UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CAMPUS DE JABOTICABAL

AMINOÁCIDOS MARCADOS APLICADOS AOS ESTUDOS DE NUTRIÇÃO E FISIOLOGIA EM FRANGOS DE CORTE

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Médico veterinário

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UNIVERSIDADE ESTADUAL PAULISTA - UNESP

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AMINOÁCIDOS MARCADOS APLICADOS AOS ESTUDOS DE NUTRIÇÃO E FISIOLOGIA EM FRANGOS DE CORTE

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutorado em Zootecnia.

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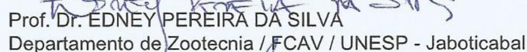

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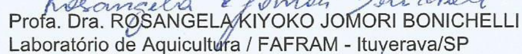

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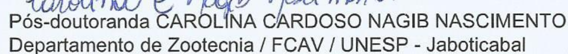

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Rafael Massami Suzuki, filho de Edson Coiti Suzuki e Marisa Tobace Suzuki, nascido no dia 6 de fevereiro de 1990 em Barretos, São Paulo. Ingressou no curso de graduação em Medicina Veterinária em 2009 na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista – UNESP - Campus de Jaboticabal, São Paulo – finalizando em 2014. Neste período, foi bolsista de iniciação científica pelo CNPq no período de 1 de novembro de 2011 a 31 de julho de 2012, sob orientação da profª. Drª. Nilva Kazue Sakomura. A seguir, durante o período de 1 de setembro de 2012 a 31 de agosto de 2013, foi bolsista de iniciação científica pela FAPESP sob orientação do prof. Dr. Luciano Hauschild. Iniciou o curso de Mestrado em Zootecnia no dia 10 de março de 2014 na mesma instituição e sob a orientação da profª. Drª. Nilva Kazue Sakomura, defendendo sua dissertação no dia 25 de fevereiro de 2016. No dia 7 de março de 2016, teve início ao curso de Doutorado em Zootecnia pela Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista - campus de Jaboticabal sob orientação da profª. Drª. Nilva Kazue Sakomura, obtendo bolsa do CNPq. Realizou um doutorado sanduíche através do programa de doutorado sanduíche no exterior (PDSE) financiado pela Capes no Institut Nationale de la Recherche Agronomique, unidade Pegase localizada em Saint-Gilles/França sob orientação do Dr. Jaap Van Milgen e Drª Nathalie Le Floc'h. Finalizou o curso de Doutorado em Zootecnia com a defesa de sua tese no dia 22 de outubro de 2019.

“I've got another confession to make

I'm your fool

Everyone's got their chains to break

Holding you

Were you born to resist

Or be abused?

Is someone getting the best

The best, the best, the best of you?

Is someone getting the best

The best, the best, the best of you?

Or are you gone and onto someone new?”

(Best of you – Foo fighters)

Dedico...

À Deus, onipotente, onipresente e onisciente.

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CERTIFICADO

Certificamos que o Protocolo nº 9999/14 do trabalho de pesquisa intitulado **"Modelagem da produção e das exigências nutricionais de aves e peixes - Metodologia para determinar a eficiência de utilização da proteína e de aminoácidos essenciais com uso do nitrogênio ^{15}N"**, sob a responsabilidade da Prof.ª Dr.ª Nilva Kazue Sakomura está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 07 de julho de 2014.

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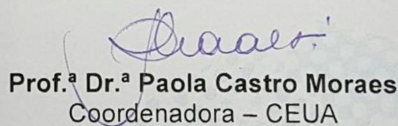

