphocytes. Differential white blood cell counts and the heterophil:lymphocyte (H:L) ratio was determined was described by Zhang et al. (2009).The results were expressed in relative numbers.

2.3. Biochemical assays

For the biochemical analyzes, the liver was collected (n=6) in liquid nitrogen and stored in the freezer at -80°C until analysis.

To determine the Thiobarbituric acid reactive substances (TBARS) content, 100mg of liver was added in 1mL of phosphate buffer (0.1M, pH 7.4) and homogenized. The homogenate was centrifuged at 4ºC for 10 minutes at 10,000 xg. After centrifugation, 500µL of the supernatant was transferred to a new tube where it was add 250µL of 28% trichloroacetic acid (TCA) diluted in HCL (0.25N), 250µL of 1% thiobarbituric acid (TBA) diluted in acetic acid 1:1, and 125µL of 5mM butylated hydroxytoluene (BHT) diluted in ethanol. The solution was briefly homogenized and heated in a 95°C water bath for 15 minutes. Then the solution was centrifuged at 4°C for 10 minutes at 10,000 xg. The reaction concentration was determined in a spectrophotometer at 535nm. This concentration was obtained by the molar extinction coefficient ε = 1.56 x 105 L.mol-1.

cm-1, according to Lambert Beer's law. The results of this analysis were expressed in nmoles/mg of protein.

The activity of the enzyme superoxide dismutase (SOD) was measured according to its ability to inhibit the auto-oxidation of pyrogallol. 100mg of liver was homogenized in 1mL of phosphate buffer (0.1M, pH 7.2). The obtained homogenate was centrifuged at 10,000 xg for 10 minutes at 4°C. 20 μL of the supernatant was collected and added in a tube containing 1.800 μL of pyrogallol (1 mM) dissolved in Tris-HCL (0.2M) and EDTA (0.02M, pH 8.2). The reading was performed at 420nm, observing the increase in absorbance for 180 seconds.

The glutathione (GSH) content was measured by fluorescence using o-phthalaldehyde (OPT) as described by Hissin and Hilf (1976) with some modifications. 100 mg of liver tissue was homogenized in 1mL of extraction medium (pH 7.2) containing 250mM sucrose, 1mM EDTA, 10mM HEPES and distilled water. The homogenate was centrifuged at 10,000 xg for 10 minutes at 4°C to obtain the supernatant. In a new tube it was added 25μL of the supernatant, 500μL of 25% TCA and 1mL of precipitation medium (125mM sucrose, 65mM KCl, 10mM HEPES and distilled water, pH 7.2). For the reading, three reactions were prepared: the negative control containing 2mL of 0.1M phosphate buffer + 5mM EDA (pH 8.0), 100μL of distilled water and 100μL OPT (1mg/mL, diluted in methanol); the standard reaction, containing 2mL of 0.1M phosphate buffer + 5mM EDA (pH 8.0), 60μL of distilled water, 40μL of GSH standard (1mg%) and 100μL of OPT; and the sample reaction, containing 2mL of 0.1M phosphate buffer + 5mM EDA (pH 8.0), 100μL of the supernatant and 100μL of OPT. After the addition of OPT to all tubes, they were incubated for 15 minutes and then samples were read in fluorimeter (350nm for excitation and 420nm for emission). After this reading it was added 40μL of GSH standard in the sample reaction, 15 minutes was waited and a new reading was performed. The results are expressed as μg of GSH / mg protein in the supernatant.

The concentration of carbonylated protein was measured by the colorimetric method using 2,4-dinitrophenylhydrazine (DNPH). For the reaction, 200mg of liver tissue was homogenized in 1mL of phosphate buffer (50mM) and EDTA (1mM) (pH 6.7). The homogenized was centrifuged at 10.000xg for 10 minutes at 4ºC and the supernatant was collected for later use. For each sample, two tubes were prepared, one as sample (S) and one as control (C). 500μL of TCA (10%), 300μL of the supernatant and 200μL of the phosphate buffer and EDTA were added to both tubes. The tubes were vortexed briefly,

centrifuged at 5,000xg for 10 minutes at 4°C. The supernatant from both tubes was discarded. 500µL of DNPH (10mM) was added to sample tube and 500µL of HCl (2.5M) was added to control tube. Both tubes were kept in the dark at room temperature for 30 minutes (vortex every 15 minutes). 500µL of TCA (10%) were added in both tubes, vortexed briefly, centrifuged at 5,000xg for 10 minutes at 4°C. Again the supernatants were discarded, 1mL of ethanol plus ethyl acetate (1: 1) was added to the two tubes. Again the tubes were homogenized and centrifuged at 5,000xg for 10 minutes at 4°C and the supernatant discarded. Then 1mL sodium dodecyl sulfate (SDS) (6%) was added to tubes A and C, ther were homogenized and centrifuged at 10,000xg for 10 minutes at 4°C. 200 µL of supernatant was used for the spectrophotometer reading at 370nm.

Protein content from each sample was performed according to the methodolog described by Lowry et al. (1951).

2.4. Gene expression

For the analysis of gene expression, liver tissue was collected (n = 4) at the end of the experiment. The samples were stored in Holder RNA (BioAgency Biotechnology, Brazil) at -20ºC until RNA extraction.

Total RNA was extracted using the Trizol® reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's recommendations, at the rate of 1mL for each 80mg tissue. RNA integrity was assessed by 1% agarose gel electrophoresis stained with ethidium bromide (10mg / mL) visualized under UV light. All RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions so that possible contamination with DNA was eliminated. For complement DNA synthesis (cDNA) the GoScript Reverse Transcription System (Promega, Madison, Wi, USA) was used using 4µL of DNase-treated RNA following the manufacturer's instructions. The concentration of the total RNA and cDNA was evaluated by spectrophotometer at a wavelength of 260 to 280 nm.

For real-time PCR reactions, SYBR® GREEN PCR Master Mix (Applied Biosystems, USA) was used based on the manufacturer's recommendations. To measure the efficiency of each primer/gene set, a serie of 25µl reactions were performed using 5µL of cDNA pool from a serial dilution (80ng/µL, 40ng/µL, 20ng/µL and 10ng/µL). The thermocycler programming for all genes was: 95°C for 10 minutes, then 40 cycles of denaturation and annealing/extension at 95°C for 15 seconds and 60°C for 1 minute. The melting curves were performed to guarantee the specificity of each primer.

The primers used in the reactions of amplification of the glutathione peroxidase (*GPx*) and glutathione synthetase (*GSS*) genes were synthesized according to the information in Del Vesco et al. (2017). The β-actin gene (accession number L08165) was used as housekeeping gene (Table 2). All analyzes were performed in a volume of 25μL and in duplicates. Amplification efficiencies were similar between the housekeeping and genes of interest, ranging from 90% to 100%.

