UNIVERSIDADE ESTADUAL DE MARINGÁ

CENTRO DE CIÊNCIAS AGRÁRIAS

DL-METIONIL-METIONINA EM DIETAS DE FRANGOS DE CORTE SUBMETIDOS À ESTRESSE TÉRMICO AOS 21 DIAS DE IDADE

Autora: Isabelle Naemi Kaneko
Orientadora: Prof.ª Dr.ª Tatiana Carlesso dos Santos
Coorientadora: Prof.ª Dr.ª Eliane Gasparino

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Estado do Paraná
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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

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“Os rios não bebem sua própria água; as árvores não comem seus próprios frutos. O sol não brilha para si mesmo; e as flores não espalham sua fragrância para si. Viver para os outros é uma regra da natureza. (...)

A vida é boa quando você está feliz; mas a vida é muito melhor quando os outros estão felizes por sua causa".

Papa Francisco

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pelo amor, incentivo e confiança,
por nunca deixarem de acreditar em mim.

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pela parceria, amizade e incentivo.

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BIOGRAFIA

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Em abril de 2018, submete-se ao exame geral de qualificação.

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RESUMO

A suplementação de DL-Metionil- DL-Metionina (Met-Met) foi avaliada na dieta de frangos de corte de 1 a 21 dias, submetidos a estresse de temperatura (32ºC) por até 48 horas. Foram utilizados 216 pintos de corte machos Cobb-Vantress® distribuídos em um fatorial 3 x 3, composto por 3 dietas (basal - sem suplementação de metionina, suplementada com DL-Met e suplementada com Met-Met) e 3 períodos de estresse de temperatura (21 dias (sem estresse) e após 24 e 48 horas a 32ºC). Para o desempenho zootécnico, as aves suplementadas com DL-Met e Met-Met apresentaram maior peso em relação a dieta basal de 1 a 21 dias (P=0,006). Para o ganho de peso (P=0,0006) e conversão alimentar (P=0,0001) de 1 a 21 dias, ambas as dietas suplementadas com metionina apresentaram melhores valores, comparadas a basal. Com relação a composição de carcaça, a proteína bruta, foi superior para animais suplementados com metionina de ambas as fontes (P=0,087). Após 24 (P<0,0001) e 48 horas (P=0,0004) de estresse por calor, as aves suplementadas com DL-Met e Met-Met apresentaram maior porcentagem de proteína bruta na carcaça comparadas a dieta basal. Após 24 horas de estresse, as aves alimentadas com dieta basal aprestaram maior porcentagem de extrato etéreo na carcaça comparadas as suplementadas com DL-Met e Met-Met (P=0,0334). Em relação ao músculo do peito, houve efeito da dieta para o peso, com aves com peito maior quando suplementados com metionina. Histologicamente as fibras musculares das aves alimentadas com DL-Met tiveram maior diâmetro em relação as outras dietas (P=0,0001). As aves nutridas com dieta basal apresentaram maior número de fibras/área em relação àquelas que receberam metionina (P<0,0001). Para as análises plasmáticas, após o estresse de 48 horas, os níveis de alanina aminotransferase foram mais elevados para as

aves da dieta basal em relação às da DL-Met (P=0,0145). Neste mesmo período, as aves que receberam dieta basal apresentaram maiores valores de creatina quinase em relação às aves que receberam as outras dietas (P=0,0028). Ao avaliar a morfologia intestinal, houve interação entre as dietas e os períodos de estresse para profundidade de cripta (P=0,011) e relação vilo/ cripta (P=0,0091) e os períodos de estresse ocasionaram menores vilos ileais (P=0.0188) e vilos duodenais mais finos (P=0,0061). Avaliando a expressão gênica de transportadores de aminoácidos na borda em escova (B^0AT1 e *PEPT1*) e na membrana basolateral (Y^+LAT1) do jejuno, houve interação entre dieta e tempo de estrese para Y^+LAT1 e B^0AT1, com maior expressão do Y^+LAT1 após 48 horas de estresse nas aves com dieta Met-Met e com maior expressão do B^0AT1 em todas as dietas após as 48 horas. A expressão do *PEPT1* foi influenciada pelo período de estresse, sendo superior após 48 horas em todas dietas. Conclui-se que a suplementação de metionina na dieta é fundamental para o desempenho, morfologia muscular, composição de carcaça e morfologia intestinal, independentemente da fonte utilizada de 1 a 21 dias de idade. A expressão gênica dos transportadores de aminoácidos, sugerem que a via de absorção primária da metionina é através dos transportadores de aminoácidos livres. O estresse por calor influi no metabolismo hepático e na expressão dos transportadores intestinais após 48 horas.

Palavras-chave: DL-Metionina, DL-Metionil-DL-Metionina, intestino, músculo peitoral.

ABSTRACT

The DL-Methionyl-DL-Methionine (Met-Met) supplementation was evaluated in the diet of broilers from 1 to 21 d, subjected to temperature stress (32ºC) for up to 48 hours. A total of 216 male Cobb-Vantress® chicks were distributed in a 3 x 3 factorial, consisting of 3 diets (basal - without methionine supplementation, supplemented with DL-Met and supplemented with Met-Met) and 3 temperature stress periods (21 days (no stress) and after 24 and 48 hours at 32 °C). For zootechnical performance, birds supplemented with DL-Met and Met-Met presented higher weight in relation to a basal diet of 1 to 21 d (P = 0.006). For the weight gain (P = 0.0006) and feed conversion (P = 0.0001) from 1 to 21 days, both diets supplemented with methionine presented better values, compared to basal. Regarding the carcass composition, the crude protein was superior for animals supplemented with methionine from both sources (P = 0.087). After 24 (P <0.0001) and 48 hours (P = 0.0004) of heat stress, birds supplemented with DL-Met and Met-Met showed a higher percentage of crude protein in carcass compared to a basal diet. After 24 hours of stress, birds fed with basal diet presented a higher percentage of ethereal extract in carcass compared to those supplemented with DL-Met and Met-Met (P = 0.0334). Regarding the breast muscle, there was dietary effect for weight, with birds with bigger chest when supplemented with methionine. Histologically, the muscle fibers of birds fed DL-Met had a larger diameter than the other diets (P = 0.0001). Birds fed a basal diet had a higher number of fibers per area compared to those receiving methionine (P<0.0001). For plasma analysis, after 48-hour stress, alanine aminotransferase levels were higher for birds on basal diet than for DL-Met (P = 0.0145). In this same period,

birds that received basal diet had higher creatine kinase values in relation to the one that received the other diets (P = 0.0028). When evaluating intestinal morphology, there was interaction between diets and stress periods for crypt depth (P = 0.011) and villus:crypt ratio (P = 0.0091) and stress periods resulted in lower ileal villi (P = 0.0188) and finer duodenal villi (P = 0.0061). By evaluating the gene expression of amino acid transporters in the brush border (*B^0AT1* and *PEPT1*) and jejunal basolateral membrane (*Y^+LAT1*), there was interaction between diet and stress periods for *Y^+LAT1* (P=0.0011) and *B^0AT1* (P=0.0007), with a higher expression of *Y^+LAT1* after 48 hours of stress in birds with Met-Met diet and with higher expression of *B^0AT1* in all diets after 48 hours. The expression of *PEPT1* was influenced by the stress period (P=0.0148), being superior after 48 h in all diets. It is concluded that methionine supplementation in diet is fundamental for performance, muscle morphology, carcass composition and intestinal morphology, regardless of the used source from 1 to 21 days of age. The amino acid transporters gene expression suggests that the primary methionine uptake pathway is through the free amino acid transporters. Heat stress influences hepatic metabolism and expression of intestinal transporters after 48 hours.

Key words: DL-Methionine, DL-Methionyl-DL-Methionine, intestine, *Pectoralis* muscle.

I. INTRODUÇÃO

Em dietas formuladas à base de milho e soja, a metionina é o primeiro aminoácido limitante para de aves. Desta forma, com o intuito de atender as necessidades nutricionais, as dietas geralmente são suplementadas com metionina nas formas de L-metionina, DL-metionina e o análogo de metionina, ácido DL-2-hidroxi-4- (metiltio) butanóico (DL-HMTBA) (Zhang et a., 2016). O DL-HMTBA não possui um grupo amino e, portanto, não é um aminoácido, mas um precursor de aminoácido, que precisa ser convertido para ser absorvido (Dibner e Knight, 1984). Considerando as características individuais dessas fontes, diferenças na absorção e no metabolismo deverão afetar a utilização da metionina pelo organismo.

Em geral, todos os precursores de metionina são convertidos em L-metionina para serem absorvidos pelos animais, a fim de atender à diversas funções metabólicas. Dentre as funções, a síntese proteica e a síntese de metabólitos de enxofre, como a cisteína, a carnitina e a taurina. A metionina também pode ser convertida em S- adenosilmetionina (SAM), que é a principal doadora de grupamentos metil. Outro papel importante está associado ao fato de a metionina ser codificada por um único códon (AUG), que também é o códon de iniciação para a síntese da maioria das proteínas (Martin-Venegas et al., 2006; Metayer et al., 2008; Agostini et al, 2016; Zhang et al., 2016).

Para serem absorvidas as proteínas dietéticas sofrem primeiramente hidrólise enzimática para gerar aminoácidos livres e peptídeos e, desta forma, seus constituintes são aproveitados como tal no lúmen intestinal (Brodin et al., 2002). Esses nutrientes são absorvidos por células epiteliais do intestino delgado por meio de transportadores da membrana plasmática. No interior dessas células, podem ser metabolizados ou

transportados para fora das células atingindo a corrente sanguínea a fim de serem utilizados em outras células e tecidos (Broer, 2008; Gilbert et al., 2008).

Em 1959, Newey e Smith, mostraram que um di ou tripeptídeo poderia ser absorvido também de maneira intacta, por meio de um transportador específico, o transportador PEPT1. Desta forma, iniciou-se a utilização destes compostos na dieta, com o intuito de promover uma absorção mais efetiva e eficiente com menor gasto energético. A DL-metionil-DL-metionina, é uma nova fonte de metionina, que difere das outras fontes por se caracterizar como um dipeptídeo de DL-metionina, tendo a possibilidade de ser metabolizada também como um dipeptídeo (EFSA- Journal, 2015).

Diversos trabalhos já compararam fontes de metionina na dieta de frangos de corte. Porém, a DL-Metionil-DL-Metionina ainda não foi utilizada para essa espécie. Essa molécula se mostra efetiva principalmente em animais aquáticos, como peixes e crustáceos, sendo caracterizada como uma molécula mais estável em água, diminuindo o potencial de lixiviação. Em hipótese, a utilização dessa molécula na dieta de frangos de corte estaria ligada, principalmente a possibilidade de utilização do transportador PEPT1 para sua absorção, pois este seria mais resistente a condições de estresse, quando comparado a outros transportadores de aminoácidos (Gilbert et al., 2008).

1. Importância dos aminoácidos no metabolismo

Os aminoácidos são essenciais no metabolismo da proteína corporal. Além de serem componentes das proteínas e polipeptídeos, alguns aminoácidos regulam vias metabólicas-chaves que são necessárias para mantença, crescimento, reprodução e imunidade (Wu, 2009). A síntese proteica (anabolismo) permite a produção de novas proteínas bem como a renovação das proteínas corporais. Enquanto a degradação de proteínas (catabolismo) e dos aminoácidos resultantes leva a produção de metabólitos que podem ser oxidados ou convertidos à glicose ou ácidos graxos. O grupo amino é excretado na forma de amônia, ureia ou ácido úrico, e a produção de moléculas com esqueleto de carbono (Nelson e Cox, 2011).

Os aminoácidos, provenientes principalmente das proteínas da alimentação ou da degradação de proteínas intracelulares, e se caracterizam por ser um nutriente secundário para a geração de energia metabólica (Nelson e Cox, 2011). A maior diferença entre os

aminoácidos e outros macronutrientes, como lipídeos e carboidratos, é que os aminoácidos contêm nitrogênio, presente em vários estados de oxidação, e desempenham funções importantes em várias vias metabólicas celulares (Wu, 2013).

Diferentemente dos carboidratos e lipídeos, que podem ser armazenados, respectivamente, na forma de glicogênio e triglicerídeos, os aminoácidos quando são fornecidos em excesso não podem ser armazenados. Todos os aminoácidos não utilizados para mantença e produção, em relação à animais em crescimento, gestantes e lactantes, por exemplo, são oxidados ou convertidos a carboidratos e lipídios (Moreira e Pozza, 2014).

Os principais tecidos envolvidos no processo de catabolismo são o fígado, o intestino, o cérebro e os músculos esqueléticos (Moreira e Pozza, 2014). Devido às diferenças nas cadeias laterais, os aminoácidos possuem particularidades em sua via metabólica. No entanto, o catabolismo de muitos aminoácidos possui alguns passos comuns para gerar alguns metabólicos como o piruvato, oxalacetato, α- cetoglutarato, fumarato, succinil-CoA, e acetil- CoA (Wu, 2013).

Diversas reações desempenham papel importante no início da degradação de aminoácidos, originando vários metabólitos como o NH_3, CO_2, ureia, ácido úrico, acetil-CoA, ácidos graxos de cadeia curta, sais ou ésteres de ácido fórmico, glicose, H_2S, corpos cetônicos, óxido nítrico, poliaminas, e outras substâncias nitrogenadas (Wu, 2013).

1.1. Metabolismo da Metionina

A metionina é metabolizada através de três vias principais: a transmetilação, a remetilação e a transsulfuração (Stipanuk, 1986; Courtney-Martin et al., 2012). A primeira etapa é a transmetilação, que ocorre por ação da enzima metionina-adenosiltransferase (MAT), que catalisa a biossíntese de S-adenosilmetionina, através da transferência de uma molécula de adenosina proveniente de um ATP para a metionina (Brosnan e Brosnan, 2006; Blom e Smulders, 2011). A S-adenosilmetionina possui um átomo de enxofre carregado positivamente, caracterizando-se como um íon sulfônio, este íon ataca o carbono 5`da ribose do ATP e, dessa forma, os três fosfatos são removidos da molécula simultaneamente (Brosnan e Brosnan, 2006; Nelson e Cox, 2014). A S-adenosilmetionina faz a doação do seu grupamento metil para um receptor que libera a S-adenosilhomocisteína, este processo é mediado por metil transferases. A S-adenosilhomocisteína é subsequentemente

hidrolisada em homocisteína e adenosina, através da enzima S-adenosil-homocisteína-hidrolase (SAHH) (Nelson e Cox, 2014).

No segundo passo, a homocisteína é remetilada para formar metionina mediante duas vias. Em uma das vias a metilação da homocisteína é catalisada pela enzima metionina sintase (MS), esta enzima, em uma de suas formas, emprega o N^5 -metil-tetraidrofolato (5-CH_2-THF) como um doador de metil, e em outra forma utiliza a metilcobalamina derivada da coenzima $B_{1\ 2}$. A metionina é então reconvertida para a S-adenosilmetionina para completar um ciclo de metil ativado (Nelson e Cox, 2014).

Em uma segunda via a homocisteína requer a betaína como doadora de grupamento metil em uma reação catalisada pela betaína-homocisteína-metil-transferase (BHMT), dando origem a metionina e a dimetilglicina (Blom e Smulders, 2011). A homocisteína pode ainda ser degradada irreversivelmente a metionina pela via de transsulfuração (terceiro passo), que consiste primeiramente na condensação da homocisteína e serina em cistationina, catalisada pela enzima cistationina-β-sintase (CBS). Então, a cistationina é hidrolisada em cisteína e α-cetobutirato, pela ação da enzima cistationina-γ-liase (CGL) (Blom e Smulders, 2011, Stipanuk e Ueki, 2011). As duas enzimas envolvidas nessas reações são dependentes da vitamina B_6 (Blom e Smulders, 2011).

O ciclo da metionina é regulado principalmente pela S-adenosilmetionina, quando a concentração de metionina é baixa, o conteúdo de S-adenosilmetionina hepática cai, liberando a inibição que esta molécula exerce na síntese de metionina através da via da remetilação em níveis normais de recuperação. No entanto, quando a concentração de metionina é elevada o conteúdo de S-adenosilmetionina hepática aumenta, causando a ativação do catabolismo da metionina através das vias de transmetilação e transsulfuração e a inibição da regeneração na metionina através da via de metilneogênese, restaurando assim o conteúdo normal da metionina (Mato et al., 2008). A S-adenosilmetionina, na sua forma descarboxilada, age como fonte de grupos propilamilo para a síntese das poliaminas espermina e espermidina (Nelson e Cox, 2014). Desta forma, essa síntese é dependente de metionina e arginina.

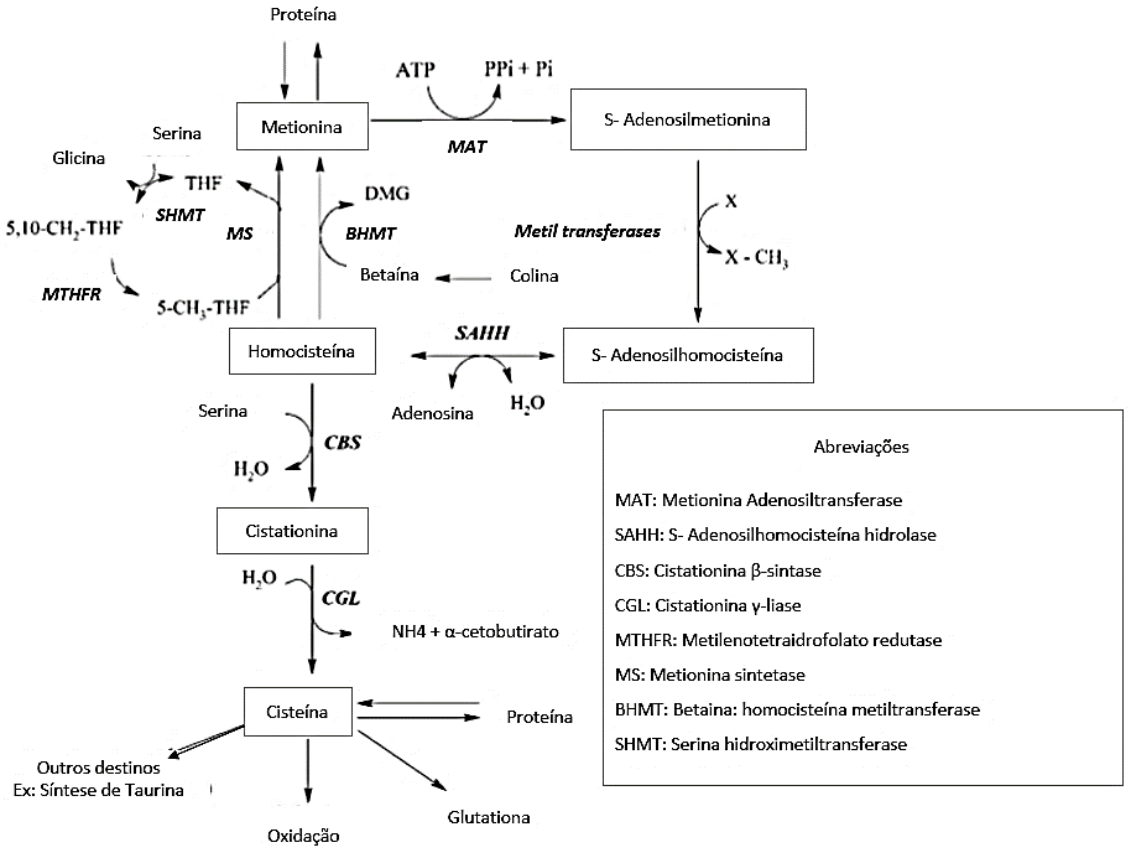

Figura 1. Metabolismo dos Aminoácidos sulfurados. Fonte: Adaptado Brosnan e Brosnan, 2006.