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CAPÍTULO 1

AMINOÁCIDOS MARCADOS APLICADOS AOS ESTUDOS DE NUTRIÇÃO E FISIOLOGIA DE FRANGOS DE CORTE

RESUMO: A evolução das análises, considerando especificamente a nutrição e fisiologia de frangos de corte, permitiram rastrear nutrientes a níveis nucleares, permitindo que novas metodologias fossem desenvolvidas e novos objetivos alcançados. Algumas das análises que merecem destaque considerando estudos voltados a exigências de aminoácidos e metabolismo proteico foram: determinação de nitrogênio total/proteína, análise da composição aminoacídica e análise isotópica, sendo que a associação destas análises permite um potencial ainda maior. O uso de compostos enriquecidos é uma excelente ferramenta para estudos fisiológicos, uma vez que as respostas obtidas são inerentes ao composto de interesse, sendo possível o rastreamento do isótopo. Contudo, esta tecnologia ainda apresenta um alto custo, parte devido à baixa produção destes produtos e devido o sigilo da tecnologia da produção de tais compostos. Deste modo, no **capítulo 2** foi padronizado uma metodologia para avaliar a adição de aminoácido marcado na dieta de aves e sua incorporação nos tecidos. A seguir, no **capítulo 3** é apresentado um estudo com o objetivo de fracionar a utilização dos aminoácidos sulfurados com a utilização de metionina e cistina marcadas, avaliando-se diferentes proporções de ambos aminoácidos e diferentes fontes de metionina. Por fim, no **capitulo 4** foi avaliado o efeito da idade sob a incorporação da L-(^{15}N) treonina em diferentes tecidos de frangos de corte. Portanto, os objetivos desta tese foram a padronização e aplicação de uma técnica com o uso de isótopos marcados para elucidar questões relacionadas a nutrição e fisiologia em frangos de corte.

PALAVRAS-CHAVE: Isótopos, metodologia, proteína.

LABELED AMINO ACIDS APPLIED TO NUTRITION AND PHYSIOLOGY STUDIES FOR BROILER CHICKENS

ABSTRACT: The evolution of the analyzes, specifically regarding nutrition and physiology of broiler chickens, allowed to track nutrients to nuclear levels, resulting in the development of new methodologies and new objectives were achieved. Some of the analyzes that deserve to be highlighted considering amino acid requirements and protein metabolism studies were: total nitrogen/protein determination, amino acid composition analysis and isotopic analysis, and the association of these analyzes allows an even greater potential. The use of enriched compounds is an excellent tool for physiological studies, since the responses obtained are inherent to the compound of interest, and it is possible to track the tracer. However, the cost of such technology is still high, partly due to the low production of these products and also due to the secrecy of the technology of the production of such compounds. Thus, in **chapter 2**, a methodology was standardized in order to evaluate the addition of labeled amino acid in the broiler diet and its incorporation into tissues. In addition, **chapter 3** presents a study in order to fractionate the use of sulfur amino acids using labeled methionine and cystine, and evaluating different proportions of both amino acids and different sources of methionine. Finally, in **chapter 4**, the effect of age on the incorporation of L-(^{15}N) threonine into different broiler tissues was evaluated. Therefore, the objectives of this thesis were the standardization and application of a technique using labeled compounds to elucidate nutrition and physiology issues in broilers.

KEY-WORDS: Methodology, isotopes, protein.

Considerações iniciais

1. INTRODUÇÃO

É possível observar que um dos objetivos mais almejados dentro da avicultura é o aumento da produção animal por meio de parâmetros de desempenho como um acréscimo no ganho de peso, uma redução na conversão alimentar, ou então em um aumento na produção de ovos. Porém, muitas vezes, aspectos fisiológicos não recebem tanta ênfase, sendo que a expansão dos conhecimentos relacionados aos processos, atividades e fenômenos fisiológicos das aves fornece a base racional para a compreensão dos mecanismos das aves para obtenção de melhores desempenhos produtivos.

A nutrição aplicada a avicultura moderna necessita de um dinamismo muito intenso, uma vez que são animais de ciclo curto e sua genética evolui rapidamente em relação as demais espécies, resultando em uma alta necessidade de pesquisas voltadas a esta área. O desenvolvimento de novos equipamentos, análises e tecnologias permite ampliar as fronteiras atuais do conhecimento, permitindo também uma maior possibilidade de estudos. Considerando a evolução de equipamentos que permitem análises cada vez mais sofisticadas, estas atualmente estão em uma escala molecular ou até mesmo atômico.