Table 2. Primer sequences used for quantitative real-time PCR

Genes	Primer sequence (5′–3′)	Amplicon (bp)
GSS	GTGCCAGTTCCAGTTTTCTTATG TCCCACAGTAAAGCCAAGAG	108
GPx7	TTGTAAACATCAGGGGCAAA TGGGCCAAGATCTTTCTGTAA	140
β-actina	ACCCCAAAGCCAACAGA CCAGAGTCCATCACAATACC	136

GSS, glutathione synthetase; *GPx7*, glutathione peroxidase 7; pb = pares de bases

2.5. DNA methylation

Genomic DNA was extracted from the liver of the same animals evaluated for gene expression (n=4) using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) following the manufacturer's recommendations.

For the measurement of DNA methylation, the EpiJET DNA Methylation Analysis Kit (Thermo Scientific, Pittsburg, PA, USA) was used. The assay was performed using the restriction enzymes Hpall and Mspl, and qPCR for amplification of the fragments following the manufacturer's recommendations. The upstream region of the *GPx* and *GSS* genes were identified using the NCBI tools. The primers were designed in the promoter region of each gene through the online software MethPrimer (Li and Dahiya, 2002). Primers for *GPx* gene amplification: 5'TGATCTTAGCCGTGCTTTCC3' and 5' ACACTGCCCTCAGGATCTA3', amplicon 175bp. Primers for *GSS* gene amplification: 5' ATCACATCCAACCTGGCTTT3' and 3'GCTCTGCGTTGCCTTCT5', amplicon 149bp.

The level of methylation in the amplified region was calculated according to the manufacturer's recommendations by the formula:% m = 100 / $(1 + E)^{Cq2-Cq1}$, where E is the efficiency of the qPCR reaction; Cq1 is the threshold cycle of undigested DNA sample, and Cq2 is the threshold cycle of DNA digested with Epi HpaII enzime.

2.6. Statical analysis

The $2^{-\Delta Ct}$ method was used for the analyzes of relative expression of the genes under study. Gene expression results are shown as arbitrary unit (AU). For all the data evaluated, the effect of temperature and diet was tested using ANOVA. When the diet effect was significant, the means were compared by the Tukey test ($P < 0.05$) (JMP software, SAS Inst. Inc., Cary, NC, USA). The data is presented as mean and *pooled* standard error.

3. Results

3.1. Relative weight and blood parameters

We evaluated the effect of methionine supplementation as free amino acid or as dipeptide on the performance of broilers raised at comfort temperature or at hight temperature (HT) (Figure 1). As expected, birds raised under HT had lower feed intake and weight gain. There was no effect of diet on feed intake, however, birds fed DL-M and DL-MM diets presented higher weight gain than birds receiving SM diet in both environments. There was no difference between sources of methionine supplementation.

Birds raised under HT also presented lower relative weight of liver, heart and spleen. In HT condition, birds fed SM diet presented higher relative weight of liver (1.41%) than birds fed DL-M and DL-MM diet, 1.13 and 1.16%, respectively. For birds raised in comfort temperature, birds fed SM diet had higher relative weight of heart (0.50%) than birds fed DL-M diet (0.42%). There was no significant difference between the sources of supplementation on the relative weight of any of the evaluated organs (Figure 2).

The results of performance and relative weight of the organs showed that chronic heat stress can cause changes in the metabolism of birds resulting in lower productive efficiency. Thus, we evaluated the heterophy/lymphocyte ratio as an indicator of physiological responses to stress (Figure 3). As expected, birds raised on HT had significantly higher values of heterophils, lower values of lymphocytes, and therefore, a higher H/L ratio. However, it is important to emphasize that there is no significative difference between lymphocytes content of birds fed DL-M raised in HT environment or raised in comfort temperature. This result is related to the trend of lower H/L ratio under

HT condition observed for birds fed DL-M (0.39) when compared to birds fed SM and DL-MM diets (0.55 and 0.42, respectively).

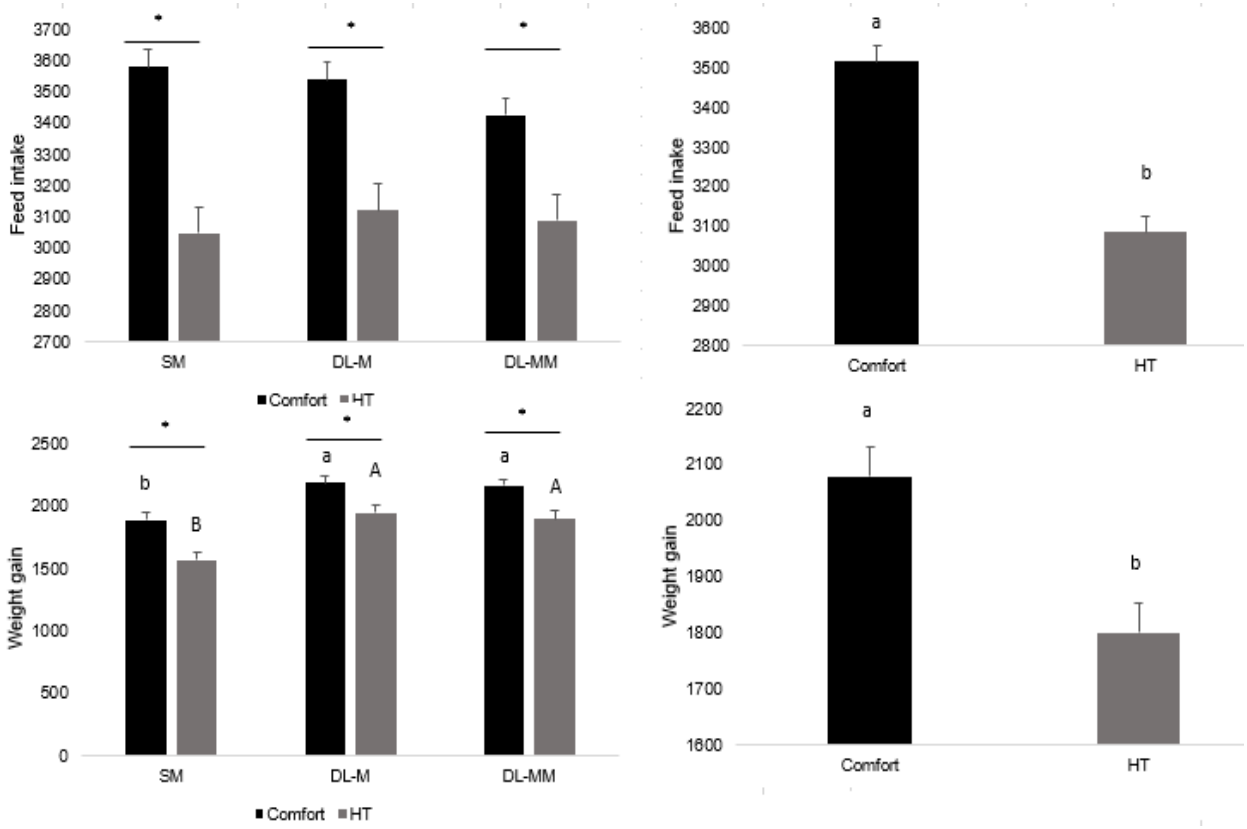


Figure 1- Feed intake (g) and weight gain (g) of broilers fed with diet without methionine supplementation (SM), diet with DL-methionine supplementation (DL-M), and diet with methionine dipeptide supplementation (DL-MM) under comfortable or high temperature (HT). Different small letters show differences between diets under comfortable temperature. Different capitalized letters show differences between diets under high temperatures. Differences between the temperatures are shown by bars and asterisks ($P<0.05$).