1.1.1. Metionina no metabolismo muscular

Os aminoácidos são importantes na constituição das proteínas de vários tecidos e órgãos, sendo particularmente responsáveis pelo metabolismo muscular. A regulação deste metabolismo é alvo de vários estudos, principalmente em relação ao músculo esquelético, quando se considera a produção de carne o crescimento muscular fundamentais. Além disso, a redução da perda muscular é essencial em algumas situações fisiológicas, como no caso do período de lactação ou outras situações em que os animais são expostos à momentos de estresse (Tesseraud et al., 2011).

O músculo peitoral do frango de corte foi submetido a intensa seleção genética, para a máxima deposição proteica (Scheuermann et al., 2003). Desta forma, esta ave passou a exigir maior aporte de aminoácidos na dieta. Na primeira semana de vida da ave, a proteína é caracterizada como o macronutriente dietético mais importante para promover o crescimento (Swennen et al., 2010). No período neonatal, as células satélites (mioblasto adulto), essenciais para o desenvolvimento muscular pós-eclosão se encontram em intensa atividade (Powell et al., 2017).

As células satélites fundem-se às fibras musculares e seus núcleos passam a compor as células. O aumento no número de núcleos permite a elevação da capacidade de síntese proteica pela fibra muscular, ocasionando a hipertrofia muscular. Durante as primeiras semanas de vida dos pintos, as células satélites são as únicas células musculares mitoticamente ativas (Velleman et al., 2014).

A metionina por ser o primeiro aminoácido limitante na dieta de frangos de corte influencia a deposição proteica no músculo peitoral (Hickling et al., 1990), apesar de dentre todos os aminoácidos essenciais, ser o que apresenta a concentração mais baixa nesse músculo (Murphy, 1994).

O desenvolvimento do músculo esquelético é regulado pelos fatores miogênicos regulatórios (MyoD, Myf5, MyoG e MRF4), fator intensificador de miócitos 2 (MEF2A, B, C e D), miostatina (MSTN) e fator de crescimento semelhante a insulina I (IGF-I) (Naya e Olson, 1999; Zanou e Gailly, 2013). O menor crescimento de frangos alimentados com dietas isentas de metionina e cisteína é causado, principalmente, pela menor taxa de síntese proteica, associada a menor eficiência de RNAm, sugerindo uma regulação translacional (Tesseraud et al., 2011). Segundo Barnes et al. (1995), a suplementação de metionina melhora o crescimento muscular e a adição de metionina a uma dieta deficiente aumenta a síntese de proteína nos músculos gastrocnêmio e peitoral em frangos.

O desempenho de crescimento e o rendimento do músculo peitoral são frequentemente utilizados para a caracterização do resultado de determinada dieta, podendo variar com gênero, idade, quantidade de nutrientes e ambiente de criação. (Chamruspollert et al., 2004, Wen et al., 2017,). Muitos trabalhos demonstraram a diferença entre as rotas metabólicas e a expressão gênica de animais expostos a dietas com deficiência e excesso de metionina (Corzo et al., 2006; Zhai et al., 2012).

Os derivados da metionina também exercem papéis importantes no metabolismo muscular. Por exemplo, a fosfocreatina, derivada da creatina, é um importante reservatório de energia no músculo esquelético (Nelson e Cox, 2014). A creatina é derivada da glicina e da arginina, e a S-adenosilmetionina, desempenha um papel importante na doação de grupamento metil. A arginina transfere o grupamento guanidino para a glicina, para a formação da glicociamina e a formação da creatina que é completada com a metilação com o auxílio da S-adenosilmetionina (Gonzales-Esquerra e Leeson, 2006).

1.1.2. Metionina no Metabolismo Lipídico

Alguns aminoácidos essenciais podem melhorar a saúde por regular algumas vias metabólicas importantes e melhorar a utilização dos alimentos, aumentando a deposição proteica e reduzindo a adiposidade (Wu, 2009). As dietas que não possuem aminoácidos essenciais levam a diminuição rápida do consumo de alimentos em 20 a 30 % (Guo e Cavener, 2007). No entanto, a redução da metionina dietética produz, em ratos, um aumento imediato no consumo de alimentos (Hasek et al., 2010). Esse aumento no consumo alimentar eleva a temperatura do organismo como resposta a alimentação exagerada, bem como, um consequente aumento na adiposidade (Hasek et al., 2013).

A metionina exerce papel no metabolismo lipídico, também por meio dos intermediários do seu metabolismo, ela participa da biossíntese de S-adenosilmetionina, um dos principais doadores de grupamento metil, necessários para a formação de fosfatidilcolina. A fosfatidilcolina, desempenha uma função importante na absorção dos lipídeos intestinais, através do aumento da solubilidade lipídica micelar e proporcionando revestimento da superfície para a formação de quilomícrons (Jiang et al., 2001). A maioria do colesterol e fosfolipídeos absorvidos do trato gastrointestinal sob a forma de quilomícrons.

A fosfatidilcolina, também é um importante componente da camada externa das partículas de lipoproteínas de muito baixa densidade (VLDL) (Vance e Vance, 1985). A escassez desses nutrientes prejudica a produção de VLDL e os triglicerídeos passam a se acumular nos hepatócitos (Kulinski et al., 2004). Sendo assim, quando um substrato lipogênico, como por exemplo a sacarose, ou um outro açúcar é inserido em uma dieta isenta de metionina e colina, ocorre aumento na esteatose hepática, levando progressivamente a lesões mais graves, inflamações e até mesmo fibroses hepáticas (Riski et al., 2006). Essas lesões são raras em aves de produção, devido a seu rápido ciclo de vida, sendo mais propensas a ocorrer em humanos e animais domésticos.

1.1.3. Metionina no metabolismo intestinal

Durante muito tempo, o fígado foi considerado o principal órgão envolvido no metabolismo dos aminoácidos, sendo o intestino responsável exclusivamente pela digestão e absorção dos outros constituintes alimentares. Entretanto, estudos revelam que o intestino

obtém uma porção significativa da sua energia metabólica por meio do catabolismo de aminoácidos absorvidos, antes que esses atinjam a circulação portal. Desta forma, o metabolismo intestinal influi sobre a disponibilidade sistêmica dos aminoácidos (Martín-Venegas et al., 2006).

O epitélio intestinal é considerado um dos locais mais dinâmicos de troca celular. O seu crescimento pode ser modulado por diversos estímulos acompanhados de alterações importantes na mucosa intestinal (Bauchart-Thevret et al, 2009b). Estudos realizados em ratos foram os primeiros a mostrar níveis significativos de enzimas envolvidas nas vias de transsulfuração no intestino (Mudd et al., 1965). Estudos em leitões, sugerem que o metabolismo da metionina intestinal pode estar ligado a ação de células não epiteliais ou a microrganismos luminais na mucosa intestinal, por encontrarem um catabolismo insignificante da metionina nos enterócitos (Chen et al., 2009).

Demonstrou-se, em suínos que o trato gastrointestinal utiliza preferencialmente a metionina da circulação arterial ao invés da metionina da dieta, sendo que o metabolismo preferencial da primeira passagem da metionina dietética ocorre no fígado e não no trato gastrointestinal. Especula-se que a taxa de transsulfuração da metionina no trato gastrointestinal é dependente das necessidades de cisteína para a síntese de glutationa, devido ao estresse oxidativo associado a alta atividade metabólica das células epiteliais em proliferação (Riedijk et al. 2007). Shoveller et al. (2003) descobriram que a cisteína é eficaz na conservação da metionina e na presença de cisteína dietética em excesso, a exigência de metionina passa a ser de cerca de 70% da exigência enteral.

A deficiência em aminoácidos sulfurados reduz o crescimento intestinal em suínos, associando a atrofia das vilosidades, redução na proliferação de células epiteliais e menor número de células caliciformes (Bauchart-Thevret et al. 2009). Esta deficiência pode afetar também a síntese de mucinas intestinais, a mucina é composta principalmente de aminoácidos não essenciais, com exceção da cistina e da treonina, (Ravindran e Hendrix, 2004). A cistina geralmente é limitada na composição das dietas, sendo a metionina utilizada como alternativa, já que são bem estabelecidos os mecanismos de conversão de metionina e cistina. No entanto, devido ao gasto metabólico ocasionado nesta conversão, as necessidades metabólicas de mucina seriam melhor atendidas pelo acréscimo direto de cistina na dieta (Moran Jr, 2016).

Em condições deficientes de aminoácidos sulfurados, o metabolismo da metionina é priorizado de modo que a síntese de proteínas é preservada sobre a transmetilação da

metionina e a associação de metionina é preservada por regulação positiva da remetilação e supressão de homocisteína da transsulfuração. A supressão da transsulfuração contribui para a diminuição das concentrações de cisteína celular e glutationa, aumentado o estresse oxidativo e afetando, preferencialmente, o crescimento intestinal (Bauchart-Thevret et al. 2009a).

Uma diminuição na ingestão de metionina ou uma deficiência em folato pode alterar o metabolismo da metionina e ter impacto nos níveis de S-adenosilmetionina intestinal, que é necessária para a síntese de poliaminas. O epitélio intestinal possui um dos tecidos de mais rápida renovação, assim possui alta demanda por poliaminas (Bauchart-Thevret et al. 2009b). São crescentes as evidências de que as poliaminas regulam a renovação das células epiteliais intestinais, em função da sua capacidade de modular a expressão de vários genes e desta forma, pode ocorrer a inibição do crescimento intestinal após a depleção de poliaminas, pela ativação de genes inibidores do crescimento ao invés de apenas a diminuição na expressão de gene promotores de crescimento (Wang, 2007).

2. Transporte de aminoácidos e peptídeos

As proteínas dietéticas primeiramente são digeridas por meio de hidrólise enzimática com o intuito de gerar produtos finais absorvíveis, incluindo os aminoácidos livres e os peptídeos. Esses nutrientes, por sua vez, são absorvidos por células epiteliais ancoradas na borda em escova do intestino delgado por uma variedade de transportadores. Uma vez dentro das células epiteliais, são usados no metabolismo celular ou transportados para fora da célula e para o sangue, com o intuito de atuar em outras células e tecidos (Daniel, 2004; Broer, 2008; Gilbert et al., 2008). Esses transportadores estão localizados na membrana da borda da escova para o transporte de aminoácidos do lúmen intestinal para o interior das células epiteliais intestinais e na membrana basolateral para o transporte de aminoácidos do interior da célula epitelial para o sangue (Zhang et al., 2016). A absorção de peptídeos também pode ocorrer através de rotas alternativas, por meio do movimento paracelular e de peptídeos penetrantes de células, capazes de mover a carga pelo interior da membrana plasmática (Figura 2) (Gilbert et al., 2008).

Os transportadores de aminoácidos podem atuar de forma independente ou dependente de Na^+ . A metionina livre é transportada na membrana da borda em escova pelos transportadores de aminoácidos neutros B^0AT1(codificado pelo gene SLC6A19), pelo

transportador de aminoácidos catiônicos ATB^0 , dependentes de Na^+ e pelo transportador de aminoácidos catiônicos e neutros $B^{0,+}$ AT, dependentes de Na (Hyde et al., 2003). Além do transportador de di e tripeptídeos dependente de H^+ , o *PEPT1* (Gilbert et al, 2008).

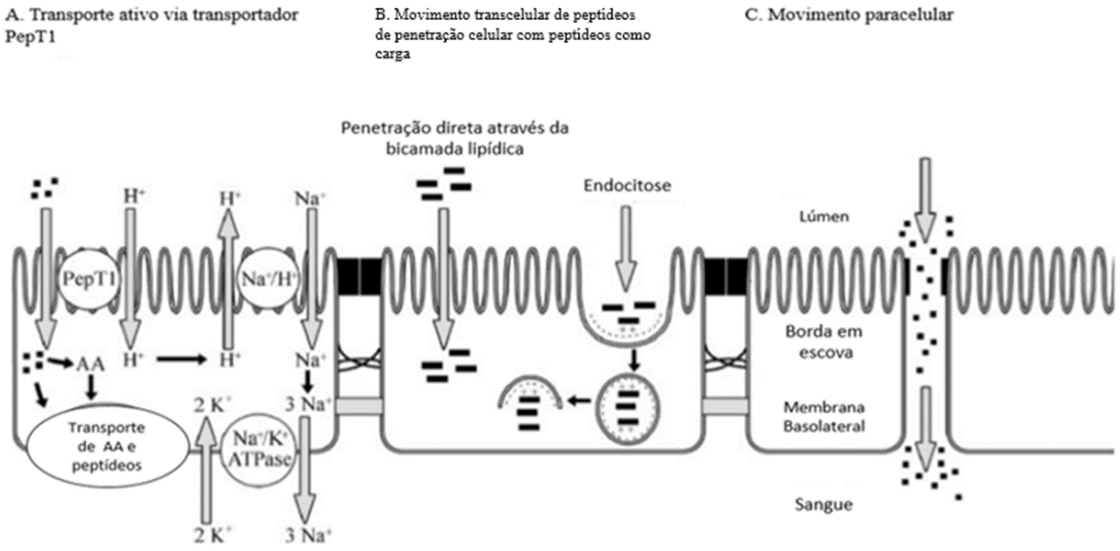

Figura 2. Rotas de absorção dos peptídeos nos enterócitos. (A) A via primária de absorção de di e tripeptídeos é através de cotransporte com H + pelo transportador de peptídeos PEPT1. (B) Os peptídeos de penetração celular (CPP) são capazes de transportar cargas, como peptídeos, para o interior das células. (C) O aumento da permeabilidade das junções apertadas entre as membranas laterais, permite a captação de peptídeos, via rota paracelular. Fonte: Adaptado Gilbert et al. (2008).

O transportador *B^0AT1*, codificado pelo gene SLC6A19, é o principal transportador apical de aminoácidos neutros nos rins e no intestino. De acordo com estudos ele é capaz de transportar todos os aminoácidos neutros, no entanto, possui afinidade variável pelos aminoácidos, demonstrando uma ordem de preferência por eles: Met - Leu - Ile - Val >Gln - Asn - Phe - Cys - Ala > Ser - Gly - Tyr - Thr - His - Pro > Trp - Lys. Tem como característica o contratransporte de um Na^+ por aminoácido, sendo assim depende da concentração de Na^+ para realizar o transporte de aminoácidos (Figura 3) (Broer, 2008).

Na membrana basolateral a metionina é transportada pelos transportadores de aminoácidos neutros SAT1, SAT2 e SAT3, que também são dependentes de Na^+ , pelos transportadores de aminoácidos neutros independentes LAT1 e LAT2 que são

independentes de Na^+ e pelos transportadores de aminoácidos catiônicos y^+ LAT1 e y^+ LAT2, que são dependentes de Na^+ (Figura 3) (Zhang et al., 2016).

O transportador y+LAT1, codificado pelo gene SLC7A7, é responsável pelo transporte de aminoácidos neutros e catiônicos através das células epiteliais. A afinidade dos aminoácidos neutros pelos transportadores aumenta cerca de duas vezes na presença do Na^+. Na ausência de Na^+ o H^+ é cotransportado. O transporte de aminoácidos catiônicos, ao contrário, é independente de Na^+. Porém, esse transportador realiza um mecanismo de antiporte obrigatório, ou seja, por causa da escassez de Na^+ intracelular, ocorre a troca de aminoácidos catiônicos por aminoácidos neutros extracelulares (Figura 3) (Broer, 2008).

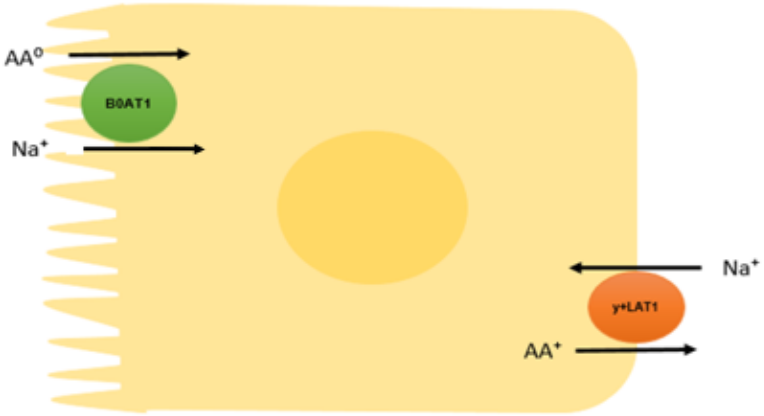

Figura 3. Transportadores de aminoácidos na borda em escova e na membrana basolateral do intestino delgado.

As primeiras evidências de transporte e absorção de dipeptídeos foram datadas por Newey e Smyth em 1959. No entanto, a absorção de dipeptídeos como contribuição no metabolismo de aminoácidos foi ignorada por muito tempo. Sendo elucidada apenas após a clonagem e caracterização do transportador intestinal de peptídeos *PEPT1* (Fei et al., 1994).

Os transportadores de aminoácidos livres são substratos específicos, já o *PEPT1* pode transportar todos os di e tripeptídeos formados pela combinação de todos os 20 diferentes aminoácidos dietéticos, desta forma, em termos de eficiência energética o transporte de aminoácidos pela *PEPT1* é muito mais efetivo, considerando que se transporta 2 ou 3 aminoácidos com o mesmo gasto energético utilizado para o transporte de um aminoácido livre (Daniel, 2004).

O transportador de peptídeos *PEPT1* é um transportador próton-dependente, de baixa capacidade e alta afinidade. Expresso principalmente na membrana apical das células intestinais e renais, sendo que nos enterócitos localiza-se de maneira restrita às junções vilos-criptas, aumentando em direção a ponta dos vilos (Gilbert et al., 2008). Uma vez dentro das células, os peptídeos podem ser hidrolisados por enzimas celulares e atravessarem a membrana basolateral como aminoácidos, ou podem ser efluídos no sangue através de um sistema de transporte peptídico basolateral. Um gradiente de prótons através da membrana apical é mantido pela atividade de um transportador Na^+ / H^+ apical, que por sua vez é energizado pela Na^+ / K^+ -ATPase basolateral. Esse gradiente apical de prótons aumenta a absorção de substratos peptídicos (Brodin et al., 2002).