Alguns dos equipamentos que merecem destaque para aplicação na nutrição e fisiologia animal são: a Cromatografia Líquida de Alta Performance (High-Performance Liquid Chromatography ou HPLC), a Cromatografia Gasosa acoplada a um Espectrômetro de Massa de Razão Isotópica (Gas Chromatography-Isotope Ratio Mass Spectrometer ou GC-IRMS), assim como a Cromatografia Liquída Acoplada a um Espectrômetro de Massa de Razão Isotópica (Liquid Chromatography-Isotope Ratio Mass Spectrometer ou LC-IRMS). Deste modo, foi possível determinar e quantificar nutrientes e/ou compostos metabólicos, sendo um grande avanço nos estudos relacionados a bioquímica, fisiologia, assim como a aplicação destes resultados na nutrição animal.

Dentre os principais aminoácidos, os sulfurados (metionina + cistina) e a treonina são focos de grande volume de estudos que envolvem a nutrição animal, uma vez que além da síntese proteica que é a função primária dos aminoácidos, apresentam outras funções biológicas essenciais como participação no sistema imune e digestório, sinalizadores celulares, entre outras.

Deste modo, os objetivos desta tese foram a padronização e aplicação de uma técnica com o uso de isótopos marcados para elucidar questões relacionadas a nutrição e fisiologia em frangos de corte.

2. Revisão de literatura

2.1 Análises

2.1.1. Determinação da proteína

A análise de determinação do nitrogênio total foi proposta por Johann Kjeldahl em 1883, até hoje é bastante utilizada e sofreu poucas modificações ao longo dos anos devido a confiabilidade dos seus resultados (VOGEL, 1992). Esta técnica possibilita a determinação indireta de proteínas em várias amostras biológicas (YASUHARA e NOKIHARA, 2001).

Este método determina o teor de nitrogênio orgânico proveniente de proteínas, ácidos orgânicos, alcaloides, lipídeos e carboidratos nitrogenados. Desta forma, é necessário fazer uma correção em relação ao nitrogênio total para se determinar a porcentagem de proteína da amostra, sendo que para carnes este fator equivale a multiplicar o valor de nitrogênio total por 6,25, ou seja, a proteína equivale a 16% do nitrogênio total de carne. Porém, dependendo do tipo de amostra, esse fator de correção pode se alterar como demonstrado na tabela 1. Contudo, essa metodologia apresenta apenas resultados quantitativos e portanto, não é capaz de determinar a composição da proteína.

Tabela 1. Fator de correção do método de Kjeldahl para transformação de nitrogênio total para proteína em diferentes tipos de amostras

Tipo de amostra	Fator de correção
Geral	N x 6,25
Gelatina	N x 5,50
Ovos	N x 6,68
Produtos lácteos	N x 6,38
Soja	N x 6,00
Farinha de trigo	N x 5,70
Arroz	N x 5,95

Carnes	N x 6,25

Fonte: GALVANI e GAERTNER (2006)

2.1.2. Determinação da composição aminoacídica

Dentre os métodos mais utilizados para a determinação da composição aminoacídica destacam-se: cromatografia iônica, cromatografia líquida de alta eficiência (HPLC) e espectroscopia de refletância do infravermelho próximo (NIRS) (DE ARAÚJO *et al.*, 2007).

A cromatografia consiste de um método físico-químico que separa os componentes de uma mistura, sendo capaz de separar fases que estão em contato íntimo e que apresentam diferenças de velocidade de transporte quando submetidas em um líquido ou gás. A técnica foi aperfeiçoada por Hamilton e Andrews, os quais introduziram uma bomba tipo pistão, semelhante as encontradas em cromatografia líquida de alta eficiência atualmente. A análise quantitativa pela HPLC pode atingir uma precisão superior a ± 0,5%, de forma que a análise qualitativa e quantitativa é levada a um alto nível de reprodutibilidade, exatidão e precisão (COLINS, 1994).