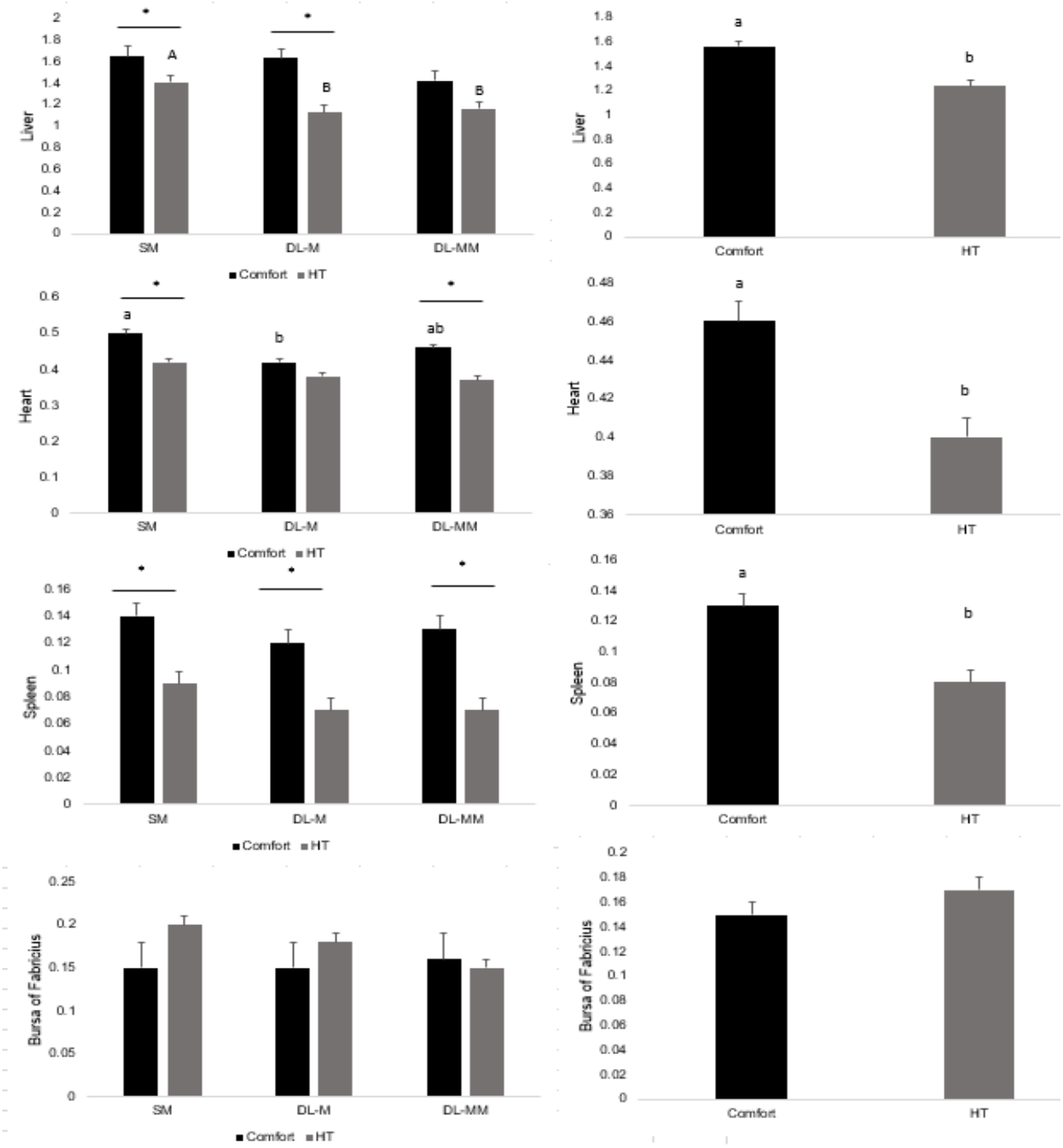


Figure 2- Relative weight (%) of liver, heart, spleen and bursa of Fabricius of broilers fed with diet without methionine supplementation (SM), diet with DL-methionine supplementation (DL-M), and diet with methionine dipeptide supplementation (DL-MM) under comfortable or high temperature (HT). Different small letters show differences between diets under comfortable temperature. Different capitalized letters show differences between diets under high temperatures. Differences between the temperatures are shown by bars and asterisks ($P<0.05$).