Além de aminoácidos, o transportador *PEPT1* pode transportar alguns compostos farmacêuticos caracterizados como peptideomiméticos, participando de sua absorção e desta forma, afetando suas características terapêuticas. Algumas dessas drogas são as cefalosporinas, penicilinas, as aminopeptidases, aciclovir, ganciclovir e inibidores da enzima conversora de angiotensina (Brodin et al. 2002; Steffansen et al. 2004).

Em pintos após a eclosão, o *PEPT1* mostrou maior expressão no intestino delgado em comparação com outros tecidos (Zwarycz e Wong, 2013). Chen et al. (1999), demonstraram ainda maior expressão de *PEPT1* no duodeno comparado ao jejuno e íleo. Speier et al. (2012) estudaram a expressão gênica de transportadores de nutrientes na membrana vitelínica e no intestino delgado de embriões Cobb e Leghorn, observando que para ambas as linhagens, a expressão de *PEPT1* na membrana vitelínica aumentou inicialmente, atingindo o pico entre os dias 13 e 15 de incubação, diminuindo os níveis no final da incubação, enquanto no intestino delgado a expressão aumentou do dia 15 para o dia 21 de incubação.

3. Metabolismo de aminoácidos e a termorregulação

Durante os primeiros dias de vida as aves agem como animais poiquilotérmicos, não sendo capazes de ajustar a sua produção de calor corporal de acordo com a temperatura do ambiente em que se encontram. Como consequência disso, baixas temperaturas ambientais podem ocasionar queda brusca na temperatura corpórea, dependendo do tamanho da ave (Weitjens et al., 1999).

A temperatura é capaz de influenciar os processos fisiológicos e bioquímicos do organismo animal, exercendo efeitos sobre diversos fatores como a atividade enzimática, a função imunológica, a contratilidade muscular, a atividade neuronal, a atividade endócrina, entre outros (Wenisch et al., 1996, Wassertrom e Vites, 1999, Aihara et al., 2001).

Alguns nutrientes geram maior incremento calórico. Em condições de estresse térmico por calor, a utilização de dietas contendo menor teor de proteína bruta, apresenta alto incremento calórico, e dietas com maior teor de lipídeos, menor incremento calórico. Contudo, todos os processos metabólicos geram calor (Ferket e Gernat, 2006).

O estresse é um fator importante a ser considerado na produção de aves, pois elas são rotineiramente expostas a situações estressoras durante os ciclos produtivos, tais como jejum, flutuações de temperatura e o estresse ocasionado pelo transporte (Burkholder et al., 2008). A exposição às temperaturas extremas é um estressor importante encontrado em ambientes sazonais, principalmente durante o verão (Bailey, 1988). A temperatura ambiente elevada mostrou influenciar a fisiologia dos frangos de corte, induzindo múltiplos distúrbios fisiológicos, tais como a desregulação imune sistêmica, distúrbios endócrinos que resultam em crescimento fraco e aumento da mortalidade (Quinteiro-Filho et al., 2012).

Um dos distúrbios mais importantes ocasionado pelo estresse por calor é a diminuição do consumo alimentar, sugerindo que as aves diminuem sua alimentação com o intuito de manter sua homeotermia (Gonzales-Esquerra e Leeson, 2006). Swennen (2004), indica que a termogênese ocasionada pela dieta representa até 23% da energia metabolizável aparente ingerida. No entanto, ainda são desconhecidos os mecanismos pelos quais a diminuição do consumo alimentar poderia auxiliar no controle da temperatura corporal. Uni et al. (2001), observaram queda nos níveis plasmáticos de T3 após 24 horas de exposição de pintos de 3 dias de idade ao calor, sugerindo que alterações nos níveis de T3 auxiliam na manutenção da homeotermia ou inversamente, poderiam ser uma resposta ao aumento da temperatura corporal (Gonzales-Esquerra e Leeson, 2006).

Em condições de estresse térmico, mesmo com acesso a água, o esvaziamento do trato gastrointestinal passa a ser mais lento, pelo fato do animal comer menos e depender mais das reservas. Neste momento, as aves passam a entrar em um período pós-absortivo, caracterizado pelo período em que o animal passa a usar suas reservas nutricionais e energéticas para a mantença (Rutz et al., 2017). Relata-se também que o animal quando

submetido a um longo período de estresse, comem durante o período noturno (Leeson e Summers, 2005).

Diante do aumento de temperatura corporal as aves vão desenvolver mecanismos de perda de calor como resposta aguda. São dois os principais meios de perda evaporativa de calor. Através das superfícies da pele e por meio das vias respiratórias superiores, sendo algumas vezes considerado também a superfície da cloaca como um mecanismo secundário de evaporação (Hoffman et al., 2007).

Outras alterações derivadas do estresse por calor são na morfologia e fisiologia do trato gastrointestinal, como a diminuição da motilidade intestinal, alterações na microflora intestinal, além de uma depressão no fluxo sanguíneo intestinal (Gonzales-Esquerra e Leeson, 2006). Estudos indicam que o estresse térmico realizado no período inicial do desenvolvimento intestinal altera a proliferação celular e os níveis plasmáticos de T3. No entanto, essas alterações modulam o trato gastrointestinal para um crescimento compensatório 48 horas após o estresse (Uni et al., 2001).

O estresse também está associado ao aumento da colonização intestinal e a eliminação fecal de patógenos em aves (Bailey, 1988). Em períodos de estresse a mucosa intestinal fica continuamente exposta a uma carga elevada de moléculas antigênicas de alimentos ingeridos e microrganismos, como bactérias e vírus residentes e invasivos (Keita e Soderholm, 2010).

O estresse por calor, sendo crônico ou agudo, reduz a função da barreira intestinal, causando respostas inflamatórias e comprometendo resultados de desempenho, indicando que os frangos que sofrem estresse gastam mais energia para regular a temperatura corporal, comprometendo a energia que seria usada para o crescimento. O estresse aumentaria também a permeabilidade intestinal à endotoxinas e bactérias. (Alhenaky et al., 2017).

Burkholder et al. (2008) observaram que galinhas submetidas ao estresse térmico agudo (30°C / 24 h) apresentaram redução das profundidades de cripta ileal, apesar de não observar diferenças na relação vilo:cripta. Frangos submetidos ao estresse térmico crônico apresentaram diminuição da altura dos vilos e no peso do jejuno (Mitchell e Carlisle, 1992). Animais submetidos ao estresse crônico de temperatura podem ainda apresentar respostas compensatórias ao longo do tempo de exposição, como melhora na integridade intestinal (Alhenaky et al., 2017).

A concentração plasmática de aminoácidos também é alterada pelo estresse por calor. Geraert et al. (1996), relataram que aves submetidas ao estresse de 32º C apresentaram menor concentração plasmática, principalmente de aminoácidos sulfurados, em relação à frangos de corte que não sofreram estresse. As aves aumentariam seu volume plasmático a fim de aumentar a dissipação de calor, por meio de resfriamento evaporativo, diluindo a concentração dos componentes sanguíneos.

4. Referências Bibliográficas

Agostini, P. S., P. Dalibard, Y. Mercier, P. Van der Aar, and J. D. Van der Klis. 2016. Comparison of methionine sources around requirement levels using a methionine efficacy method in 0 to 28 day old broilers. Poult. Sci. 95:560-569.

Aihara, H., Y. Okada, and N. Tamaki. 2001. The effects of cooling and rewarming on the neuronal activity of pyramidal neurons in guinea pig hippocampal slices. Brain Res. 893:36-45.

Alhenaky, A., A. Abdelqader, M. Abuajamieh, and A.R. Al-Fataftah. 2017. The effect of heat stress on intestinal integrity and Salmonella invasion in broiler birds. J. Therm. Biol. 70:9-14.

Bailey, J. S. 1988. Integrated colonization control of Salmonella in poultry. Poult. Sci. 67:928-932.

Barnes, D.M., C.C. Calvert, and K.C. Klasing. 1995. Methionine deficiency decreases protein accretion and synthesis but not tRNA acylation in muscles of chicks. J. Nutr. 125: 2623-30.

Bauchart-Thevret, C., B. Stoll, S. Chacko, D.G. Burrin. 2009a. Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. Am. J. Physiol. Endocrinol. Metab. 296:1239-1250.

Bauchart-Thevret, C., B. Stoll, D.G. Burrin. 2009b. Intestinal metabolism of sulfur amino acids. Nutr. Res. Rev. 22:175-187.

Blom, H.J., and Y. Smulders. 2011. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. J. Inherit. Metab. Dis. 34:75-8.

Brodin, B., C.U. Nielsen, B. Steffansen, and S. Frokaer. 2002. Transport of Peptidomimetic Drugs by the Intestinal Di/tri-peptide Transporter, PEPT1. Pharmacol. Toxicol. 90:285-296.

Broer, S. 2008. Amino acid transport across mammalian intestinal and renal epithelia. Physiol. Rev. 88:249-286.

Brosnan, J.T., and M.E. Brosnan. 2006. The Sulfur-Containing Amino Acids: An Overview. J. Nutr.136:1636S-1640S.

Burkholder, K. M., K. L. Thompson, M. E. Einstein, T. J. Applegate, and J. A. Patterson. 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers. Poult. Sci. 87:1734-1741.

Burrin, D. G., and B. Stoll. 2007. Emerging aspects of gut sulfur amino acid metabolism. Curr. Opin. Clin. Nutr. Metab. Care. 10: 63-68.

Chamruspollert, M., G. M. Pesti, and R. I. Bakalli. 2004. Influence of temperature on the arginine and methionine requirements of young broiler chicks. J. Appl. Poult. Res. 13: 628–638.

Chen, H., E. A. Wong, and K. E. Webb Jr. 1999. Tissue distribution of a peptide transporter mRNA in sheep, dairy cows, pigs, and chickens. J. Anim. Sci. 77:1277–1283.

Chen, L.X., P. Li, J.J. Wang, X.L. Li, H.J. Gao, Y.L. Yin, Y.Q. Hou, G. Wu. 2009. Catabolism of nutritionally essential amino acids in developing porcine enterocytes. Amino Acids. 37:143-152

Corzo, A., M. T. Kidd, W.A. Dozier III, A. Shack, and S. C. Burgess. 2006. Protein expression of pectoralis major muscle in chickens in response to dietary methionine status. Br. J. Nutr. 95:703-708.

Courtney-Martin, G., R. O-Ball, and P.B. Pencharz. 2012. Sulfur amino acid metabolism and requirements. Nutr. Rev. 70:170-175.

Daniel, H. 2004. Molecular and integrative physiology of intestinal peptide transport. Annu. Rev. Physiol. 66:361-384.

Dibner, J. J., and C. D. Knight. 1984. Conversion of 2-hydroxy4-(methylthio) butanoic acid to l-methionine in the chick: A stereospecific pathway. J. Nutr. 114:1716-1723.

D'Mello, J.P.F. 2003. Amino acids as multifunctional molecules. Pages 1-14. in: Amino acids in animal nutrition. D'Mello, J.P.F. (Ed.). 2 ed. Cabi Publishing, Wallingford, UK.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). 2015. Scientific Opinion on the safety and efficacy of DL-methionyl-DL-methionine for all aquatic animal species. EFSA Journal. 13(2):4012-4026.

Fei, Y. J., Y. Kanai, S. Nussberger, V. Ganapathy, F. Leibach, M. Romero, S. Singh, W. Boron, and M. Hediger. 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature. 368:563-566.

Ferket, P.R. and A.G. Gernat. 2006. Factors that affect feed intake of meat birds: a review. Int. J. Poult. Sci. 5: 905-911.

Geraert, P. A., J. C. F. Padilha, and S. Guillaumin. 1996. Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: growth performance, body composition and energy retention. Br. J. Nutr. 75:195-204.

Gilbert, E. R., E. A. Wong, and K. E. Webb Jr. 2008. Board-Invited Review: Peptide absorption and utilization: Implications for animal nutrition and health. J. Anim. Sci. 86:2135-2155.

Gonzales-Esquerra, R., and S. Leeson. 2006. Physiological and metabolic responses of broilers to heat stress - implications for protein and amino acid nutrition. Worlds Poult. Sci. J. 62: 282-295.

Guo, F., and D.R. Cavener. 2007. The GCN2 eLF2α Kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. Cell Metab. 5: 103–114.

Guyton, A.C. 2006. Tratado de Fisiologia Médica. Elsevier . Rio de Janeiro. RJ.

Hasek, B.E., L.K. Stewart, T.M. Henagan, A. Boudreau, N. R. Lenard, C. Black, J. Shin, P. Huypens, V. L. Malloy, E.P. Plaisance, R. A. Krajcik, N. Orentreich and T.W. Gettys. 2010. Dietary methionine restriction enhances metabolic flexibility and increases uncoupled respiration in both fed and fasted states. Am. J. Physiol. Regul. Integr. Comp. Physiol. 299: R728-R739.

Hasek, B.E., A. Boudreau, J. Shin, D. Feng, M. Hulver, N. T. Van, A. Laque, L. K. Stewart, K. P. Stone, D. Wanders, S. Ghosh, J. E. Pessin and T. W. Gettys. 2013. Remodeling the integration of lipid metabolism between liver and adipose tissue by dietary methionine restriction in rats. Diabetes. 62: 3362-3372.

Hickling, D., W. Guenter, and M. E. Jackson. 1990. The effects of dietary methionine and lysine on broiler chicken performance and breast meat yield. Can. J. Anim. Sci. 70: 673-678.

Hoffman, T.C.M., G.E. Walsberg, D.F. DeNardo. 2007. Cloacal evaporation: an important and previously undescribed mechanism for avin thermorregulation. J. Exp. Biol. 210:119-135.

Hyde, R., P.M. Taylor, and H. S. Hundal.2003. Amino acid transporters: roles in amino acid sensing and signalling in animal cells. Biochem. J. 373:1–18.

Jiang Y., S.K. Noh and S. I. Koo. 2001. Egg phosphatidylcholine decreases the lymphatic absorption of cholesterol in rats. J. Nutr. 131: 2358-2363.

Keita, A.V., and J.D. Soderholm. 2010. The intestinal barrier and its regulation by neuroimmune factors. Neurogastroenterol. Motil. 22: 718–733.

Kulinski, A., D. E. Vance and J. E. Vance. 2004. A choline-deficient diet in mice inhibits neither the CDP-choline pathway for phosphatidylcholine synthesis in hepatocytes nor apolipoprotein B secretion. J. Biol. Chem. 279:23916-23924.

Leeson, S., and J.D. Summers. 2005. Comercial Poultry Nutrition. 3rded. Canadá:University Books. Guelph, Ontario.

Martın-Venegas, R., J. F. Soriano-Garcıa, M. P. Vinardell, P. A. Geraert, and R. Ferrer. 2006. Oligomers are not the limiting factor in the absorption of DL-2-Hydroxy-4-(methylthio) butanoic acid in the chicken small intestine. Poult Sci. 85:56-63.

Mato, J. M., M. L. Martinez-Chantar, and S.C. Lu .2008. Methionine metabolism and liver disease. Annu. Rev. Nutr. 28:273–93.

Métayer, S., I. Seiliez, A. Collin, S. Duchêne, Y. Mercier, P.A. Geraert, S, Tesseraud. 2008. Mechanisms throught which sulfur amino acids control protein metabolism and oxidative status. J. Nutr. Biochem. 19: 207-215.

Mitchell, M.A., and A. Carlisle. 1992. The effects of chronic exposure to elevated environmental temperature on intestinal morphology and nutrient absorption in the domestic fowl (Gallus domesticus). Comp. Biochem. Physiol. A. 101: 137-142.

Moran Jr, E.T. 2016. Nutrients central to maintaining intestinal absorptive efficiency and barrier integrity with fowl. Poult Sci. 0:1–16.

Moreira, I., and P.C. Pozza, 2014. Metabolismo Proteico. Pages 112-125 in Nutrição de Não-Ruminantes. N.K. Sakomura and J.H.V. Silva, F.G.P Costa et al. Funep. Jaboticabal.

Mudd, S.H., J.D. Finkelstein, F. Irreverre, and L. Laster. 1965. Transsulfuration in mammals: microassays and tissue distribution of three enzymes of the pathway. J. Biol. Chem. 240: 4382-4392.

Murphy, M. E. 1994. Amino acid compositions of avian eggs and tissues: Nutritional implications. J. Avian Biol. 25:27–38.

Naya, F. J., and E. Olson. 1999. MEF2: A transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. Curr. Opin. Cell Biol. 11:683–688.

Nelson, D.L., Cox, M.M., 2014. Princípios de Bioquímica de Lehninger. 6th ed., Artmed, Porto Alegre.

Newey, H., and D. H. Smyth. 1959. The intestinal absorption of some dipeptides. J. Physiol. 145:48-56.

Powell, D. J., S. G. Velleman, A.J. Cowieson, and W. I. Muir. 2017. Methionine concentration in the pre-starter diet: its effect on broiler breast muscle development. Anim. Prod. Sci. 57: 448-457.

Quinteiro-Filho, W.M., M.V. Rodrigues, A. Ribeiro, V. Ferraz-de-Paula, M.L. Pinheiro, L. R. Sá, J. Palermo-Neto. 2012. Acute heat stress impairs performance parameter and induces mild intestinal enteritis in broiler chickens: Role of acute hypothalamic pituitary-adrenal axis activation. J. Anim. Sci. 90:1986-1994.

Quinteiro-Filho, W. M., A. Ribeiro, V. Ferraz-de-Paula, M. L. Pinheiro, M. Sakai, L. R. M. Sá, A. J. P. Ferreira, and J. Palermo-Neto. 2010. Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. Poult Sci. 89:1905–1914.

Ravindran, V., and W. H. Hendriks. 2004. Endogenous amino acid flows at the terminal ileum of broilers, layers and adult roosters. Animal Sci. 79:265-271.

Riedijk, M. A., B. Stoll, S. Chacko, H. Schierbeek, A. L. Sunehag, J. B. Van Goudoever, and D. G. B. Sansonetti. 2007. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. Proc. Natl. Acad. Sci. U S A. 104: 3408–3413.

Riski, G., L. Arnaboldi, B. Gabrielli, J. Yan, G. S. Lee, R. K. Ng, S. M. Turner, T. M. Badger, R. E. Pitas and J. J. Maher. 2006. Mice fed a lipogenic methionine-choline-deficient diet develop hypermetabolism coincident with hepatic suppression of SCD-1. J. Lipid Res. 4: 2280-2290.