Por outro lado, a espectroscopia do infravermelho próximo ou NIRS foi utilizada primariamente em 1976 com a finalidade de se predizer o valor nutritivo das forragens. Trata-se de um método rápido e não destrutivo, o qual foi aprovado pela “Association of Official Analytical Chemists” (AOAC) como método de mensuração de nitrogênio total. Sua tecnologia se baseia na existência de relações entre as características físicas, químicas e sensoriais de uma amostra de acordo com a absorbância e comprimentos de onda específicos da região do "infravermelho próximo" (CAMPESTRINI, 2005; BARTON II, 2002).

A associação da determinação da composição aminoacídica juntamente com a produção de aminoácidos sintéticos e com a origem e aplicação do conceito de proteína ideal, possibilitou a formulação de dietas com baixos teores de proteína bruta, de modo que a excreção nitrogenada foi reduzida significamente, mantendo-se o mesmo desempenho animal (DARI *et al.*, 2005; FERKET *et al.*, 2002).

2.1.3. Análises isotópicas

Os isótopos estáveis do carbono, hidrogênio, oxigênio, nitrogênio e enxofre, também conhecidos como bioelementos, são encontrados na natureza e sua

abundância varia de acordo com o elemento e com o isótopo, sendo que normalmente a forma predominante encontrada naturalmente é o isótopo leve. A tabela 2 contém as proporções das abundâncias naturais dos isótopos estáveis de cada um destes elementos químicos encontradas na natureza.

Tabela 2. Abundância natural (atm%) dos isótopos de H, C, N, O e S.

Isótopo estável	Abundância (atm%)	Isótopo estável	Abundância (atm%)
^{1}H	99,9844	^{16}O	99,7628
^{2}H	0,0156	^{17}O	0,0372
^{12}C	98,8890	^{18}O	0,2000
^{13}C	1,1110	^{32}S	95,0180
^{14}N	99,6340	^{33}S	0,7500
^{15}N	0,3660	^{34}S	4,2150
		^{35}S	0,0170

Fonte: Adaptado de DUCATTI (2007).

Isótopos podem ser detectados por meio de sua massa com o auxílio de um espectrômetro de massa de razão isotópica (Figura 1), pelo modo vibracional por meio da eletroscopia por ressonância magnética nuclear ou através de seu decaimento radioativo para isótopos instáveis com o uso de um cintilador (SHARMA e MISHRA, 2013).

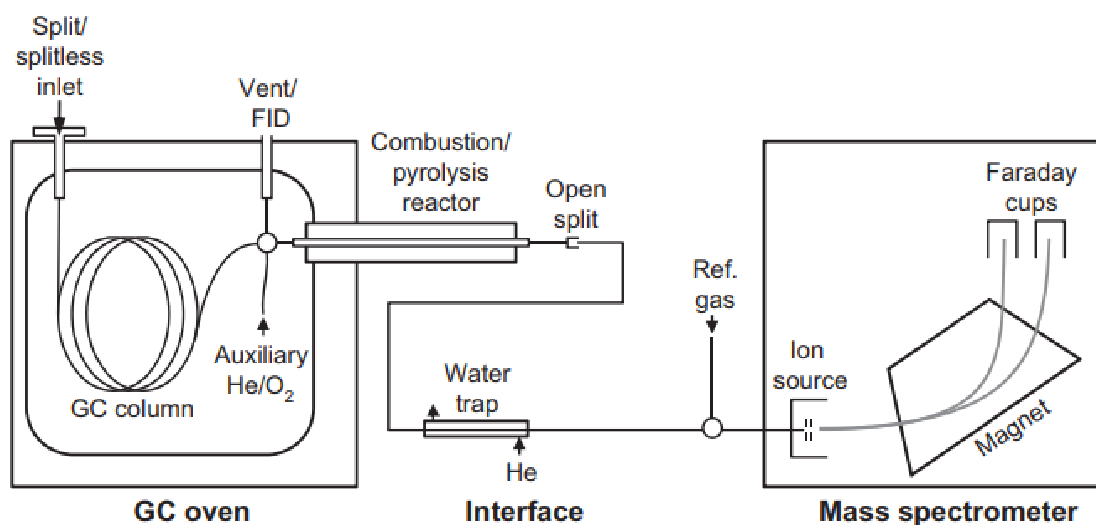

Figura 1. Esquematização de um sistema “cromatógrafo gasoso acoplado a um espectrômetro de massa de razão isotópica” ou GC-IRMS (**Fonte:** SESSIONS, 2006)