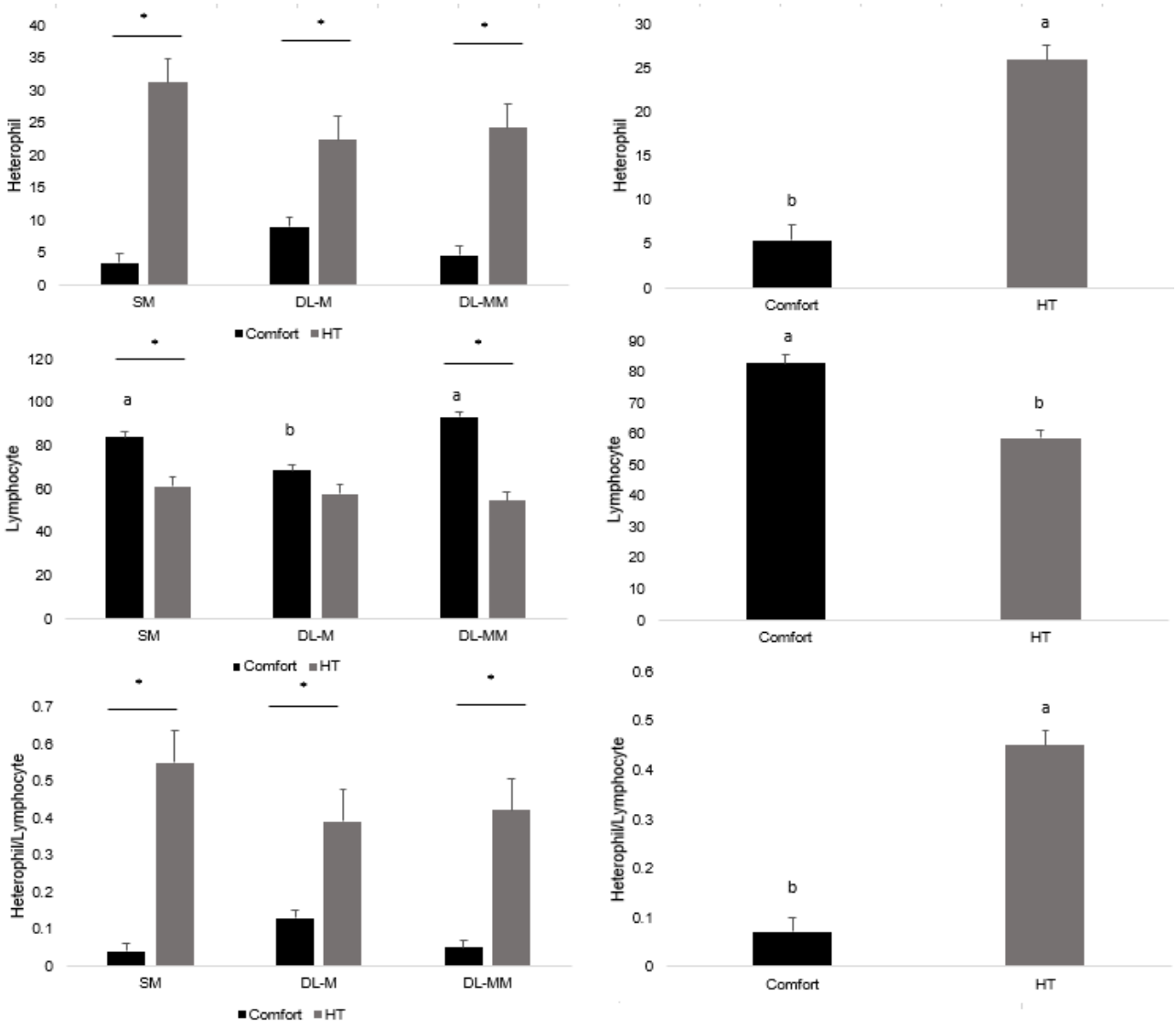


Figure 3- Heterophil and Lymphocyte numbers and Heterophil/Lymphocyte (H/L) ratio of broilers fed with diet without methionine supplementation (SM), diet with DL-methionine supplementation (DL-M), and diet with methionine dipeptide supplementation (DL-MM) under comfortable or high temperature (HT). Different small letters show differences between diets under comfortable temperature. Different capitalized letters show differences between diets under high temperatures. Differences between the temperatures are shown by bars and asterisks ($P<0.05$).

3.2. Biochemica assays

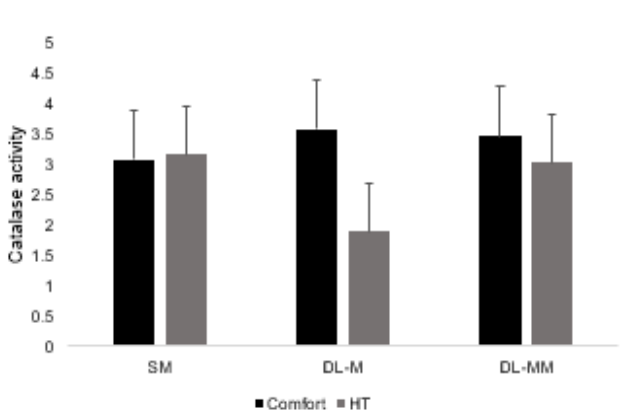
As birds that were fed with methionine supplementation presented better performance and trend to lower H/L ratio, we evaluated the effect of supplementation of both sources of methionine on the profile of components related to antioxidant capacity

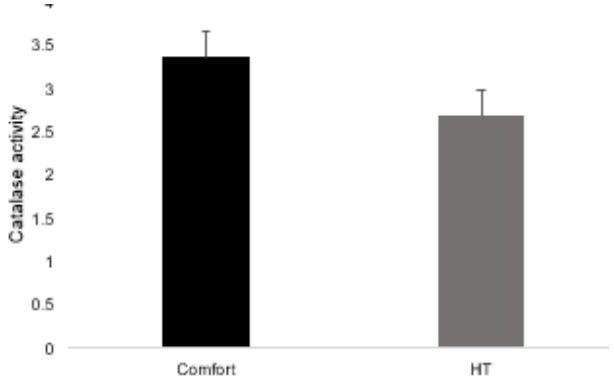
(Figure 4). Higher concentration of carbonylated proteins and lower concentration of glutathione (GSH) were significantly observed in birds raised under HT compared to birds of comfort.

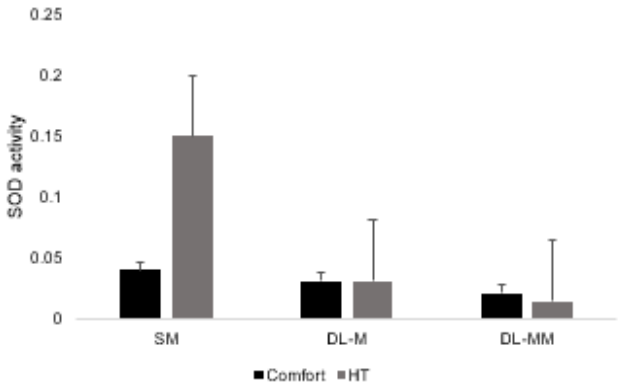
Comparing birds raised in HT environment, we observed that birds fed DL-M diet had significantly lower concentration of TBARS and carbonylated proteins than birds receiving SM diet. For birds from comfort group, the highest concentration of GSH was observed in birds receiving DL-M diet ($P < 0.05$). There was no significant difference between the sources of supplementation.

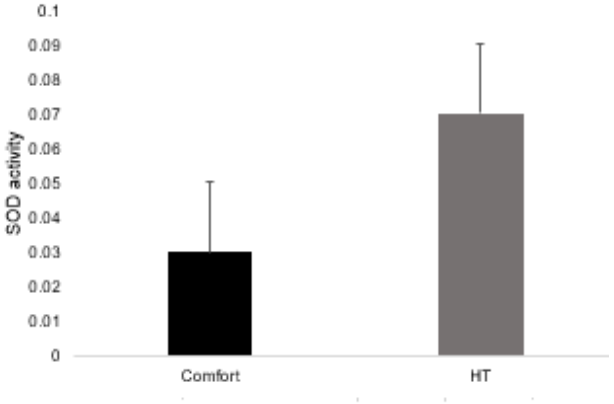
There was no significant difference in the content of carbonylated proteins between birds fed DL-M diet under comfort or HT environment (0.0007 and 0.0012 nmol/mg protein, respectively).