Scheuermann, G. N., S. F. Bilgili, J. B. Hess, and D. R. Mulvaney. 2003. Breast muscle development in commercial broiler chickens. Poult. Sci. 82:1648-1658.

Shoveller, A.K., J.A. Brunton, P.B. Pencharz, and R.O. Ball. 2003. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J. Nutr. 133:1390–1397.

Speier, J. S., L. Yadgary, Z. Uni, and E. A. Wong. 2012. Gene expression of nutrient transporters and digestive enzymes in the yolk sac membrane and small intestine of the developing embryonic chick. Poult Sci. 91:1941–1949.

Steffansen, B., C. Nielsen, B. Brodin, A. Eriksson, R. Anderson, and S. Frokjaer. 2004. Intestinal solute carriers: An overview of trends and strategies for improving oral drug absorption. Eur. J. Pharm. Sci. 21:3-16.

Stipanuk, M.H, and I. Ueki. 2011. Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. J. Inherit. Metab. Dis. 34:17-32.

Stipanuk, M. H. 1986. Metabolism of Sulfur-Containing Amino acids. Ann. Rev. Nutr. 6:179-209.

Swennen, Q. 2006. The role of diet-induced thermogenesis in the regulation of voluntary feed intake in the chicken nutritional and genetic influences. Leuven: Katholiec Universiet Leuven. 171p. PhD Thesis - Katholieke Universiet Leuven.

Swennen, Q., N. Everaert, M. Debonne, I. Verbaeys, C. Careghi, K. Tona, G.P. Janssens, E. Decuypere, V. Bruggeman, and J. Buyse. 2010. Effect of macronutrient ratio of the pre-starter diet on broiler performance and intermediary metabolism. J Anim Physiol Anim Nutr. 94: 375–384.

Tesseraud, S., N. Everaert, S. Boussaid-Om Ezzine, A. Collin, S. Métayer-Coustard, and C. Berri. 2011. Manipulating tissue metabolism by amino acids. Worlds Poult. Sci. J. 67: 243-251.

Uni, Z., O. Gal-Garber, A. Geyra, D. Sklan, and S. Yahav. 2001. Changes in growth and function of chick small intestine epithelium due to early thermal conditioning. Poult. Sci. 80:438–445.

Vance, J.E. and D.E. Vance. 1985. The role of phosphatidylcholine biosynthesis in the secretion of lipoproteins from hepatocytes. Can. J. Biochem. Cell Biol. 63: 870-881.

Velleman, S. G., C. S. Coy, and D.A. Emmerson. 2014. Effect of the timing of post-hatch feed restrictions on broiler breast muscle development and muscle transcriptional regulatory factor gene expression. Poult Sci. 93:1484-1494.

Vieira, S.L., and C.R. Angel. 2012. Optimizing broiler performance using different amino acid density diets: What are the limits?. J. Appl. Poult. Res. 21:149-155.

Vieira, S.L., and C. Stefanello. Carboidratos: digestão e absorção. Pages 220-237. in: Fisiologia das aves comerciais. M. Macari. and Maiorka A. Funep. Jaboticabal, São Paulo, Brasil.

Wang, J.Y. 2007. Polyamines and mRNA stability in regulation of intestinal mucosal growth. Amino Acids. 33:241-252.

Wassertrom J.A., and A.M. Vites.1999. Activation of contration in cat ventricular myocytes: effects of low Cd(2+) concentration and temperature. Am. J. Physiol. 277: H488-H498.

Weitjens, S., R. Meijerhof, J. Buyse, and E. Decuypere. 1999. Thermoregulation in chicks originating from breeder flocks of two different ages. J. Appl. Poultry Res. 8:139-145.

Weinisch, C., E. Narzt, D.I. Sessler, B. Parschalk, R. Lenhard, A. Kurz, and W. Graninger.1996. Mild intraoperative hypothermia reduces production of reactive oxygen intermediates by polymorphonuclear leucocytes. Anesth. Analg. 4:810-816.

Wen, C., X. Jiang, L. Ding, T. Wang, and Y. Zhou. 2017. Effects of dietary methionine on breast muscle growth, myogenic gene expression and IGF-I signaling in fast- and slow-growing broilers. Sci. Rep. 7:1924.

Wu, G. 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids. 37:1-17.

Wu, G. 2013. Amino Acids: Biochemistry and Nutrition. Page 480. Taylor & Francis Group.

Zanou, N., and P. Gailly. 2013. Skeletal muscle hypertrophy and regeneration: interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways. Cell. Mol. Life Sci. 70: 4117-4130.

Zhai, W., L.F. Araujo, S. C. Burgess, A. M. Cooksey, K. Pendarvis, Y. Mercier, and A. Corzo. 2012. Protein expression in pectoral skeletal muscle of chickens as influenced by dietary methionine. Poult. Sci. 91:2548-2555.

Zhang, S., B. Saremi, E. R. Gilbert, and E. A. Wong. 2016. Physiological and biochemical aspects of methionine isomers and a methionine analogue in broilers, Poult. Sci. 0:1-15.

Zwarycz, B., and A.Wong. 2013. Expression of the peptide transporters PEPT1, PepT2, and PHT1 in the embryonic and posthatch chick. Poult Sci. 92:1314–1321

II. OBJETIVOS GERAIS

Avaliar a utilização de DL-Metionil-DL-Metionina e do estresse térmico aos 21 dias de idade sobre o desempenho, as características intestinais e musculares de frangos de corte.

2.1. Objetivos Específicos

- Determinar o desempenho de frangos de corte de 1 a 21 dias submetidos a dietas com duas fontes do aminoácido metionina;
- Medir a altura e a largura das vilosidades intestinais e a profundidade das criptas aos 21 dias de idade e após 24 e 48 horas de estresse térmico de 32º C;
- Analisar os níveis séricos de alanina aminotransferase, aspartato aminotransferase, ácido úrico e creatina quinase aos 21 dias de idade e após 24 e 48 horas de estresse térmico de 32º C;
- Quantificar a expressão dos genes PEPT1, $Y^{+}LAT$, $B^{0}AT1$ no jejuno de frangos de corte aos 21 dias de idade e após 24 e 48 horas de estresse térmico de 32º C;
- Mensurar o diâmetro das fibras musculares e o número de células por área no músculo peitoral aos 21 dias de idade e após 24 e 48 horas de estresse térmico de 32º C;
- Avaliar a composição corporal aos 21 dias de idade e após 24 e 48 horas de estresse térmico de 32º C;

III. Effect of DL-Methionyl-Methionine supplementation on muscle development and body composition of broiler chickens submitted to heat stress at 21 days old

ABSTRACT: The DL-Methionyl-DL-Methionine (Met-Met) supplementation was evaluated in diet of broiler chickens from 1 to 21-d, subjected to heat stress (32ºC) for up to 48 hours. A total of 216 male Cobb-Vantress® chicks were distributed in a completely randomized experimental design with 3 x 3 factorial scheme, consisting of 3 diets (without methionine supplementation – basal, and supplemented with DL-methionine (DL-Met) and Met-Met) and 3 stress periods of temperature at 21 days (no stress and after 24 and 48 hours of 32 °C). For performance, broilers supplemented with DL-Met and Met-Met had higher weight in relation to a basal diet of 1 to 21 d ($P = 0.006$). For weight gain ($P = 0.0006$) and feed conversion ($P = 0.0001$) from 1 to 21 days, both diets supplemented with methionine had better values, compared to basal. Regarding the carcass composition, the crude protein was higher for broilers supplemented with methionine from both sources in all stress period ($P < 0.05$). After 24 hours of heat stress, birds fed with basal diet presented a higher percentage of ether extract compared to those supplemented with DL-Met and Met-Met ($P = 0.033$). Regarding the breast muscle, there was dietary effect for weight, with bigger breast in broiler supplemented with methionine. Histologically, the muscle fibers of broilers fed DL-Met had a larger diameter than the other diets ($P = 0.0001$). Plasma alanine aminotransferase values was higher after 48-hs of heat stress in broilers with basal diet compared to DL-Met ($P = 0.014$) and plasma creatine kinase values were higher in basal broiler compared with both supplemented diets ($P = 0.003$). It is concluded that methionine supplementation in diet of broilers is essential for performance, muscle morphology and carcass composition, and the 48 hs heat stress causes initial liver damage and changes in carcass lipid deposition.

Key words DL-Methionine, DL-Methionil-DL-Methionine, Pectoral muscle, crude protein, ether extract.

INTRODUCTION

The structural and metabolic characteristics of the pectoral muscle, as well as carcass quality and composition are closely associated to muscle fibers development in broilers. The genetic selection employed in these birds allowed the growth and, also the highest pectoral development by increasing the diameter and length muscle fibers (Guernec et al., 2003; Berri et al., 2007).

Body weight of broiler chickens in the first week of life may increase about 3 to 4 times as a result of muscle and gastrointestinal growth, which are extremely accelerated at this time (Murakami et al., 1992; Uni, 2006). The neonatal period coincides with the moment of greater activity of muscle satellite cells in birds. Satellites cells contribute to the muscle hypertrophy, since they play a role of nucleus donor for the fibers, contributing to their growth (Powell et al., 2016).

Studies in turkeys, evaluating the effect of amino acid levels in pre-initial diet on the dynamics of muscle satellite cells, observed that deficiencies in crude protein, lysine, methionine, cysteine and threonine temporarily increase the mitotic activity of satellite cells, affecting muscle development (Nierobisz et al., 2007).

Methionine is characterized as being the first limiting amino acid in poultry diets, so is supplemented by several sources and the most commonly used are L-methionine, DL-methionine and DL-2-hydroxy-4-methylthiobutyrate (DL-HMTBA). Due to the individual characteristics of these molecules, differences in absorption and metabolism may affect the methionine availability to the body (Zhang et al., 2016).

DL-Methionyl-DL-Methionine is a new methionine source, characterized as a DL-methionine dipeptide, having the possibility of being absorbed either as a dipeptide using the PEPT1 dipeptide transporter or as L-methionine, via transporters of free amino acids, in the same way as the other sources used in supplementation (EFSA-Journal, 2015).

Free amino acid transporters are specific substrates, whereas PEPT1 can carry all di and tripeptides formed by the combination of the 20 different dietary amino acids. Thus, in terms of energy efficiency the amino acids transports by PEPT1 would be much more effective considering that it would carry 2 or 3 amino acids with the same energy expenditure used to transport a free amino acid (Daniel, 2004).

Several studies showed that a diet with inadequate methionine levels, especially under heat stress conditions, may result in lower protein deposition in the breast muscle and that

satellite cells, responsible for muscle proliferation, are significantly affected by this amino acid restriction (Corzo et al., 2006, Powell et al., 2014 Zhai et al., 2012, Wen et al., 2014).

In view of these aspects, the use of DL-Methionyl-DL-Methionine could provide a more efficient methionine absorption, providing more of this amino acid for protein synthesis and muscle fibers hypertrophy. This leads to reduced muscle loss and muscle degradation, characterized by a post-absorptive period, in which the birds begin to use nutritional and energy reserves for maintenance (Rutz et al., 2017).

The objective of this study was to compare the effect of two methionine sources, DL-Methionine and DL-Methionyl-DL-Methionine on performance, muscle fiber development and carcass composition in broilers at 21 days of age, subjected to temperature stress of 32°C for up to 48 hours.

MATERIAL AND METHODS

Animals and diets

The Committee of Ethical Conduct on the Use of Animals for Experimentation of the State University of Maringá approved the experimental procedure, under protocol number 4000170615. A total of 216 male broilers from commercial Cobb-Vantress® line were distributed in 3 diets: basal (below methionine exigence – 0.585), Met-Met and DL-Met (methionine digestible 0.856) and 3 evaluation periods: at 21 days (before heat stress) and at 22 and 23 days, respectively, with 24 and 48 hours of heat stress of 32 ºC. Broilers and diets were weighed weekly from 1 to 21 days to determine the productive performance. The diets were formulated based on the recommendations of Rostagno et al. (2011) according to the species requirements, being isoenergetic and isonutritives with the exception of the methionine and methionine + cystine levels (Table 1). The birds were housed in an air-conditioned room, distributed in 18 metal cages with an area of 1 m^2, 12 birds per cage, 6 replicates per used diet. The environmental temperature was adequate to the birds age, according to the lineage manual until the 21 days-old, after this period, birds were submitted to heat stress (32ºC), during 24 and 48 hours and were evaluated in the periods of 0 and after 24 and 48 hours of stress. For caloric stress implementation, the birds were fasted for 4 hours and then the temperature of the climatic room was raised gradually within a period of 2 hours, until the room temperature reached 32 °C.

Table 1. Centesimal and nutritional composition of experimental diets for broilers at 1 to 21 days-old with different methionine sources.

Ingredients	Basal	Met-Met 97%	Dl-Met 99%
Corn 7.8%	54.89	54.89	54.89
Soybean meal 46%	37.30	37.30	37.30
Soybean oil	3.80	3.80	3.80
Salt	0.45	0.45	0.45
Limestone 38% Ca	1.16	1.16	1.16
Dicalcium phosphate 20%	1.53	1.53	1.53
DL-Met-Met 97%	-	0.295	-
DL - Methionine 99%	-	-	0.28
L-Treonine 98.5%	0.03	0.03	0.03
L-Lisine HCl 78%	0.15	0.15	0.15
PREMIX[1]	0.40	0.40	0.40
Inerte (washed sand)	0.30	0.005	0.02
Composition calculated			
CP. %	22	22	22
ME, Kcal/Kg	3052	3052	3052
SID Met + Cys, %	0.585	0.856	0.856
SID Lys, %	1.199	1.199	1.199
SID Trp, %	0.244	0.244	0.244
SID Thr, %	0.780	0.780	0.780
SID Ile, %	0.856	0.856	0.856
SID Val, %	0.924	0.924	0.924
SID Arg, %	1.385	1.385	1.385
Na, %	0.200	0.200	0.200
Ca, %	0.876	0.876	0.876
P, %	0.450	0.450	0.450

[1] Mineral and vitamin supplementation (guarantee levels per Kg of diet): Vit. A: 9,080 IU; Vit. E: 33.32 IU; Vit. B1: 2.36 mg; Vit. B2: 5.96 mg; Vit. B6:2.63 mg; Vit. B12: 14 mcg; Vit. K3: 1.8 mg; Ca-pantothenate: 11.904; Niacin: 35.28 mg; Folic Acid: 0.8 mg; Biotin: 0.08 mg; Choline: 0.344 mg; Zn: 0.076 mg; F: 0.056 mg; Mn: 0.08 mg; Cu: 12.16 mg; I: 1.16 mg; Co: 0.2 mg; Se: 0.352 mg; Ethoxyquin: 0.1 mg; BHA: 0.08 mg; Vehicle: 4mg.

Performance

Weekly, the birds and food were weighed, for body weight, weight gain, feed intake and feed conversion determination. At 0 hours and after 24 and 48 hours of heat stress, 6 birds per treatment were weighed, and the rectal temperature was measured than they were anesthetized by intravenous sodium thiopental (10 $mg.kg^{-1}$), and slaughtered through

cervical dislocation to collect the pectoral muscle fragments for morphophysiological characterization of muscle fibers.

Muscular Morphology

Pectoral muscle samples were frozen in N-Hexane, previously cooled to - 70ºC in liquid nitrogen (Chayen et al., 1969) and immediately conditioned in liquid nitrogen. The frozen muscle fragments were cut transversely with respect to fibers direction in a cryostat microtome (10μm) for the preparation of histological slides stained with hematoxylin and eosin. For the morphological evaluation of muscle fibers, digital images were taken through a camera (Motican® 5MP) coupled to a light microscope. The images were analyzed using Motic Image Plus 2.0 image analysis software (Motic® China Group Co. Ltd., Xiamen, China). Ten images were captured per bird, with each image covering an area of 59,000 μm^2. For the evaluation of fiber concentration by area, all the muscle fibers present within each image were counted, and fibers that were partially contained in the lower and right margins were discounted, and those contained partially in the upper and left margins, included. For the evaluation of the muscular fibers diameter, 20 fibers per image were measured in their smaller diameter, totaling 200 fibers per bird and 1200 fibers per treatment.

Plasma analyzes

At the same time, another 6 birds per treatment were submitted to a 6-hour fasting, then weighed and submitted to blood collection through the jugular, to obtain plasma and determine the concentrations of UA (uric acid), CK (creatine kinase), ALT (alanine aminotransferase) and AST (aspartate aminotransferase). Plasma was stored at -20 ° C, until spectrophotometer (UV-VIS Evolution 300) analysis using commercial kits (Gold Analisa Diagnostica Ltda- Belo Horizonte, Brazil).

Carcass composition

These same birds were dissected and had its breast weighed to determine the relative breast weight and then frozen for body composition determination: dry matter (DM), mineral matter (MM), crude protein (CP) and ether extract (EE). The frozen carcasses (n = 6 / treatment) were milled in an industrial meat mill with feathers, viscera, feet and head. The samples were homogenized and an average aliquot of 60g was weighed and taken to the lyophilizer for 36 hours for the determination of DM. Afterwards, they were ground in a ball

mill, to perform the analyzes of MM, CP and EE. For determination of MM, aliquots of lyophilized samples were weighed in porcelain crucibles and oven-dried at 105 °C for 24 hours, and then taken to the muffle at 550 °C for hours, and by incineration the value of ashes were obtained.

CP was obtained using the Kjeldahl nitrogen method (crude protein = nitrogen x 6.25). EE was obtained by extraction in Soxhlet extractor. The methodologies used for the analyzes were described in detail by AOAC (2016).

Statistical analysis

The data were analyzed using the GLM procedure, and the means were compared using Tukey's test (SAS Inst. Inc., Cary, NC, USA). The results are expressed as means and standard error to describe the effects of the dietary on each period of stress. For rectal temperature, the interaction between diets and heat stress was considered.

RESULTS

The temperature of birds after 24 hs of heat stress was higher than the ones that did not suffer heat stress and those submitted to 48 hours of heat stress at 32ºC (P=0.0003) (Figure1-A).

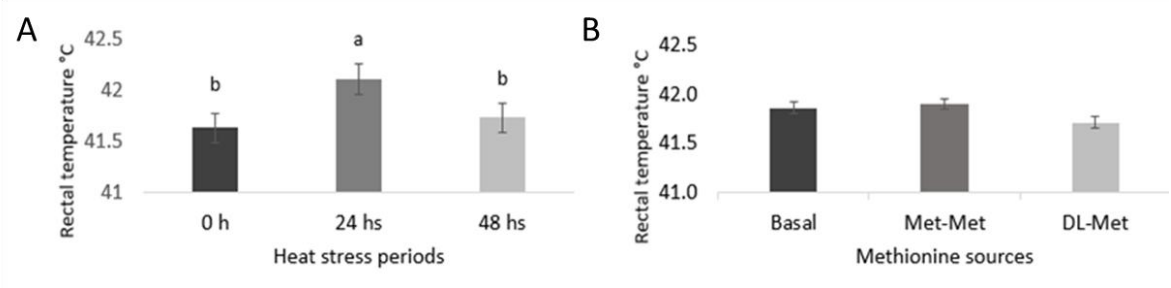

Figure 1. Effects of heat stress periods (A), and methionine sources (B) on rectal temperature in broilers at 21days-old. Values are means ± SEM;$^{a, b}$: means within the same time point with different letters differ significantly (P<0.05).

The body weight, weight gain and feed conversion were affected by diets at 1 to 7, 7 to 14 and 14 to 21 days. Birds submitted to diets supplemented with methionine from both sources had better values compared to basal diet for body weight in all periods, and for weight gain at 1 to 7 days and feed conversion at 7 to 14 and 14 to 21 days. The birds supplemented with Met-Met presented best values for weigh gain compared to basal dietary at 7 to 14 and 14 to 21 days (Table 2).

During the period when birds were submitted to temperature stress, they presented prostrate and panting, with the breast supported on the floor of the cage and open wings, greatly reducing the water consumption and feed intake. Thus, feed intake in all heat stress period was on average ±0.180 kg per bird, with no significant difference between treatments.

Table 2. Performance of broilers submitted to diets with different sources of methionine from 01 to 21 d-old.

	Body weight 21d (kg)	Feed intake (kg)	Weight gain (kg)	Feed conversion (kg/kg)
		1 a 7 d		
Basal	0.152[b]	0.144	0.105[b]	1.368
Met-Met	0.167[a]	0.135	0.122[a]	1.108
Dl-Met	0.168[a]	0.132	0.121[a]	1.097
EPM	0.015	0.027	0.048	0.235
P value	0.0215	0.940	0.022	0.087
		7 a 14 d		
Basal	0.355[b 1]	0.334	0400[b]	1.651[a]
Met-Met	0.407[a]	0.361	0.466[a]	1.451[b]
Dl-Met	0.417[a]	0.356	0.443[ab]	1.485[b]
EPM	0.030	0.028	0.052	0.090
P value	<.0001	0.047	0.011	<0.0001
		14 a 21 d		
Basal	0.755[b]	0.648	0.401[b]	1.623[a]
Met-Met	0.850[a]	0.692	0.466[a]	1.484[b]
Dl-Met	0.884[a]	0.666	0.443[ab]	1.507[b]
EPM	0.072	0.067	0.053	0.122
P value	0.0006	0.229	0.011	0.015

[1]Means followed by different letters, in the column, differ from each other by the Tukey test.

Regarding carcass composition analyzes the percentages of DM and MM presented no significant difference between treatments. After 24 (P<0.0001) and 48 hours (P=0.0004) of heat stress, the birds supplemented with DL-Met and Met-Met presented higher CP percentage in the carcass compared to basal diet (Figure 2). After 24 hours of heat stress, the birds fed with basal diet had a higher EE percentage in carcass compared to those supplemented with DL-Met (P=0.0334) (Figure 2).

Comparing the breast relative weight, birds fed diets supplemented with DL-Met and Met-Met showed statistically higher values than birds fed with basal diet at 0 (P=0.0002), and 24 (P=0.0001) and 48 hs (P<0.0001) of stress (Figure 3). In relation to the morphology of breast muscle fibers (Figure 3), it was observed that the birds fed with the DL-Met expressed fibers with larger diameter in relation to those submitted to the diet composed of Met-Met, and these birds presented values greater than those submitted to basal diet at all stress periods (P=0.0001). These results, on the other hand, result in a greater number of

muscle fibers per area for birds fed with basal diet (P<0.0001). However, histologically, they presented qualitatively greater amount of fat around the muscular fibers (Figure 4).

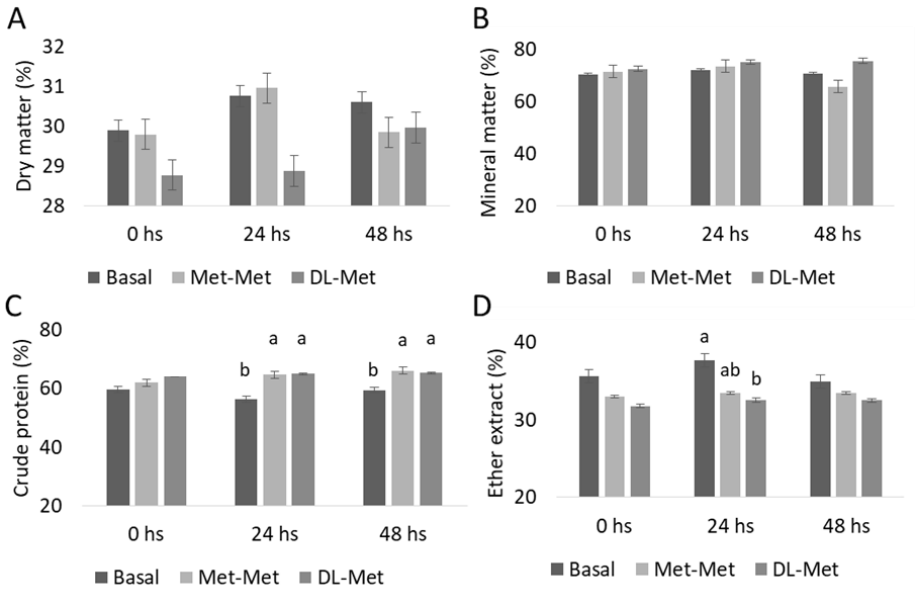

Figure 2. Effects of methionine supplementation sources on Dry matter % (A), Mineral Matter % (B), Crude protein % (C), and Ether extract % (D) in carcass of 21 days-old broilers submitted to different period of heat stress. Values are means ± SEM;$^{a, b}$: means within the same time point with different letters differ significantly (P<0.05).

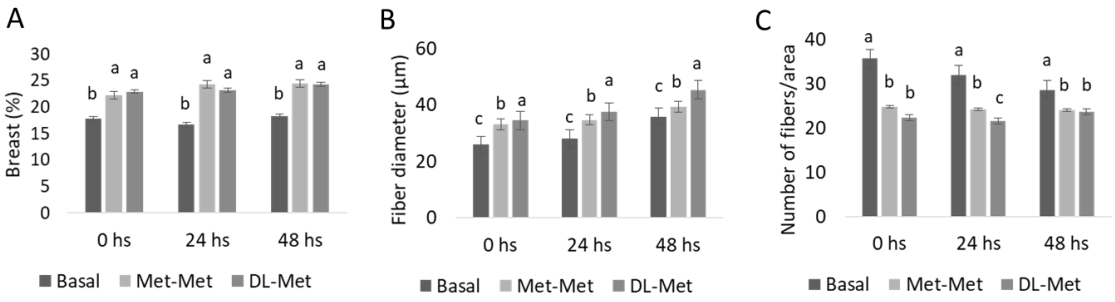

Figure 3. Effects of methionine supplementation sources on breast% (A), breast fiber diameter (µm) (B), and number of fibers (C) of 21 days-old broilers submitted to different period of heat stress. Values are means ± SEM;$^{a, b}$: means within the same time point with different letters differ significantly (P<0.05).

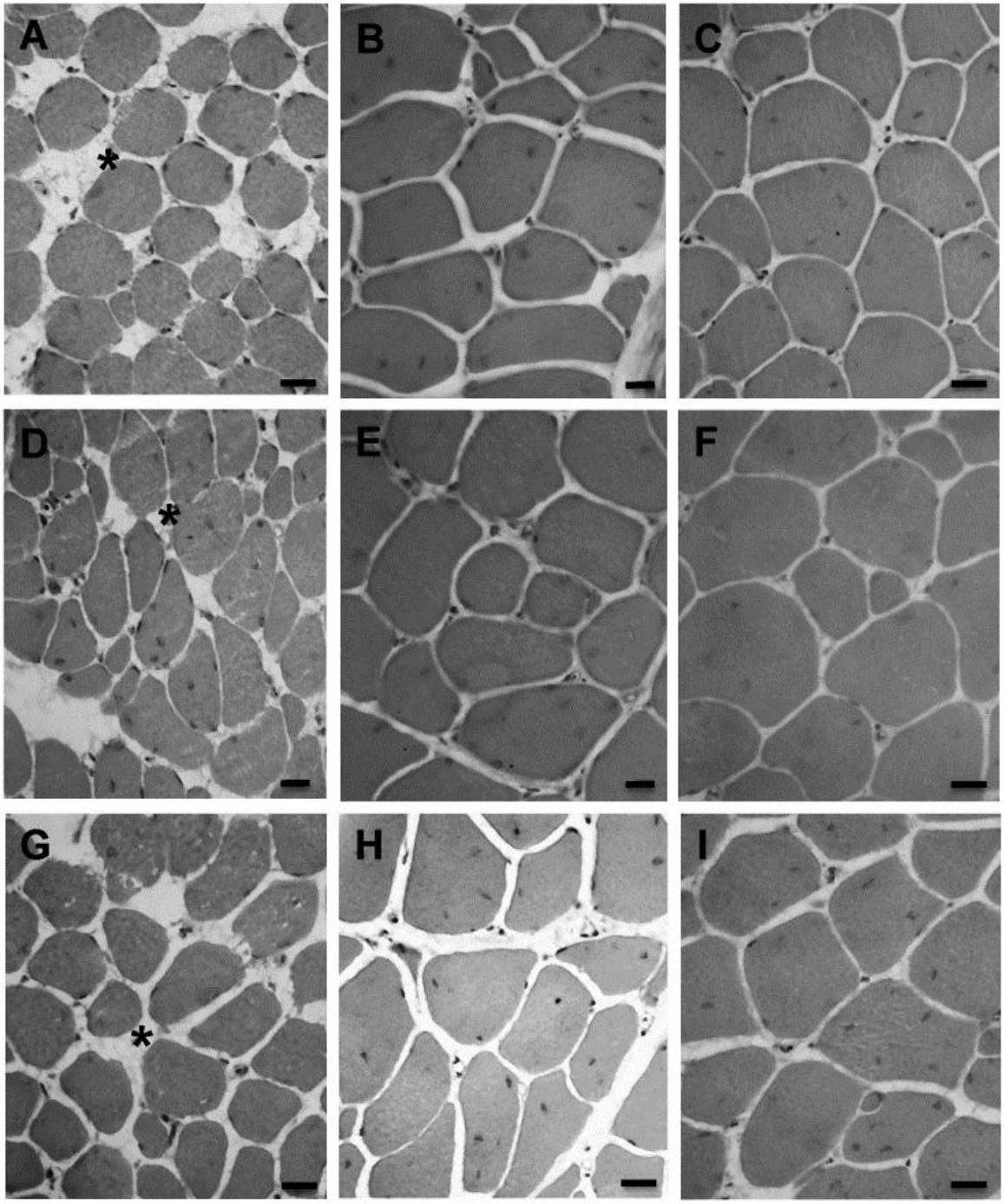

Figure 4. Cross-sectional histological images of muscle fibers of broilers submitted to diets with different methionine sources at 21 days-old, 0 hours of stress (A: Basal, B: Met-Met, C: DL- Met), 24 hours of stress (D: Basal, E: Met-Met, F: DL-Met), and 48 hours of stress (G: Basal, H: Met-Met, I: DL-Met). A, D, G: Fat layer between muscle fibers (*). Scale bar: A, B, C, D, E, F, G, H and I) 20 μm.

For the plasmatic ALT, there was dietary effect at 48 hs of heat stress, and the birds fed with DL-Met supplemented diet presented higher values than those fed with basal diet and supplemented with Met-Met (P=0.0145). For no stress and after 24 hs of heat stress there was no significant effect (Figure 5).

AST were not influenced by diet in all heat stress periods. For CK, there was a significant effect of the diet at 48 hours of thermal stress (P=0.0028), in which birds receiving a basal diet had higher values than those receiving diets supplemented with DL-Met and Met-Met (Figure 5).

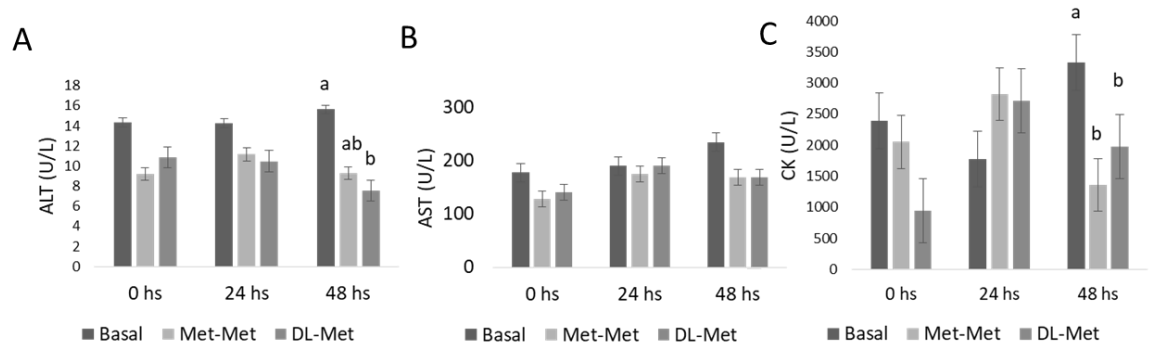

Figure 5. Effects of methionine supplementation sources on ALT (A), AST (B) and CK (µm) (C) of plasma in 21 days-old broilers submitted to different periods of heat stress. Values are means ± SEM;$^{a, b}$: means within the same time point with different letters differ significantly (P<0.05).

DISCUSSION

Methionine is the first limiting amino acid for birds, in corn and soybean meals based diets, which are commonly supplemented to meet the requirements of these animals (Dozier and Mercier, 2013). Considering this aspect, in the present study, the values of body weight, feed conversion and weight gain for the animals that received the basal diet, are in line with that expected for birds receiving diets above the methionine requirements.

It is known that protein is important in controlling satiety in several species. However, among the amino acids, methionine is not known as a direct influence on food intake. In our study, the evaluated diets had the same amount of protein, differing only in relation to the SID methionine content, compared to the basal diet. Therefore, they presented similar crude protein content, so as not to present significant differences in relation to food intake. Sklan and Plavnik (2002) reported that the higher crude protein content (28%), compared to a basal

diet (18%), caused a decrease in feed intake in 7-day-old chicks. Birds receiving methionine in the diet, whether in the form of Met-Met or DL-Met showed significantly, higher CP contents in the body, compared to the one that received a basal diet, especially in the periods after 24 and 48 hours of heat stress.

In our study, it was observed that the birds had an increase in body temperature after 24 hs of heat stress, regardless of diet. However, after 48 hs exposed to 32 ° C it returned to the temperature of when they were not subject to stress. Demonstrating a possible adaptation to the environment in question. Del Vesco et al. (2015), when subjected broilers to 24 hours of heat stress, observed the same rectal temperature increase. However, the authors did not evaluate the birds after 48 hours of stress.

The difference between acute and chronic heat stress is tenuous, acute heat stress is a consequence of exposure to high ambient temperatures for several hours, inducing physiological and metabolic changes to support the survival of the animals. Chronic heat stress is induced by high cyclic or continuous temperatures over a long period (days to weeks), allowing acclimatization to the environment (Loyal et al., 2015).

There are no studies capable of defining the moment when the animal crosses from one stage to another, perhaps because this event is marked only by physiological changes, which can be influenced by the age of animal, by the productive period in which it is and by environment temperature. The change in body temperature from 24 to 48 hours of heat stress may be one of the indications that this animal has crossed from acute stress to chronic, so it would not mean that the birds have adapted to the heat stress.

Geraert et al. (1996) studied the chronic heat stress (32 °C) for two different periods of stress (2 to 4 weeks-old and 4 to 6 weeks-old), which reported that the high environment temperature significantly decreased body protein content and energetic retention, regardless of the decrease in food intake, suggesting that only the increase in temperature is sufficient, to reduce protein synthesis and increase the catabolic rate. However, in our study, evaluating a period of lower stress, it was observed that even the 48 hours of caloric stress were not enough to affect the crude protein percentage in carcass

Temin et al. (2000) reported that at 32ºC, broilers at 42 days presented greater damage in protein synthesis compared to proteolysis, in the pectoral and gastrocnemius muscles, resulting in a reduction in muscle protein deposition. The authors attributed these characteristics to a decrease in ribosomal capacity, which would lead to a reduction in the rate of protein synthesis.

Take into account the muscle fibers diameter, it was observed that the birds consuming DL-Met had a higher diameter of the pectoral muscle fibers in all the evaluated periods, superior to those presented by the Met-Met supplemented animals, that presented higher values in relation to basal diet.

Powell et al. (2014) by *in vitro* cells cultures demonstrated that the activity of satellite cells is significantly affected by the methionine and cystine availability. Thus, adjusting the methionine levels in the pre-initial period can increase the activity of the satellite cells activity, consequently increasing the muscular development in broilers.

Muscle growth after hatching is dependent on the addition of new nucleus of satellite cells from adult myoblasts to muscle fibers, which were formed during the embryonic period through myoblastic hyperplasia (Velleman et al., 2014). The role of satellite cells is the hypertrophy of muscle cells through nucleus, promoting mionuclear addition and increasing protein synthesis (Moss and Le Blond, 1971).

Zhai et al. (2012) found that dietary methionine supplementation at recommended levels increased the pectoral muscle development and attributed this increase to sarcoplasmic muscle hypertrophy characterized by the sarcoplasm growth, an interfibrillar substance that is not composed of contractile proteins, thus, the muscle fibers would increase in diameter, but would not have greater contractile force.

Analyzes regarding the number of satellite cells, expression of genes responsible for protein deposition, as well as the extension of the experimental period, could answer questions about the mechanisms responsible for these alterations and clarify if these results would entail effective responses regarding muscle development.

The morphological muscular breast fibers evaluation showed that birds received basal diet presented higher amount of fat around the muscular bundles. As well as in the period after 24 hours of stress, the carcass of birds that received the basal diet presented higher EE percentage in relation to the other diets. This result may characterize the interaction of methionine with lipid metabolism. By stimulating the oxidative catabolism of fatty acids, S-Adenosylmethionine is the main donor of methyl groups, essential for carnitine synthesis. Carnitine is a cofactor involved in the transport of long chain fatty acids into the mitochondrial membrane. Therefore, adequate amount of dietary methionine has the potential to reduce the lipid concentration in the carcass (Hutte et al., 1997, Ahmed and Abbas, 2011).

Regarding the plasma analyzes, there was a change in the serum levels of CK and ALT at 48 hours of stress. Birds fed with basal diet showed higher levels compared to those receiving the other diets, these indexes may indicate that birds wich did not consume methionine in diet were more susceptible to stress and had higher levels of muscle proteolysis. Both enzymes were quantified in plasma. Therefore, it may originate from all animal metabolism; however, both CK and ALT are highly present in muscle tissue, with a greater probability of indicating changes in this tissue. The AST value was not altered, this enzyme is indicative of more severe lesions, if altered together with ALT, it could point to possible hepatic lesions.