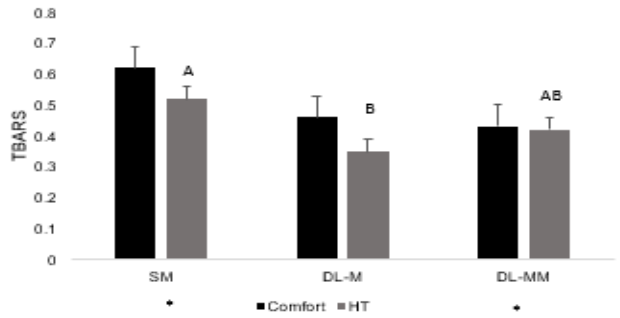
There was no effect of the treatments on the activity of the enzymes catalase and superoxide dismutase.

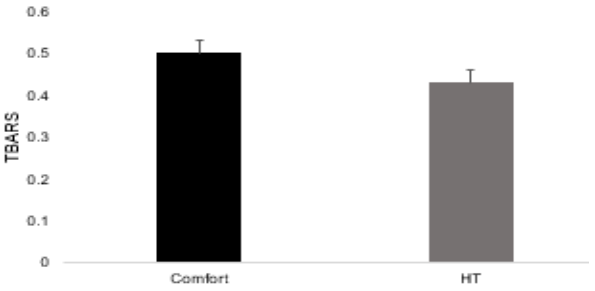

Catalase activity
SM
DL-M
DL-MM
Comfort
HT

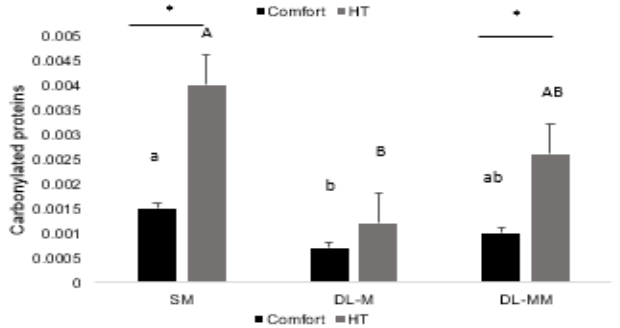

Catalase activity
Comfort
HT

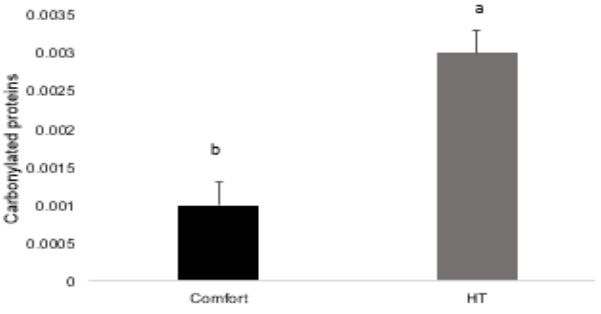

SOD activity
SM
DL-M
DL-MM
Comfort
HT

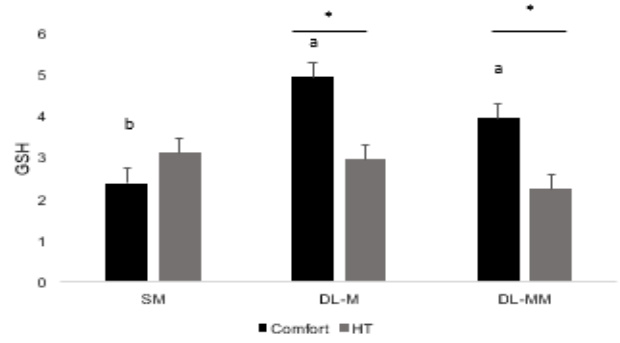

SOD activity
Comfort
HT


TBARS
A
B
AB
SM
DL-M
DL-MM
Comfort
HT


TBARS
Comfort
HT


Carbonylated proteins
A
B
AB
a
b
ab
SM
DL-M
DL-MM
Comfort
HT


Carbonylated proteins
a
b
Comfort
HT


GSH
a
b
a
SM
DL-M
DL-MM
Comfort
HT

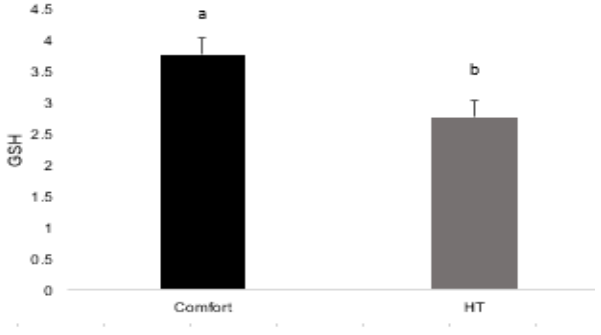

GSH
a
b
Comfort
HT

Figure 4- Thiobarbituric acid reactive substances (TBARS), carbonylated proteins and glutathione (GSH), and catalase and superoxide dismutase enzyme activity in the liver of broilers fed with diet without methionine supplementation (SM), diet with DL-methionine supplementation (DL-M), and diet with methionine dipeptide supplementation (DL-MM) under comfortable or high temperature (HT). Different small letters show differences between diets under comfortable temperature. Different capitalized letters show differences between diets under high temperatures. Differences between the temperatures are shown by bars and asterisks($P<0.05$).

3.3. Gene expression

Since we did not observe effect of heat stress on the activity of catalase and SOD enzymes, and as methionine supplementation was able to attenuate the effects caused by stress on the values of TBARS, carbonylated proteins and GSH, we evaluated the expression of glutathione peroxidase (*GPx*) and glutathione synthetase (*GSS*) genes. Glutathione peroxidase and GSS act in the glutathione cycle during the elimination of H_2O_2, and in the endogenous glutathione biosynthesis, respectively (Figure 5). As we expected, due to the role of glutathione in the elimination of ROS produced during heat stress, we observed a significant higher expression of *GPx* and *GSS* in broilers raised in HT than in birds raised in comfort.

Regarding *GPx* expression, broilers raised under HT receiving SM diet presented the highest expression of *GPx*. No significant difference was observed between birds receiving DL-M and DL-MM diets when raised in comfort or HT environments.

Regarding *GSS* expression, broilers fed SM diet in HT environment had higher expression of *GSS* than broilers receiving SM diet in comfort temperature. Birds receiving DL-M diet raised in comfort or HT environment had similar values of *GSS* expression (0.009 and 0.011 AU, respectively).