According to Rizki et al. (2006), low levels of dietary methionine, increase liver fat levels by the β-oxidation pathway. Rinella and Green (2004), characterize that the absence of methionine in the diet interferes in the synthesis and secretion of very low density lipoproteins (VLDL), because it is necessary for phosphatidylcholine synthesis, an essential component of plasma lipoproteins, that transport triacylglycerols out of the hepatocytes, leading to hepatic steatosis (Serviddio et al., 2011). McLean et al. (2014) further suggests that an additional effect of methionine as a source of sulfur sulfate maintains a metabolic balance of liver cells under adverse conditions.

CONCLUSION

Methionine supplementation independent of the source used was essential for muscle development and protein deposition in birds. The diameter of the muscle fibers was altered by methionine source in the diet, being superior for the birds that received DL-Methionine. However, this result did not influence broilers performance, and considering these indices, any of the sources of methionine used would be adequate for the supplementation of broiler until 21 days-old.

REFERENCES

Ahmed, M.E., and T.E. Abbas. 2011. Effects of Dietary Levels of Methionine on Broiler Performance and Carcass Characteristics. Int J Poult Sci. 10 (2): 147-151.

AOAC. 2016. Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists, Washington, DC.

Berri, C., E. Le Bihan-Duval, M. Debut, V. Santé-Lhoutellier, E. Baéza, V. Gigaud, Y. Jégo and M. J. Duclos. 2007. Consequence of muscle hypertrophy on Pectoralis major characteristics and breast meat quality of broiler chickens. J.Anim. Sci. 85-2005-11.

Chayen, J., L. Bitensky, R.G. Butcher; L.W. Poulter.1969. A guide to pratical histochemstry in Oliver e Bould, London.

Corzo, A., M. T. Kidd, W. A. Dozier, L. A. Shack, and S. C. Burgess. 2006. Protein expression of pectoralis major muscle in chickens in response to dietary methionine status. British Journal of Nutrition. 95: 703–706.

Daniel, H. 2004. Molecular and integrative physiology of intestinal peptide transport. Annu. Rev. Physiol. 66:361-384.

Del Vesco, A.P., E. Gasparino, D.O. Grieser, V. Zancanella, D.M. Voltolini, A.S. Khatlab, S.E.F. Guimarães, M.A.M. Soares, and A.R. Oliveira-Neto. 2015. Effects of methionine supplementation on the expression of protein deposition-related genesin acute heat stress-exposed broilers. PloS One. 10 (2):e0115821.

Dozier, W.A., and Y. Mercier. Ratio of digestible total sulfur amino acids to lysine of broiler chicks from 1 to 15 days of age. 2013. J. Appl. Poult. Res., 22: 862–871.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). 2015. Scientific Opinion on the safety and efficacy of DL-methionyl-DL-methionine for all aquatic animal species. EFSA Journal. 13(2):4012-4026.

Geraert, P. A., J.C.F. Padilha, and S. Guillaumin. 1996. Metabolic and endocrine changes iduced by chronic heat exposure in broiler chickens: growth performance, body composition and energy retention. Br J Nutr. 75: 195-204.

Guernec, A., C. Berri, B. Chevalier, N. Wacrenier-Ceré, E. Le Bihan-Duval, and M. J. Duclos. 2003. Muscle development, insulin-like growth factor-I and myostatin mRNA levels in chickens selected for increased breast muscle yield. Growth Horm. IGF Res. 13:8-18.

Loyau, T., L. Bedrani, C. Berri, S. Métayer-Coustard, C. Praud, V. Coustham, S. Mignon-Grasteau, M. J. Duclos, S. Tesseraud, N. Rideau, C. Hennequet-Antier, N. Everaert, S. Yahav, A. Collin. 2015. Cyclic variations in incubation conditions induce adaptive responses to later heat exposure in chickens: a review. Animal. 9:76-85.

Maclean, K.N., L.S. Greiner, J.R. Evans, S.K Sood, S. Lhotak, N.E. Markham, S.P. Stabler, R.H. Allen, R.C. Austin, V. Balasubramaniam, and H. Jiang. 2012. Cystathionine Protects against Endoplasmic Reticulum Stress-induced Lipid Accumulation, Tissue Injury and Apoptotic Cell Death. J Biol Chem. 38: 31994-32005.

Moss, F.P., and C.P. Leblond. 1971. Satellite cells as source of nuclei in muscles of growing rats. Anat. Rec. 170: 421-436.

Murakami H, Y. Akiba, M. Horiguchi .1992. Growth and utilization of nutrients in newly-hatched chick with or without removal of residual yolk. Growth Dev. Aging. 56: 75-84.

Nierobisz, L.S., V. Felts, and P.E. Mozdziak. 2007. The effect of early dietary amino acid levels on muscle satellite cell dynamics in turkeys. Comparative Biochemistry and Physiology. Part B. Biochem. Mol. Biol. Educ. 148:286–294.

Powell, D.J., D.C. McFarland, A.J. Cowieson, W.I. Muir, and S.G. Velleman. 2014. The effect of nutritional status and muscle fiber type on myogenic satellite cell fate and apoptosis. Poult Sci. 93: 163–173.

Powell, D.J., S.G. Velleman, A.J. Cowieson, and W. I. Muir. 2017. Methionine concentration in the pre-starter diet: its effect on broiler breast muscle development. Anim. Reprod. Sci. 57: 448–457.

Rinella, M.A., and R.M. Green. 2004. The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. J. Hepatol. 40: 47–51.

Rizki, G., L. Arnaboldi, B. Gabrielli, J. Yan, G.S. Lee, R K. Ng, S.M. Turner, T.M. Badger, R. E. Pitas, and J.J. Maher. 2006. Mice fed a lipogenic methionine-choline-deficient diet develop hypermetabolism coincident with hepatic suppression of SCD-1. J. Lipid Res**.**47: 2280-2290.

Rostagno, H.S., L.F.T Albino, J.L. Donzele, P.C. Gomes, R.F., Oliveira, D.C. Lopes, A.S. Ferreira, and, S.L.T. Barreto. 2011. Brazilian tables for poultry and swine: Composition of feedstuffs and nutritional requirements. Pages 225. Viçosa, MG, Brasil.

Rutz, F., V.F.B. Roll, and E.G. Xavier. Metabolismo Intermediário. Pages 340-377. in: Fisiologia das aves comerciais. M. Macari. and Maiorka A. Funep. Jaboticabal, São Paulo, Brasil.

SAS Institute. 2011. SAS User's Guide: Version 9.2 ed. SAS Inst. Inc., Cary, NC.

Serviddio, G., A.D. Romano, T. Cassano, F. Bellanti, E. Altomare, and G. Vendemiale. 2011. Principles and therapeutic relevance for targeting mitochondria in aging and neurodegenerative diseases. Curr Pharm Des. 17(20):2036–2055.

Sklan, D., and I. Plavnik. 2002. Interactions between dietary crude protein and essential amino acid intake on performance in broilers. Br. Poult. Sci. 43:442-449.

Temim, S, A. M. Chagneau, R. Peresson, and S. Tesseraud .2000. Chronic heat exposure alters protein turnover of three different skeletal muscles in finishing broiler chickens fed 20 or 50% protein diets. J. Nutr. 130:813–819

Uni, Z. 2006. Early development of small intestinal function. Pages 29–42 in Avian Gut Function in Health and Disease. G. C. Perry, ed. CAB Int., London, UK.

Velleman, S. G., C. S. Coy, and D. A. Emmerson. 2014. Effect of the timing of post-hatch feed restrictions on broiler breast muscle development and muscle transcriptional regulatory factor gene expression. Poult. Sci. 93:1484–1494.

Wen, C., X. Chen, G.Y. Chen, P. Wu, Y.P. Chen, Y. M. Zhou, and T. Wang. 2014. Methionine improves breast muscle growth and alters myogenic gene expression in broilers. J. Anim. Sci. 92:1068–1073.

Zhai, W., L.F. Araujo, S.C. Burgess, A. M. Cooksey, K. Pendarvis, Y. Mercier, and A. Corzo. 2012. Protein expression in pectoral skeletal muscle of chickens as influenced by dietary methionine. Poult Sci. 91:2548–2555.

Zhang, S., B. Saremi, E. R. Gilbert, and E.A. Wong. 2016. Physiological and biochemical aspects of methionine isomers and a methionine analogue in broilers. Poult Sci. 0:1–15.

IV. DL-Methionil-Methionine supplementation on intestinal morphology and gene expression of amino acid transporters in 21 days-old broilers submitted to heat stress

ABSTRACT: The DL-Methionyl-DL-Methionine (Met-Met) supplementation was evaluated on diet of broilers from 1 to 21 d, subjected to temperature stress (32ºC) for up to 48 hours. A total of 216 male Cobb-Vantress® chicks were distributed in a completely randomized experimental design with 3 x 3 factorial scheme, consisting of 3 diets (without methionine supplementation – basal, and supplemented with DL-methionine (DL-Met) and Met-Met) and 3 heat stress periods at 21 days (no stress and after 24 and 48 hours of 32 °C). In intestinal morphology, there was interaction between diets and heat stress periods for crypt depth (P = 0.011) and villus:crypt ratio (P = 0.009) and heat stress periods resulted in lower ileal villi (P = 0.01) and thinner duodenal villi (P = 0.006). The analysis of amino acid transporters gene expression in the brush border (B^0AT1 and PEPT1) and basolateral membrane (Y^+LAT1) of jejunum, was performed. There was interaction between diet and heat stress periods for Y^+LAT1 (P=0.001) and B^0AT1 (P=0.0007), with a higher Y^+LAT1 expression after 48 hours of heat stress in birds with Met-Met diet and with higher B^0AT1 expression in all diets after 48 hours. The PEPT1 expression was influenced by the heat stress period (P=0.015), being superior after 48 hs in all diets. In broilers without heat stress, B^0AT1 and PEPT1 transporters were more expressed in basal diet. The intestinal morphology results evidenced the importance of methionine supplementation in diet of broilers from 1 to 21 d and also the negative effects caused by heat stress. Gene expression of intestinal amino acid transporters suggested that the primary pathway of methionine absorption was through the free amino acid transporters.

Key words: DL-Methionine, DL-Methionil-DL-Methionine, duodenum, jejunum, ileum.

INTRODUCTION

The first two post hatching weeks are characterized as a period of rapid intestinal development in broilers. Occur an intensification in the digestive enzymes activity, an increase in the number and in the rate of enterocytes proliferation, villi length and width and crypts depth (Uni et al., 1998, 2000; Sklan and Noy, 2000; Moran, 2007, Gilbert et al., 2010).

The rapid growth of broilers, affected by genetic selection, requires an adequate dietary balance. Methionine is the first limiting amino acid in poultry diet, this amino acid is encoded by a single codon in the standard genetic code (AUG), and this codon plays an important role in the translation of the mRNA protein, since it signals the beginning of the protein translation in this way. Methionine is incorporated into the N-terminal position of all proteins during translation and plays a determinant role in the proteins deposition in animal metabolism (Berg et al., 2002).

For a long time, the liver was considered the main organ responsible for the amino acids metabolism, being the intestine responsible only for the digestion and absorption of other constituents of diet. However, studies reveal that the intestine obtains a significant portion of its metabolic energy through the catabolism of dietary amino acids, absorbing the amino acids even before it enters the portal circulation, previously determining its systemic availability (Brosnan, 2003, Shoveller et al., 2006, Martín-Venegas et al., 2006).

The amino acids absorption in the intestine is mediated by protein transporters located in enterocytes (Zeng et al., 2011). Methionine is a neutral amino acid that is transported on the brush border membrane by the B^0AT neutral amino acid transporters, by the Na dependent cationic amino acid transporters ATB^0 and the $B^{0,+}$ AT cationic amino acid transporter, dependent of Na (Hyde et al., 2003), as well as by the dipeptide transporter PEPT1, H^+ dependent (Gilbert et al, 2008).

In the basolateral membrane, methionine is transported by the neutral amino acid transporters SAT1, SAT2 and SAT3, which are also dependent on Na^+ , by the neutral amino acid carriers LAT1 and LAT2 which are independent of Na^+ and by the cationic amino acid carriers Y^+ LAT1 and Y^+ LAT2, which are Na dependent (Broer, 2008). This can be regulated in the small intestine by several factors, such as genetic selection, intestinal development, and the quantity and quality of protein in diet (Daniel, 2004, Gilbert et al., 2007, 2010).

Environmental temperature also affects gut characteristics. The increase in body temperature leads to a decrease in feed intake by animals in order to maintain the homeothermy, causing morphological and physiological changes in the gastrointestinal tract, such as decreased intestinal motility, changes in intestinal microflora, and a decrease in blood flow (Gonzales-Esquerra and Leeson, 2006).

The DL-Methionyl-DL-Methionine molecule has been used in animal production in diet of aquatic animals, not yet studied in birds, its use in poultry diets was considered different from other sources of methionine mainly in relation to its form of absorption. By being characterized as a DL-methionine dipeptide, it would be possible to be metabolized not as a free amino acid, as for other sources of the amino acid methionine, but also through the transported PEPT1, in the form of a readily available dipeptide (EFSA-Journal, 2015).

Thus, our hypothesis would be that the use of a methionine dipeptide in diet may influence the intestinal villi development and determine changes in the growth of the animals. As well as, to influence the mRNA expression of free amino acids transporters, di and tripeptides in the brush border and in the basolateral membrane, mainly in face of thermal and sanitary challenges. The objective of this study was to evaluate the role of DL-Methionyl-DL-Methionine in diet on the intestinal morphology and gene expression of PEPT1, Y^{+}LAT1 and B^{0}AT1 amino acid transporters in the jejunum of broilers at 21 days submitted to heat stress of 0, 24 and 48 hours.

MATERIAL AND METHODS

The Committee of Ethical Conduct on the Use of Animals for Experimentation of the State University of Maringá approved the experimental procedure, under protocol number 4000170615. A total of 216 male broilers from commercial Cobb-Vantress® line were distributed in 3 diets: basal (below methionine exigence – 0.585), Met-Met and DL-Met (methionine digestible 0.856) and 3 evaluation periods: at 21 days (before heat stress) and at 22 and 23 days, respectively, with 24 and 48 hours of heat stress of 32 ºC. The diets were formulated based on the recommendations of Rostagno et al. (2011) according to the requirements for the species, being isoenergetic and isonutritives with the exception of the methionine and methionine + cystine levels (Table 1).

The birds were housed in an air-conditioned room, and distributed in 18 metal cages with an area of 1 m^2, 12 birds per cage, 6 replicates per diet used. The climatic temperature

of the room was adequate to the birds age, according to the lineage manual until the 21 days-old, after this period, birds were submitted to ambient heat stress (32ºC), for 24 and 48 hours and were evaluated in the periods of 0 and after 24 and 48 hours of stress. For caloric stress implementation, the birds were fasted for 4 hours and then the temperature of the climatic room was raised gradually within a period of 2 hours, until the room temperature reached 32 °C.

In each evaluation period, 6 birds per treatment were weighed, anesthetized by thiopental sodium (10mg.kg^{-1}) and slaughtered by cervical dislocation. Then the intestine was dissected and the small intestine was measured along its length and the segments (duodenum, jejunum and ileum) were weighed and collected for the histological slides preparation and intestinal morphophysiology determination. Jejunum intestinal segments were also collected for expression determination of the following transporters: *PEPT1*, *Y^+LAT1* and *B^0AT1*.

Table 1. Centesimal and nutritional composition of experimental diets for broilers at 1 to 21 days-old with different methionine sources.

Ingredients	Basal	Met-Met 97%	Dl-Met 99%
Corn 7.8%	54.89	54.89	54.89
Soybean meal 46%	37.30	37.30	37.30
Soybean oil	3.80	3.80	3.80
Salt	0.45	0.45	0.45
Limestone 38% Ca	1.16	1.16	1.16
Dicalcium phosphate 20%	1.53	1.53	1.53
DL-Met-Met 97%	-	0.295	-
DL - Methionine 99%	-	-	0.28
L-Treonine 98.5%	0.03	0.03	0.03
L-Lisine HCl 78%	0.15	0.15	0.15
PREMIX[1]	0.40	0.40	0.40
Inert (washed sand)	0.30	0.005	0.02
Composition calculated			
CP. %	22	22	22
ME, Kcal/Kg	3052	3052	3052
SID Met + Cys, %	0.585	0.856	0.856
SID Lys, %	1.199	1.199	1.199
SID Trp, %	0.244	0.244	0.244
SID Thr, %	0.780	0.780	0.780
SID Ile, %	0.856	0.856	0.856
SID Val, %	0.924	0.924	0.924
SID Arg, %	1.385	1.385	1.385
Na, %	0.200	0.200	0.200
Ca, %	0.876	0.876	0.876
P, %	0.450	0.450	0.450

[1] Mineral and vitamin supplementation (guarantee levels per Kg of diet): Vit. A: 9,080 IU; Vit. E: 33.32 IU; Vit. B1: 2.36 mg; Vit. B2: 5.96 mg; Vit. B6:2.63 mg; Vit. B12: 14 mcg; Vit. K3: 1.8 mg; Ca-pantothenate: 11.904; Niacin: 35.28 mg; Folic Acid: 0.8 mg; Biotin: 0.08 mg; Choline: 0.344 mg; Zn: 0.076 mg; F: 0.056 mg; Mn: 0.08 mg; Cu: 12.16 mg; I: 1.16 mg; Co: 0.2 mg; Se: 0.352 mg; Ethoxyquin: 0.1 mg; BHA: 0.08 mg; Vehicle: 4mg.

Intestinal Morphology

The duodenum, jejunum and ileum intestinal fragments were fixed in 4% paraformaldehyde buffered in 0.1 M PBS pH 7.4 and subsequently processed in the histological routine, embedded in paraffin, cut 5 micrometers thick and stained with hematoxylin and eosin. Digital images were taken through a camera (Motican® 5MP) coupled to a light microscope, for the morphometric study of the mucosa. The images were

analyzed using Motic Image Plus 2.0 image analysis software (Motic® China Group Co. Ltd., Xiamen, China). In the intestinal segments villus height and width and crypts depth, were obtained, as well as the calculation of the villus: crypt ratio. For each variable, 15 measurements were performed per bird, and 6 birds per treatment were used.

Gene Expression

For gene expression determination, the jejunum intestinal samples were collected and frozen immediately in liquid nitrogen and later stored in freezer - 80 ºC until the total RNA extraction.

The jejunum of 5 birds per treatment was analyzed. Total RNA extraction was performed according to the manufacturer's instructions, 1ml of Trizol (Invitrogen, Carlsbad CA, USA) was added for each 100 mg of jejunum, the sample was ground and then 200 µl of chloroform were added, the samples were manually homogenized for 1 minute and the material was centrifuged for 15 minutes at 12,000 rpm at 4 ° C. The liquid phase of these tubes was transferred to a new tube with the addition of 500 µl of isopranol, homogenizing and centrifuging again for 15 minutes at 12,000 rpm at 4 ° C. The supernatant was discarded and the precipitate was washed with 1 mL of 75% ethanol, centrifuged again at 12,000 rpm for 5 minutes and the supernatant was discarded. The obtained pellet was dried for 15 minutes and then was resuspended in RNAse free ultrapure water. The RNA concentration was measured using the NanoDrop 2000™ spectrophotometer (Thermo Scientific), at the wavelength of 260 nm.

For the manufacture of the cDNA the Super Scrippt ™ III First-Strand Syntesis Super Mix kit (Invitrogen Corporation, Brazil) was used according to the manufacturer's standards. Into a sterile and RNA free tube, were added 6 µl of total RNA, 1 µl of oligo (dT) (50 µM oligo (dT) and 1 µL of annealing buffer). The reaction was incubated for 5 minutes at 65 ° C and then placed on ice for 1 minute, then 10 µl of 2x First-Strand Reaction Mix solution and 2 µl of solution containing the Super Script III reverse transcriptase enzyme and the RNAse inhibitor were added. The solution was incubated for 50 minutes at 50 °C and then incubated for 5 minutes at 85 °C and immediately placed under ice, then samples were stored at -20 °C until the time of use.

The primers used in the reactions were designed according to the sequences of the transport genes PEPT1, Y^{+}LAT1, B^{0}AT1 (Table 2), which were deposited on the website

www.ncbi.nlm.nih.gov. ß-actin was used as endogenous control and all analyzes were performed in a volume of 25 μL and in duplicates.

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

Protein	Primers sequence (5'-3')
PEPT1	F- CCCCTGAGGAGGATCACTGTT
	R- CAAAAGAGCAGCAGCAACGA
Y+LAT1	F- TGTTGGAGCCAGAGAAGGA
	R- CACAAGGAGAGATAAAGCAAAGTC
B^0AT1	F-TCTATTGAAGATTCGGGCAC
	R-AATGGTAAGCACAAGGTATGG
β-Actin	F- GCCAACAGAGAGAAGAAGATGAC
	R- CACCAGAGTCCATCACAATAC

Statistical analysis

Data were submitted to variance analysis, and means were compared by Tukey test using the statistical program SAS Institute Inc. (2011), to describe the effects of the different sources of methionine, the different periods of heat stress and the interaction between them, the villus height and width, the crypts depth and the villus: crypt ratio of duodenum, jejunum and ileum and the PEPT1, Y^+LAT1 and B^0AT1 expression in jejunum.

RESULTS

For body weight there was an isolated effect of the heat stress periods ($P<0.0001$) and diet ($P<0.0001$), in which the birds that suffered stress of 24 and 48 hours and those that received methionine supplementation from both sources in the diet, presented higher body weight. The small intestine length did not present significant difference. Regarding the duodenum relative weight, the animals that underwent 24 and 48 hours of stress presented lower weight in relation to the basal ($P<0.0001$), already in relation to the jejunum ($P=0.056$) and ileum ($P=0.008$) relative weight of birds that were not submitted to the stress presented higher values when compared to those submitted to 48 hours of stress. For ileum relative weight, there was an isolated effect of the diet ($P=0.004$), in which the animals that consumed methionine had a lower relative weight (Table 3).

Table 3. Effects of different methionine sources of and periods of heat stress on body weight, small intestine length (duodenum, jejunum and ileum) and relative weight of duodenum, jejunum and ileum of 21 days old broilers.

		Body weight (g)	Small intestine Length (cm)	Duodenum %	Jejunum %	Ileum %
0 hs	Basal	719 ± 26.62	133 ± 2.21	1.16 ±0.04	3.07 ± 0.11	0.29 ± 0.020
	Met-Met	798 ± 35.00	135 ± 6.17	1.06 ± 0.09	2.69 ± 0.13	0.27 ±0.010
	DL-Met	856 ± 43.45	134 ± 5.96	0.99 ± 0.07	2.85 ± 0.13	0.24 ± 0.020
24 hs	Basal	845 ± 22.26	130 ± 3.95	1.13 ± 0.06	2.67 ± 0.19	0.25 ±0.009
	Met-Met	911 ± 58.89	132 ± 4.86	1.06 ± 0.09	2.65 ± 0.36	0.22 ± 0.022
	DL-Met	985 ± 31.32	134 ± 6.57	0.85 ± 0.03	2.34 ± 0.10	0.20 ± 0.023
48 hs	Basal	820 ± 18.14	140 ± 3.66	1.12 ± 0.07	2.89 ± 0.09	0.23 ± 0.008
	Met-Met	984 ± 29.86	131 ± 5.48	0.95 ± 0.03	2.61 ± 0.08	0.23 ± 0.021
	DL-Met	944 ± 32.90	129 ± 4.71	0.81 ± 0.02	2.44 ± 0.09	0.20 ± 0.015
Main Effects						
Stress periods	0 hs	795^{b} ± 18.03	134 ± 2.14	1.14^{a} ± 0.03	2.88^{a} ± 0.08	0.26^{a} ± 0.009
	24 hs	898^{a} ± 29.86	133 ± 3.04	1.03^{b} ± 0.04	2.65^{ab} ± 0.12	0.24^{ab} ± 0.011
	48 hs	928^{a} ± 23.63	132 ± 3.19	0.88^{b} ± 0.03	2.54^{b} ± 0.08	0.21^{b} ± 0.012
Diets	Basal	791^{b} ± 23.65	134 ± 2.92	1.07 ± 0.04	2.87 ± 0.08	0.27^{a} ± 0.011
	Met-Met	914^{a} ± 26.02	132 ± 2.86	1.01 ± 0.04	2.56 ± 0.14	0.22^{b} ± 0.011
	DL-Met	916^{a} ± 22.60	133 ± 2.77	0.96 ± 0.04	2.64 ± 0.07	0.22^{b} ± 0.009
Probabilities						
Stress periods		<.0001	0.8514	<.0001	0.0564	0.0078
Diets		<.0001	0.8719	0.0962	0.0731	0.0040
Interaction		0.4653	0.6509	0.7094	0.6725	0.9424

[1] Mean followed by different letters in the column differ from each other by the Tukey test

For intestinal morphology data, there was no significant effect on the villus height of the duodenum and jejunum. However, periods of 24 and 48 hours of stress caused lower villus in the ileum (P=0.019), compared to non-stressed birds (Table 4). Stress periods also affected the villus width of the duodenum, and birds that did not suffer stress had thicker villus (P=0.006) (Table 5).

Table 4. Effects of different methionine sources of and periods of heat stress on the villus height of the duodenum, jejunum and ileum of 21 days old broilers.

		Duodenum (µm)	Jejunum (µm)	Ileum (µm)
0 hs	Basal	1630 ± 20.96	862 ± 14.04	598 ± 11.16
	Met-Met	1555 ± 23.95	800 ± 15.05	614 ± 9.01
	DL-Met	1659 ± 38.57	812 ± 14.13	695 ± 18.92
24 hs	Basal	1466 ± 24.30	954 ± 15.70	549 ± 15.97
	Met-Met	1686 ± 44.36	883± 15.60	556 ± 9.93
	DL-Met	1631 ± 29.82	813± 29.58	532 ± 15.92
48 hs	Basal	1584 ± 25.76	896± 15.34	520 ± 7.74
	Met-Met	1560 ± 20.62	849 ± 19.16	549 ± 10.76
	DL-Met	1601± 41.34	748 ± 12.93	546 ± 17.15
Main Effects				
Stress periods	0 hs	1608 ± 15.99	827± 8.47	636[a] ± 7.96
	24 hs	1586 ± 19.93	888± 12.32	541[b] ± 8.31
	48 hs	1581± 16.80	831 ± 10.07	538 [b] ± 6.59
Diets	Basal	1556 ± 14.54	904 ± 9.00	555 ± 7.00
	Met-Met	1595± 17.48	846 ± 9.84	574 ± 6.02
	DL-Met	1628 ± 21.22	791± 11.87	593 ± 11.24
Probabilities				
Stress periods		0.8125	0.4089	0.0188
Diets		0.9831	0.0793	0.7663
Interaction		0.6321	0.8716	0.7220

[1] Mean followed by different letters in the column differ from each other by Tukey test.

Table 5. Effects of different methionine sources of and periods of heat stress on the villus width of the duodenum, jejunum and ileum of 21 days old broilers.

		Duodenum (µm)	Jejunum (µm)	Ileum (µm)
0 hs	Basal	162 ± 6.00	125 ± 5.16	110 ± 3.00
	Met-Met	129 ± 5.40	108 ± 4.25	112 ± 4.02
	DL-Met	109 ± 2.74	87 ± 2.30	114 ± 18.82
24 hs	Basal	97 ± 1.51	101 ± 4.23	93 ± 1.83
	Met-Met	106 ± 2.10	85 ± 2.57	118 ± 4.37
	DL-Met	109 ± 2.26	84 ± 1.88	107 ± 3.19
48 hs	Basal	114 ± 2.41	95 ± 2.53	104 ± 2.93
	Met-Met	116 ± 4.52	88 ± 1.73	112 ± 2.93
	DL-Met	97 ± 1.40	89 ± 1.49	101 ± 3.90
Main Effects				
Stress periods	0 hs	134[a] ± 3.32	109 ± 2.65	112 ± 2.17
	24 hs	103[b] ± 1.16	91 ± 1.91	106 ± 2.00
	48 hs	110[b] ± 1.92	91 ± 1.15	106 ± 1.86
Diets	Basal	122 ± 2.63	108 ± 2.59	103 ± 1.62
	Met-Met	117 ± 2.61	94 ± 1.85	114 ± 2.18
	DL-Met	105 ± 1.29	87 ± 1.11	108 ± 2.19
Probabilities				
Stress periods		0.0061	0.0944	0.7659
Diets		0.2314	0.1494	0.3281
Interaction		0.1352	0.5318	0.7567

[1] Means followed by different letters in the column differ from each other by Tukey test.

For crypt depth, there was interaction between stress periods and diets (P=0.011), in the stress-free period, birds receiving supplemented diets with methionine from both sources had deeper crypts and the birds that received a basal diet, presented deeper crypts after 24 hours of stress compared to the stress-free period. The jejunum (P=0.0001) and ileum (P<0.0001) of birds that did not suffer stress, and the jejunum of those that went through 24 hours of stress presented deeper crypts (Table 6).

Table 6. Effects of different methionine sources of and periods of heat stress on the crypt depth of the duodenum, jejunum and ileum of 21 days old broilers.

		Duodenum (µm)	Jejunum (µm)	Ileum (µm)
0 hs	Basal	145[b] ± 4.16	113 ± 3.31	105 ± 3.01
	Met-Met	184[a] ± 5.44	117 ± 2.66	103 ± 2.66
	DL-Met	173[a] ± 4.66	112 ± 3.32	100 ± 1.89
24 hs	Basal	191[a] ± 4.23	112 ± 3.48	69 ± 2.47
	Met-Met	157[ab] ± 4.51	95 ± 2.59	79 ± 1.99
	DL-Met	149[ab] ± 3.57	90 ± 3.72	79 ± 4.60
48 hs	Basal	156[ab] ± 3.64	77 ± 2.72	68 ± 2.12
	Met-Met	128[b] ± 3.52	68 ± 1.37	70 ± 1.78
	DL-Met	132[b] ± 4.33	69 ± 2.26	77 ± 3.48
Main Effects				
Stress periods	0 hs	168[a] ± 3.05	114[a] ± 1.82	102[a] ± 1.51
	24 hs	167[a] ± 2.67	100[a] ± 1.98	76[b] ± 1.92
	48 hs	139[b] ± 2.33	71[b] ± 1.28	71[b] ± 1.38
Diets	Basal	165 ± 2.61	102 ± 2.13	81 ± 1.83
	Met-Met	156 ± 3.02	94 ± 1.85	84 ± 1.56
	DL-Met	150 ± 1.62	90 ± 2.16	86 ± 2.18
Probabilities				
Stress periods		0.0002	<.0001	<.0001
Diets		0.2431	0.1800	0.8735
Interaction		0.0011	0.6776	0.8263

[1] Means followed by different letters in the column differ from each other by Tukey test.

There was interaction between stress periods and diets for the villi:crypt ratio of the duodenum (P=0.009), in which birds fed with diets supplemented with DL-methionine when it passed by 48 hours of stress had higher values compared to Met-Met birds stress-free and basal birds passed by 24 hours of heat stress. As for jejunum (P=0.0001) and ileum (P=0.020), there was an isolated effect of stress periods, and stress time increased the villi:crypt ratio for jejunum and ileum, birds that went through 48 hours of stress presented higher villi:crypt ratio compared to the one that were not stressed (Table 7).

Table 7. Effects of different methionine sources and periods of heat stress on the villi:crypt ratio of the duodenum, jejunum and ileum of 21 days old broilers.

		Duodenum	Jejunum	Ileum
0 hs	Basal	11.37^{ab}±0.70	7.92±0.79	5.89±0.25
	Met-Met	8.58^{b}±0.43	6.39±0.66	6.03±0.19
	DL-Met	9.35^{ab}±1.32	7.55±0.98	6.97±0.77
24 hs	Basal	7.70^{b}±0.36	8.64±0.44	7.95±0.49
	Met-Met	10.97^{ab}±1.54	9.35±0.28	7.15±0.54
	DL-Met	11.07^{ab}±0.40	8.94±0.58	6.93±0.81
48 hs	Basal	10.24^{ab}±0.74	11.54±0.88	7.94±0.81
	Met-Met	12.35^{ab}±0.66	9.92±0.92	7.99±0.58
	DL-Met	13.98^{a}±1.75	11.00±0.61	7.11±0.50
Main Effects				
Stress periods	0 horas	9.69^{b}±2.20	7.27^{c} ±1.91	6.28^{b} ±1.09
	24 horas	9.69^{b}±2.69	8.98^{b} ±1.01	7.38^{ab} ±1.28
	48 horas	11.97^{a}±2.60	11.52^{a} ±1.91	7.80^{a} ±1.54
Diets	Basal	9.68±2.09	9.37±2.32	7.30±1.65
	Met-Met	10.62±2.62	9.22±2.80	7.05±1.34
	DL-Met	11.30±3.30	9.16±2.13	7.00±1.39
Probabilities				
Stress periods		0.0053	0.0001	0.0201
Diets		0.1204	0.9378	0.8642
Interaction		0.0091	0.4396	0.3855

[1] Means followed by different letters in the column differ from each other by Tukey test.

For gene expression, the heat stress period influenced the expression of the *PEPT1* transporter (P=0.015), the birds submitted to 48 hours of stress presented greater expression when compared to 0 hours of stress (Table 8). There was interaction between the diets used and the stress periods for the $Y^{+}LAT1$ (P=0.001) and $B^{0}AT1$ (P=0.0007) transporters. After the 48 hours of stress, the birds that consumed Met-Met had a higher expression of the $Y^{+}LAT1$ transporter (Table 8).

The birds that passed through 48 hours of stress presented greater $B^{0}AT1$ gene expression compared to the other in periods of stress, regardless of the diet received. In addition, when it was considered only the birds that did not suffer stress, there was a greater expression of $B^{0}AT1$ when consuming basal diet than the diets supplemented with Met-Met and DL-Met (Table 8).

Table 8. Effects of different methionine sources of and periods of heat stress on jejunum gene expression of *PEPT1*, *Y^+LAT1* and *B^0AT1* in 21 days old broilers.

		PEPT1	Y+LAT1	B^0AT1 [2]
0 hs	Basal	0.028 ± 0.006	0.00018^{b} ± 0.00006	0.047^{b} ± 0.008
	Met-Met	0.017 ± 0.002	0.00018^{b} ± 0.00006	0.020^{c} ± 0.004
	DL-Met	0.019 ± 0.004	0.00023^{b} ± 0.00007	0.019^{c} ± 0.006
24 hs	Basal	0.023 ± 0.0003	0.00012^{b} ± 0.00001	0.016^{c} ± 0.001
	Met-Met	0.040 ± 0.010	0.00012^{b} ± 0.00001	0.024^{c} ± 0.005
	DL-Met	0.040 ± 0.010	0.00020^{b} ± 0.00004	0.033^{c} ± 0.006
48 hs	Basal	0.038 ± 0.008	0.00054^{b} ± 0.00016	0.988^{a} ± 0.001
	Met-Met	0.051 ± 0.009	0.00110^{a} ± 0.00018	0.983^{a} ± 0.003
	DL-Met	0.042 ± 0.009	0.00040^{b} ± 0.00009	0.977^{a} ± 0.004
Main Effects				
Stress periods	0 hs	0.022^{b} ± 0.003	0.00019^{b} ± 0.00003	0.030^{b} ± 0.005
	24 hs	0.034^{ab} ± 0.006	0.00015^{b} ± 0.00001	0.025^{b} ± 0.003
	48 hs	0.044^{a} ± 0.005	0.00068^{a} ± 0.00011	0.983^{a} ± 0.002
Diets	Basal	0.030 ± 0.004	0.00028^{b} ± 0.00007	0.351 ± 0.120
	Met-Met	0.036 ± 0.006	0.00047^{a} ± 0.00013	0.342 ± 0.121
	DL-Met	0.034 ± 0.043	0.00028^{b} ± 0.00043	0.343 ± 0.120
Probabilities				
Stress periods		0.015	<.0001	<.0001
Diets		0.627	0.027	0.085
Interaction		0.407	0.001	0.001

[1] Means followed by different letters in the column differ from each other by Tukey test.[2]Expressed as arbitrary units (AU).

DISCUSSION

Regarding the body weight of the birds, it was observed the isolated effect of periods of stress and diet. For these results we must considered that the natural growth of birds occurs, even though the animals are suffering a period of stress. When considering the relative weight of the intestinal segments, we observed that despite this physiological growth, stress directly affected the intestinal growth, with the decrease of relative weight of the duodenum, jejunum and ileum of birds submitted to a longer stress period.

The intestine muscular growth occurred in parallel to the growth of the animal throughout its life. However, the growth of the intestinal villi happens intensely only in the first week of bird's life. Noy and Sklan (1998) characterize the peak growth of the small intestine segments (duodenum, jejunum and ileum) in broilers as between 6 and 8 d-old. Therefore, at 21 d-old, when we evaluated the intestinal morphology, the villi had stabilized their growth. In consequence, it is possible to compare the results obtained at 21 d with the results after 24 and 48 hours of heat stress. Assuming that the differences between treatments in this period occurred in function of heat stress and diet.