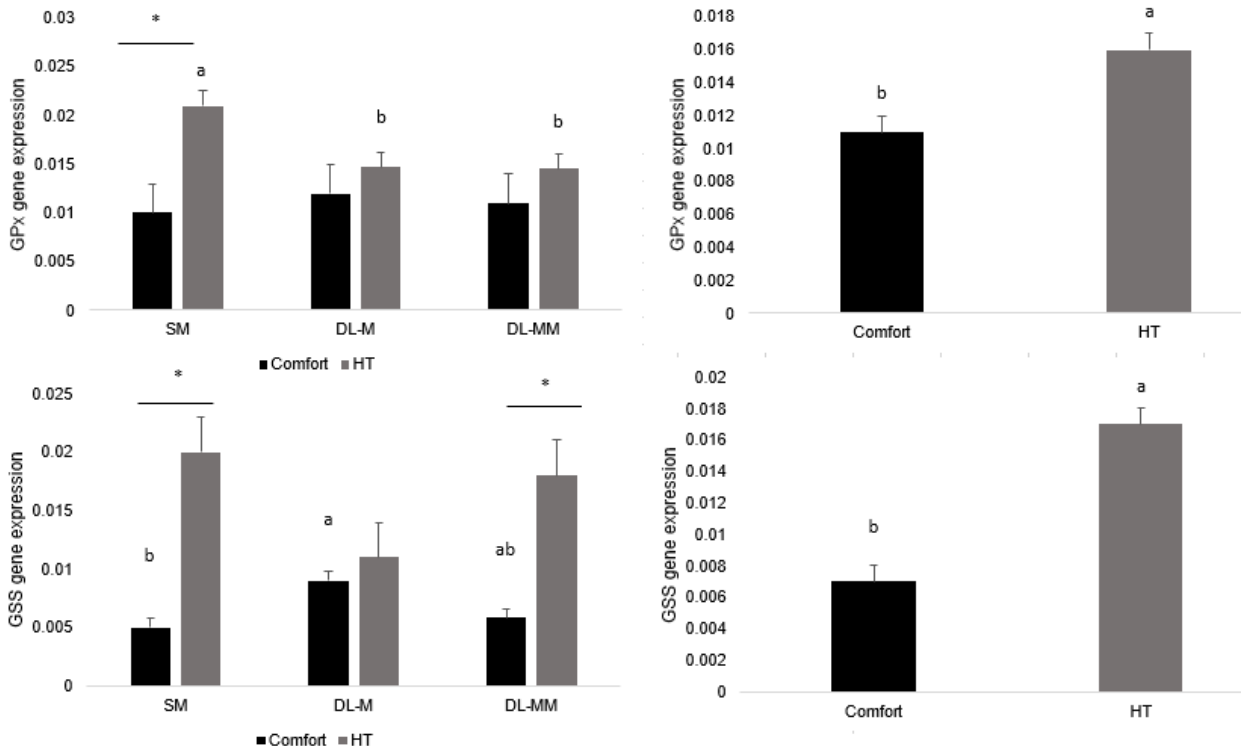


Figure 5- Glutathione peroxidase (*GPx*) e glutathione synthetase (*GSS*) gene expression (AU) in the liver of broilers fed with diet without methionine supplementation (SM), diet with DL-methionine supplementation (DL-M), and diet with methionine dipeptide supplementation (DL-MM) under comfortable or high temperature (HT). Different small letters show differences between diets under comfortable temperature. Different capitalized letters show differences between diets under high temperatures. Differences between the temperatures are shown by bars and asterisks ($P<0.05$).

3.4. DNA methylation

To evaluate the effect of environmental temperature and methionine supplementation on mechanisms related to gene regulation, we evaluated the level of DNA methylation in the promoter region of *GPx* and *GSS* genes. In HT conditions, birds fed DL-MM diet had the highest DNA methylation level in the promoter region of the *GPx* and *GSS* genes (Figure 6).

We also observed a negative effect between methylation levels and gene expression (Figure 7). With regard to the *GPx* gene, birds fed DL-M and DL-MM diets under HT had lower values of DNA methylation accompanied by higher gene expression values. This same inverse relationship was observed in the *GSS* gene results, broilers from HT group had lower methylation and higher expression values than birds from comfort temperature for all diets.

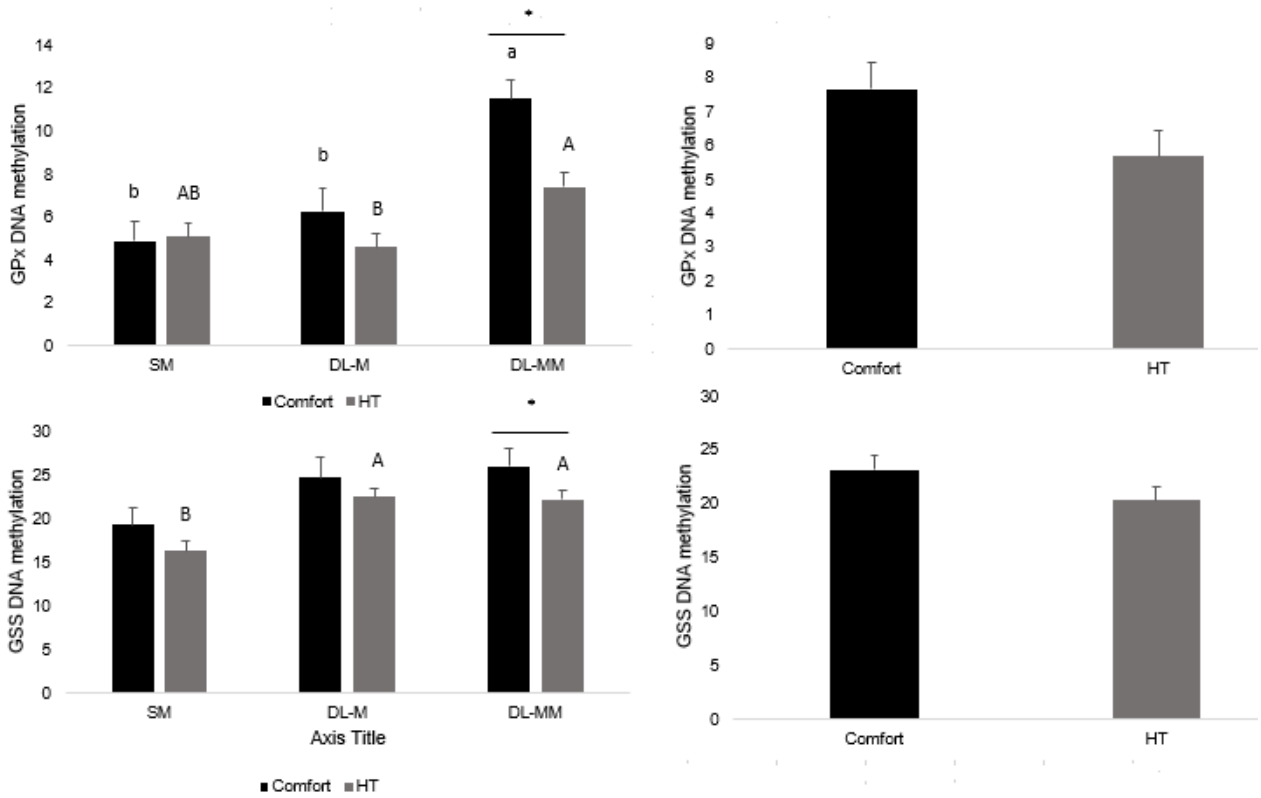


Figure 6- DNA methylation (%) in the promoter region of Glutathione peroxidase (*GPx*) e glutathione synthetase (*GSS*) genes in the liver of broilers fed with diet without methionine supplementation (SM), diet with DL-methionine supplementation (DL-M), and diet with methionine dipeptide supplementation (DL-MM) under comfortable or high temperature (HT). Different small letters show differences between diets under comfortable temperature. Different capitalized letters show differences between diets under high temperatures. Differences between the temperatures are shown by bars and asterisks ($P<0.05$).