In relation to intestinal morphology, the villus height was affected only by heat stress periods in ileum segment. It was observed a decrease in height with increment in stress period. Some factors may affect the villus height, the smaller height may be caused by an increased rate of cell loss or a reduced rate of cell turnover, these aspects are associated with an increase in crypt cell production (Pluske et al., 1997). In this way, the behavior of the villus and crypts are always associated (Uni et al., 2001).

The intestinal epithelium is composed of a cells population that undergoes continuous renewal. The stem cells located in the crypt region are responsible for the production of enterocytes that migrate towards the villus top and are then extruded into the intestinal lumen. This movement is regulated by a number of factors, such as cytokines, growth hormones and nutrients, which are found in the intestinal lumen (Uni et al., 2001).

Often, an increase in crypt depth may compensate the reduction in villi proliferation level (Geyra et al., 2001, Brudinick, 2017). As well as this increase may result in a high production of regenerative cells for the rapid villus growth (Awad et al., 2009). In our study there was interaction between the stress period and diet for the duodenum crypt depth, in which birds that had not been exposed to stress but receiving supplementation

of Met-Met and DL-Met presented crypts deeper. Demonstrating a higher rate of cell proliferation for birds receiving methionine.

The binding of methionine to the development of intestinal villus lies in the fact that it gives rise to cysteine, an amino acid that plays a key role in cellular antioxidant function, determining cell proliferation and survival. However, it cannot be stated how the methionine can affect the availability of cysteine in epithelial cells through the transsulfuration reaction (Shoveller et al., 2006).

The methionine transsulfuration rate in the gastrointestinal tract is dependent on the need for cysteine for glutathione synthesis, due to oxidative stress associated with high metabolic activity of proliferating epithelial cells (Moran Jr, 2016). In our study, the broilers were exposed to the oxidative stress process and thus would require the transsulfuration process of methionine for of cysteine and glutathione synthesis to maintain the proliferation and survival of intestinal cells. In this way the broilers that consumed basal diet and pass through heat stress, would require more methionine, to respond to the oxidative process.

Under poor sulfur amino acid conditions, methionine metabolism is prioritized so that protein synthesis is preserved by methionine transmethylation and methionine poll is preserved by regulation of remethylation and suppression of homocysteine transsulfuration. Transsulfuration suppression contributes to the decrease of cellular cysteine and glutathione concentrations and the increase of oxidative stress, which affects intestinal growth preferentially (Bauchart-Thevret et al., 2009).

However, our study demonstrated that under heat stress conditions, the duodenum crypt depth increased for birds receiving a basal diet, and decreased its depth for birds receiving methionine supplementation from both sources. This result may have been due to the fact that the birds submitted to the basal diets, were not receiving methionine supplementation from the beginning of their life and therefore, dietary methionine deficiency could be causing a physiological stress in these animals, before the stress caused by the rise in temperature, causing a more rapid response to heat stress. The jejunum and ileum presented a decrease in crypt depth with increased heat stress, showing a decrease in cellular production in this period

The longest stress period also led to an increase in the villi:crypt ratio, this indicates an increase in the absorption area of the intestinal villi, which in this case probably occurred in response to heat stress (Gonzales-Esquerra and Leeson, 2006). It is known

that the bird under heat stress decreases food intake and intestinal metabolism, in order to dissipate heat and maintain proper body temperature. In response to this situation, the birds in this experiment increased the surface of intestinal absorption in order to capture more nutrients for maintenance.

With respect to the expression of amino acids specific transporters from the small intestine, it may be regulated by a multitude of factors, including intestinal development, genetic selection and the quantity and quality of dietary protein (Gilbert et al., 2010). As hypothesis we take into account that changes in the methionine source in dietary, being Met-Met a dipeptide, and whether or not methionine supplementation within the requirements for the species, when comparing the basal diet with the other diets, could alter the transporters expression involved with this metabolism. Based on the assertion of some researchers that peptide transport would be a faster and more efficient route compared to free amino acid uptake (Steinhardt and Adibi, 1986, Gilbert et al., 2008).

When we analyzing the expression of *PEPT1* peptide transporter and B^0AT1 neutral amino acid transporter, we did not observe significant differences between the diets. However, we must consider that these carriers, despite possessing high affinity for the amino acid methionine, are not exclusive to the transport it. The PEPT1 transporter may carry some pharmaceutical compounds characterized as peptideomimetics, participating in their absorption and can affect their therapeutic characteristics. Some of these drugs are cephalosporins, penicillins, aminopeptidases, acyclovir, ganciclovir and angiotensin converting enzyme inhibitors (Brodin et al., 2002; Steffansen et al., 2004). And the B^0AT1 is capable of carrying all neutral amino acids, however, has variable affinity for amino acids, demonstrating an order of preference for them: Met-Leu-Ile-Val> Gln-Asn-Phe-Cys-Ala> Ser-Gly-Tyr-Thr His-Pro> Trp-Lys (Broer, 2008).

However, the B^0AT1 transporter showed interaction between the stress period and the diet, in which independent of the methionine source, gave a transport expression 30 times more transporter in the period of 48 hours of stress. Similarly, there was isolated stress time effect for the *PEPT1* transporter, being the highest expression with 48 hours of stress. This effect is very similar to that presented by Thamotharan et al. (1999) that studied the intestinal *PEPT1* expression in rats submitted to fasting of 24 hours, in which there was a great increase in the expression of this transporter, being this increase considered pre-translational due to its magnitude. In our study, although the birds were not fasting, they greatly reduced their feed intake when subjected to heat stress.

When we observe the transporters expression in the stress-free period, we see that the *B^0AT1* transporter is more expressed for birds receiving a basal diet compared to those supplemented with DL-Met and Met-Met. The diets used differ only in relation to methionine. This result probably stems from the fact that the lower amount of methionine in diet leads the bird to a greater transporters expression, in order to absorb the greater amount of methionine present in the intestinal lumen to meet their requirements.

In relation to the *Y^+LAT1* transporter expression, located in the basolateral membrane of the enterocyte, we observed higher expression in the jejunum of the animals submitted to a diet supplemented with Met-Met, after the stress of 48 hours. The *Y^+LAT1* transporter has as a characteristic the transport of free amino acids in the basolateral membrane of the enterocytes into the bloodstream.

Supported by this idea, the greater expression of this gene would be given if there were a greater amount of free amino acids for this transport. Comparing the diet supplemented with Met-Met to the others, the basal diet would have less amount of amino acid available, and the diet supplemented with DL-Met would provide the same amount of amino acid. Considering that *Y^+LAT1* is not an exclusive transporter of methionine and that there is no evidence of breakdown of dipeptides into free amino acids within the enterocyte, it is possible that the use of Met-Met has allowed a greater passage of other neutral and cationic amino acids.

For a long time the di and tripeptides transport through the basolateral membrane was not recognized, but studies confirm the occurrence of this passage, and there is evidence that the 112 kDa protein may be responsible for this transport (Sawada et al., 2001; Shepherd et al., 2002). However, in our work, we observed that birds fed diets supplemented with Met-Met within the requirements for the species, submitted 48 hours of heat stress presented greater expression of the *Y^+LAT1* transporter, responsible for the transport of free amino acids by the basolateral membrane.

The result in question would raise hypothesis that birds fed with diets supplemented with Met-Met could in the long term present some advantage of performance in relation to those that received other diets if they were submitted to situations of heat stress. Mitchell and Carlisle (1992), showed that heat stress caused higher absorption of L-methionine in the jejunum of birds. In vitro assay carried out by Dibner et al. (1992) determined that the flow of DL-2-hydroxy-4-methylthiobutanoic acid was higher in

intestinal segments prepared from heat-stressed birds, compared to those from birds raised in thermoneutral environment.

Garriga et al. (2006) showed an increase in the *SGLT-1* (responsible for the sodium-glucose transport) expression in the jejunum of broilers subjected to 14 days of heat stress. Suggesting that the animals developed a compensatory physiological response that promoted the glucose absorption as a way to guarantee an energy source for the animal organism. Sun et al. (2015) when studying the mRNA expression of the *SGLT-1*, *PEPT1* and some amino acid transporters (*Y^+LAT1*, *CAT1* and *R-BAT*), did not observe significant differences in the jejunum of birds subjected to heat stress.

It is still unclear in the literature how long birds expend to develop a compensatory response against environmental stress. However, in our study we realized that 48 hours were enough in order to produce significant result to gene expression of *PEPT1*, *Y^+LAT1* and *B^0AT1*. Evaluations in the expression of other genes and an extended time of experiment could be enlightening to draw conclusions about these physiological characteristics

CONCLUSION

The results of intestinal morphology evidenced the importance of methionine supplementation in the diet of broilers from 1 to 21 d-old and the negative effects caused by heat stress. While the gene expression of the amino acid transporters suggests that the main route of absorption of the mono or dipeptide methionine is through the free amino acid transporters.

REFERENCES

Awad W. A., K. Ghareeb, S. Abdel-Raheem, and J. Böhm. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult Sci. 88:49-55.

Bauchart-Thevret, C., B. Stoll, S. Chacko, and D.G. Burrin. 2009. Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. Am J Physiol Endocrinol Metab. 296: E1239-E1250.

Berg, J. M., J. L. Tymoczko, and L. Stryer. 2002. Biochemistry. 5th ed. W. H. Freeman and Company, New York, NY.

Berri, C., E. Le Bihan-Duval, M. Debut, V. Santé-Lhoutellier, E. Baéza, V. Gigaud, Y. Jégo and M. J. Duclos. 2007. Consequence of muscle hypertrophy on Pectoralis major characteristics and breast meat quality of broiler chickens. J.Anim. Sci. 85-2005-11.

Brodin, B., C.U. Nielsen, B. Steffansen, and S. Frokaer. 2002. Transport of Peptidomimetic Drugs by the Intestinal Di/tri-peptide Transporter, PEPT1. Pharmacol. Toxicol. 90:285-296.

Broer, S. 2008. Amino acid transport across mammalian intestinal and renal epithelia. Physiol. Rev. 88:249-286.

Brosnan, J.T. 2003. Interorgan Amino Acid Transport and its Regulation. J. Nutr. 133: 2068S-2072S.

Brudnicki, A., W. Brudnicki, R. Szymeczko, M. Bednarczyk, D. Pietruszyńska, and K. Kirkiłło-Stacewicz. 2017. Histo-morphometric adaptation in the small intestine of broiler chicken, after embryonic exposure to α– galactosides. J. Anim. Plant. Sci. 27(4): 1075-1082.

Daniel, H. 2004. Molecular and integrative physiology of intestinal peptide transport. Annu. Rev. Physiol. 66:361-384.

Dibner, J. J., and C. D. Knight. 1984. Conversion of 2-hydroxy4-(methylthio) butanoic acid to l-methionine in the chick: A stereospecific pathway. J. Nutr. 114:1716-1723.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). 2015. Scientific Opinion on the safety and efficacy of DL-methionyl-DL-methionine for all aquatic animal species. EFSA J. 13(2):4012-4026.

Garriga C., R. R. Hunter, C. Amat, J. M. Planas, M. A. Mitchell, and M. Moreto. 2006. Heat stress increases apical glucose transport in the chicken jejunum. Am. J. Physiol. Regul. Integr. Comp. Physio. 290: R195-R201.

Geyra, A., Z. Uni, and D. Sklan. 2001. The effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick. Br. J. Nutr. 86: 53-61.

Gilbert, E. R., H. Li, D. A. Emmerson, K. E. Webb Jr., and E. A. Wong. 2007. Developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. Poult. Sci. 86:1739-1753.

Gilbert, E. R., E. A. Wong, and K. E. Webb Jr. 2008. Board-Invited Rewiew: Peptide absorption and utilization: Implications for animal nutrition and health. J. Anim. Sci. 86:2135-2155.

Gilbert E. R., H. Li, D.A. Emmerson, K. E. Webb Jr., and E. A. Wong. 2010. Dietary protein composition influences abundance of peptide and amino acid transporter messenger ribonucleic acid in the small intestine of 2 lines of broiler chicks. Poult. Sci. 89:1663-1676.

Gonzales-Esquerra, R., and S. Leeson. 2006. Physiological and metabolic responses of broilers to heat stress - implications for protein and amino acid nutrition. Worlds Poult. Sci. J. 62: 282-295.

Hyde, R., P.M. Taylor, and H. S. Hundal. 2003. Amino acid transporters: roles in amino acid sensing and signalling in animal cells. Biochem. J. 373:1-18.

Martín-Venegas R., P. A. Geraert, and R. Ferrer. 2006. Conversion of the Methionine Hydroxy Analogue DL-2-Hydroxy-(4-Methylthio) Butanoic Acid to Sulfur-Containing Amino Acids in the Chicken Small Intestine. Poult Sci. 85:1932-1938.

Mitchell, M.A., and A. Carlisle. 1992. The effects of chronic exposure to elevated environmental temperature on intestinal morphology and nutrient absorption in the domestic fowl (Gallus domesticus). Comp. Biochem. Physiol A. 101: 137-142.

Moran Jr, E.T. 2007. Nutrition of the developing embryo and hatchling. Poult. Sci., 86: 1-7.

Moran Jr, E.T. 2016. Nutrients central to maintaining intestinal absorptive efficiency and barrier integrity with fowl. Poult. Sci. 0:1-16.

Noy, Y. and D. Sklan. 1998. Metabolic responses to early nutrition. J. Appl. Poutry Res. 7:437-431.

Pluske J. R., I. David, J. Hampson, and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Sci. 51: 215-236.

Rostagno, H.S., L.F.T Albino, J.L. Donzele, P.C. Gomes, R.F., Oliveira, D.C. Lopes, A.S. Ferreira, and, S.L.T. Barreto. 2011. Brazilian tables for poultry and swine: Composition of feedstuffs and nutritional requirements. Pages 225. Viçosa, MG, Brasil.

SAS Institute.2011.SAS Proprietary Software Release 9.2. SAS Inst. Inc., Cary, NC.

Sawada, K., T. Terada, H. Saito, and K. Inui. 2001. Distinct transport characteristics of basolateral peptide transporters between MDCK and Caco-2 cells. Pflügers Arch.-Eur. J. Physiol. 443:31–37.

Shepherd, E.J. N. Lister, J.A. Affleck, J.R. Bronk, G.L. Kellett, I.D. Collier, P.D. Bailey, and C.A.R. Boyd. 2002. Identification of a candidate membrane protein for the basolateral peptide transporter of rat small intestine. Biochem. Biophys. Res. Comm. 296:918-922.

Shoveller A. K., B. Stoll, R. O. Ball, and D. G. Burrin. 2006. Nutritional and Functional Importance of Intestinal Sulfur Amino Acid Metabolism. J. Nutr. 1609-1612.

Sklan, D., and Y. Noy. 2000. Hydrolysis and absorption in the small intestines of posthatch chicks. Poult. Sci. 79:1306–1310.

Smith, M. E., and D. G. Morton. 2010. The digestive system: basic science and clinical. 2nd ed. Churchill Livingstone, Edinburgh, New York.

Steinhardt, H. J., and S. A. Adibi. 1986. Kinetics and characteristics of absorption from an equimolar mixture of 12 glycyl-dipeptides in human jejunum. Gastroenterol. 90:577-582.

Steffansen, B., C. Nielsen, B. Brodin, A. Eriksson, R. Anderson, and S. Frokjaer. 2004. Intestinal solute carriers: An overview of trends and strategies for improving oral drug absorption. Eur. J. Pharm. Sci. 21:3-16.

Sun, X., H. Zhang, A. Sheikhahmad, Y. Wang, H. Jiao, H. Lin, Z. Song. 2015. Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens (Gallus gallus domesticus). Int. J. Biometeorol. 59:127-135.

Thamotharan, M., S.Z. Bawani, X. Zhou, S.A. Adibi. 1999. Functional and molecular expression of intestinal oligopeptide transporter (Pept-1) after a brief fast. Metabolism. 48: 681–684.

Uni, Z., 1998. Identification and isolation of chicken sucrose isomaltase cDNA sequence. Poult. Sci. 77:140-144.

Uni Z., O. Gal-Garber, A. Geyra, D. Sklan, and S. Yahav. 2001. Changes in growth and function of chick small intestine epithelium due to early thermal conditioning. Poult. Sci. 80:438–445.

Wu, C., S.A. Khan, and A.J. Lange. 2005. Regulation of glycolysis-role of insulin. Exp. Gerontol. 40:894-899.

Zeng, P.L., X.G. Li, X.Q. Wang, D.X. Zhang, G. Shu, and Q. B. Luo. 2011. The relationship between gene expression of cationic and neutral amino acid transporters in the small intestine of chick embryos and chick breed, development, sex, and egg amino acid concentration. Poult. Sci. 90:2548–2556.

Zhang S., B. Saremi, E. R. Gilbert, and E. A. Wong. 2016. Physiological and biochemical aspects of methionine isomers and a methionine analogue in broilers. Poult. Sci. 0:1–15.

V. CONSIDERAÇÕES FINAIS

De acordo com este estudo, a suplementação de metionina na formulação das dietas de frangos de corte de 1 a 21 dias de idade é fundamental, pois interfere no desempenho, desenvolvimento muscular e intestinal, independente da fonte do aminoácido utilizada. O estresse por calor, demonstrou danos hepáticos e intestinais efetivos, no entanto, estudos mais aprofundados devem ser realizados.

Assim, para resultados mais adequados seriam necessários que fossem realizados três ensaios simultâneos em câmaras climáticas, cada um submetido a um dos períodos de estresse testado, para que os animais pudessem ser abatidos todos com a mesma idade, eliminando assim a questão do desenvolvimento natural do animal, que no caso dos frangos de corte ocorre de forma acelerada.

Em relação a expressão gênica dos genes *PEPT1, $Y^{+}LAT1$ e $B^{0}AT1$,* o prolongamento do período experimental, poderia responder, se os resultados identificados trariam consequências no desempenho e na constituição muscular do animal. Outro fator importante a ser considerado, seria a expressão de genes ligados ao metabolismo de glicose, por exemplo, principalmente ao levar em conta os resultados apresentados comparando os períodos de estresse. Alguns estudos relatam que a falta de um nutriente na dieta, faz com que os animais ativem uma resposta compensatória, levando a estimulação do metabolismo de outros nutrientes com o intuito de compensar a falta de um determinado composto.

A princípio esta tese seria composta por mais um experimento, que investigaria a fundo o transportador de di e tripeptídeos *PEPT1*. Entretanto, os resultados apresentados, em relação a expressão desse transportador, derrubaram nossa hipótese inicial de que o

dipeptídeo DL-Metionil-DL-Metionina, poderia ser mais ativo em relação a esse transportador comparado a outras fontes de metionina. Contudo, a expressão dos transportadores *B^0AT1* aos 21 dias e *Y^+LAT1* às 48 horas de estresse, nos instigam a realizar novos estudos em relação ao outros transportadores de aminoácidos localizados principalmente na membrana basolateral do enterócito.