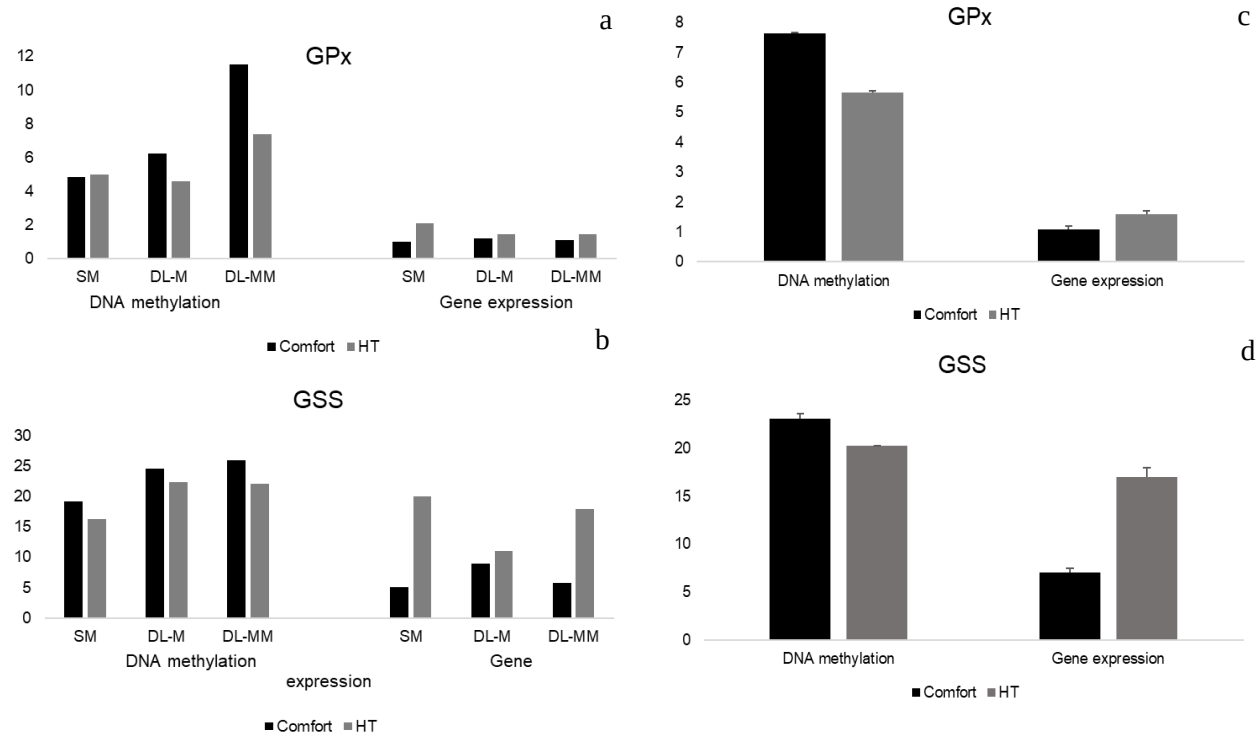


Figure 7- DNA methylation level and expression of glutathione peroxidase (*GPx*) e glutathione synthetase (*GSS*) genes in the liver of broilers fed with diet without methionine supplementation (SM), diet with DL-methionine supplementation (DL-M), and diet with methionine dipeptide supplementation (DL-MM) under comfortable or high temperature (HT). Under HT condition, broilers fed DL-MM had higher DNA methylation and lower expression of *GPx* gene than broilers fed SM diet (a); Under HT condition, broilers fed with DL-M and DL-MM diet had the highest DNA methylation level and the lowest expression of *GSS* gene (b). No relation between DNA methylation and gene expression level was observed in broilers under comfort (a-b). Broilers under HT had lower DNA methylation and higher expression of *GPx* and *GSS* gene than broilers under comfort (c-d).

4. Discussion

Several studies have shown the negative effect of chronic stress on the metabolism and performance of broiler chickens (Jahanian et al. 2015; Xu et al. 2018, Sahin et al. 2017; Rimoldi et al. 2015). In this work, the heterophy/lymphocyte ratio was evaluated as an indicator of physiological responses to stress. Birds in stress condition have high level of plasma corticosterone that is used to guarantee energy to resist the challenge (Quinteiro-Filho et al. 2010); the highest level of corticosterone causes lymphocytopenia (reduction in lymphocyte numbers) and granulocytosis (production of heterofilans), resulting in increased H/L ratio (Davison and Rowell 1983; Cotter et al. 2015) (Figure 3). Broilers under HT fed DL-M diet had lymphocyte level similar to the broilers fed DL-M diet raised in comfort condition. This result is related to the trend of lower H/L ratio for birds in HT condition receiving diet with methionine supplementation.

Some studies have been performed to evaluate the effect of different peptides supplementation for humans cells and mices under different challenge conditions (Je et al. 2015; Chen et al. 2018), however, there are not enought researches showing the role of peptides in diets of livestock animals. Some differences that occurs during the process of absorption of free amino acids and dipeptides (Wu 2013) could indicate that dipeptides are more efficiently available for animal metabolism under stress conditions than free amino acids. However, in our study we did not observe difference in the performance of broilers fed methionine as free amino acid or dipeptide. This result may be related to the bioefficacy of methionine sources, since similar values of bioefficacy between DL-M and DL-MM for broiler's weight gain was found by Silva et al. (2016) and Mencalha et al. (2016).

Regarding the effect of chronic heat stress, animals raised under HT had lower weight gain than birds from comfort group, as expected. In addition to lower weight gain, broilers raised in HT also had lower relative weight of spleen, an important secondary immune organ, liver and heart. Lower spleen weight and reduction in immune function is generally observed in birds raised under stress conditions (Ohtsu et al. 2015). The thermoregulation process triggered by chronic thermal stress is also responsible for behavioral and physiological changes that affect and cause damage to different organs and tissues, such as liver (Jastrebski et al. 2017) and cardiac tissue (Akbarian et al. 2014). Increased respiratory rate and heat dissipation through peripheral vasodilation depend on the increase in heart rate (Daghir 2008). During heat stress, this process is amplified through the action of the renin-angiotensin-aldosterone system, which stimulates cardiac contraction and increases heartbeat resulting in damage to heart tissue (Xu et al. 2018). According to Zhang et al. (2017), the reduction in cell cycle activity and the increase in apoptosis are related to the lower relative weight of the heart of birds subjected to stress. It should be point out that for birds fed DL-M or DL-MM diet, there was no significant difference between broiler raised in comfort or in HT environment for relative weight of heart and liver, respectively. Different actions may have contributed to this result, such as the higher expression of heat shock proteins (HSPs) induced by methionine supplementation (data not shown) that acts to protect the heart (Ranek et al. 2017), as well as the highest antioxidant capacity observed in birds receiving diet supplemented with both sources of methionine (Figures 4 and 5).

We also evaluated some markers of oxidative stress as an indicator of response to HT. During heat stress, changes in the activities performed by mitochondria contribute to

higher ROS production; higher activity of the electron transport chain with higher superoxide production can be observed shortly after a short period of stress. The higher O_2^- is followed by reduction of the uncoupling protein (UCP) activity, mitochondrial dysfunction and tissue damage when the effect of heat stress is prolonged (Akbarian et al. 2016). Antioxidants, enzymatic and non-enzymatic, act to eliminate the ROS produced during heat stress and to ensure equilibrium in the oxidative state (Kuss 2005; Akbarian et al. 2016). Methionine may act directly on ROS elimination, preventing them from damaging other molecules such as lipids, proteins and nucleic acids (Review by Luo and Levine 2009), or serving as a precursor for the production of glutathione (Piovacari et al. 2008).

The antioxidant system of glutathione has as one of the main functions to eliminate the hydrogen peroxide (H_2O_2) and to prevent this of being converted into the highly reactive, the hydroxyl radical. During this process, glutathione passes from its reduced form (GSH) to its oxidized form (GSSG) through the action of glutathione peroxidase (GPx), thus, the GSH concentration can be used as an indicator of the cellular oxidative state (Ballotori 2009). The lower GSH conten observed in the liver of broilers from HT grouo (Figure 4) suggests that higher concentration of GSH was used to combat ROS in birds of this treatment. It is also important to note that in comfort environment, birds fed SM diet showed lower GSH content than birds receiving DL-M and DL-MM diets. However, there was no significant difference in GSH content between birds of different diets reared under HT. This result suggests that under stress condition, higher production of glutathione is required even when methionine levels are not met, and shows the importance of the glutathione system as antioxidant in birds under chronic heat stress conditions. These results are also corroborated by the *GSS* expression results: for birds from comfort environment, lower *GSS* expression was observed in birds fed SM diet, however, there is no difference between diets when birds are under HT condition (Figure 5). Glutathione synthetase (GSS) acts on the synthesis of endogenous glutathione from the precursors γ-glutamylcysteine and glycine (Lu 2014).

Broilers from HT environment had higher *GSS* and *GPx* expression than broilers raised under comfort. The higher expression of *GPx* in broilers raised under HT and fed with SM diet suggests that the effect of chronic heat stress can be amplified by methionine deficiency and thus greater antioxidant action is required for this birds. The results of carbonylated proteins and TBARS, which respectively show the level of protein and lipid oxidation, confirm that the negative effects of stress can be amplified by methionine

defficiency or attenuated by supplementation of both sources of methionine; in HT condition, birds fed DL-M diet showed significantly lower concentration of TBARS and carbonylated proteins than birds fed SM diet. There was no significant difference in the content of carbonylated proteins between birds fed DL-M diet under comfort or HT environments.

The environment can act on gene expression through epigenetic mechanisms of gene regulation. Studies have suggested that chromatin structure may be a determinant factor in the control of transcription, and that epigenetic factors such as histone modifications and DNA methylation are involved in this process, which involves chromatin condensation and gene silencing (Donkena et al. 2010). In this work we evaluated for the first time the effects of methionine supplementation as free amino acid and as dipeptide and chronic heat stress on the level of DNA methylation in the promoter region of the *GPx* and *GSS* genes in the liver of broilers.

DNA methylation occurs by the addition of a methyl group to the 5' carbon of a cytosine, which results in a 5-methylcytosine. Methylation occurs preferentially in cytosines preceding guanines (5'CG3') (Baylin 2005) in GC rich regions, the CpG islands (Laird 2003). The CpG islands are often found in promoter regions and in the first exon of many genes, and thus are directly related to transcriptional control (reviewed in Tesseraud et al. 2009). Methionine acts in this process through the donation of the methyl group through S-adenosylmethionine (SAM) which is produced by the metabolic pathway of methionine during the process of homocysteine synthesis (Stipanuk 2004). Much of the metabolism of methionine occurs in the liver. Nevertheless, results that show the direct relationship between the level of methionine available to the animal and the content of methionine found in the liver, as well as the relationship between the level of methionine and the level of DNA methylation are still controversial (reviewed by Zhang 2018 and Tesseraud et al. 2009). The results appear to be dependent on the level of methionine, evaluated tissue and also the function of the gene in question (Zhang 2018).

DNA methylation has a important function during prenatal development. Besides that, the relationship between diet and epigenetic changes affecting gene expression are better described early in the postnatal period (Zhang 2015). Here, we observed that methionine supplementation may also influence the level of DNA methylation in specific regions in growing birds. Different results were observed for broilers in comfort or HT environments, methionine supplementation did not cause changes in the methylation level of broilers from comfort group. However, for HT animals, broilers fed DL-M and DL-

MM diets had higher levels of methylation in the promoter region of the *GSS* gene than animals fed SM diet (Figure 6). Interaction between environment and diet is also observed in the results that show the inverse relationship between the level of DNA methylation and gene expression in broilers under HT, as birds with higher DNA methylation also had lower gene expression (Figure 7). These results suggest that the effect of methionine supplementation on the control of the expression of genes related to antioxidant capacity may depend on other environmental factors that also cause changes in the cellular oxidative status.

In general, our results show that chronic thermal stress can cause changes in the metabolism of growing broiler chickens, and that methionine supplementation as free amino acid or dipeptide may help to attenuate the effects of stress through the action of genes related to the antioxidant mechanism of glutathione. The Methionine effects could be found at gene regulation, gene expression and at post-translational levels.

Acknowledgment

This study was performed with support from the National Council for Scientific and Technological Development (CNPq), grant number: 407669/2016-7 (APDV).

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CONSIDERAÇÕES GERAIS

Nossos resultados de forma conjunta nos permitem concluir (rever a frase, usar 3ª pessoa), que o estresse térmico prejudica o desempenho e alterar o metabolismo de frangos de corte, os efeitos negativos do estresse podem ser observados de maneira distinta entre o estresse agudo e crônico. No entanto, observa-se também que a suplementação de metionina na forma livre ou como dipeptídeo obteve melhores resultados de desempenho, qualidade de carne e atenuou os efeitos do estresse por calor, sobre as respostas fisiológicas e a capacidade antioxidante, por meio da maior expressão de genes relacionados à atividade antioxidante.