Os espectrômetros de massa de razão isotópica (Isotope Ratio Mass Spectrometer - IRMS) podem ser acoplados a outros equipamentos com a finalidade de aprimorar o tipo de análise almejada. Quando acoplado a um cromatógrafo e a um analisador elementar, esta associação de equipamentos é capaz de analisar as concentrações isotópicas das amostras. O processo inicia-se com a combustão total da amostra geralmente contida em uma cápsula resultando em diversos gases (N_2, CO_2, NO_x, SO_2, H_2O). No caso do nitrogênio, o gás de interesse mensurado é o N_2 e os demais são filtrados em uma série de *traps*. A seguir, os gases são impulsionados pelo gás hélio de alta pureza e são purificados para que reste somente N_2. Uma pequena parte das moléculas de N_2 são ionizadas e sob um campo magnético, os íons ($^{14}N^{14}N^+$; $^{14}N^{15}N^+$; e $^{15}N^{15}N^+$) são separados de acordo com suas relações massa/carga. A esquematização do processo de separação dos íons está representada pela figura 1.

Além disso, outra possível associação seria o cromatógrafo liquído acoplado a um espectrômetro de massa de razão isotópica (LC x GC/C/IRMS) o qual possibilita a análise não somente da concentração isotópica do elemento de interesse contido na amostra, mas também na detecção das concentrações isotópicas dos compostos marcados de interesse (YARNES e HERSZAGE, 2017). A figura 2 ilustra a etapa de separação dos aminoácidos na cromatografia líquida previamente a análise de sua composição isotópica no GC/G/IRMS.

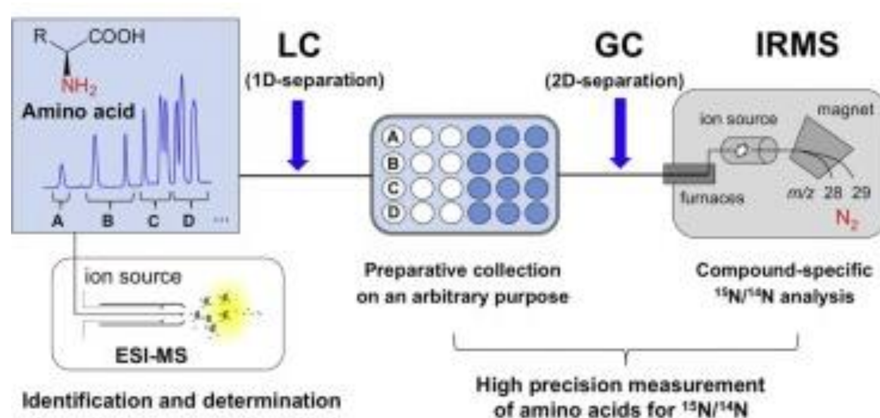

Figura 2. Esquematização de um cromatográfo líquido associado a um espectrômetro de massa de razão isotópica acoplada a um cromatógrafo gasoso ou LC x GC/G/IRMS (**Fonte:** TAKANO *et al.*, 2015)

Os resultados da abundância isotópica podem ser expressos em termos absoluto ou relativo. Quando se utiliza compostos enriquecidos, predomina a abundância absoluta que é expressa em átomos %. No caso do nitrogênio, esta concentração é obtida a partir das relações das intensidades dos feixes de íons 28 e 29. A precisão analítica é da ordem de 0,1%. Por outro lado, a terminologia delta (δ) é comumente utilizada para compostos com abundâncias isotópicas naturais e é determinada de acordo com a comparação da abundância da amostra em relação a de padrões internacionais que variam de acordo com o elemento de interesse, sendo que para o nitrogênio é adotado a razão isotópica do nitrogênio atmosférico como padrão internacional. A medida adotada para estas unidades relativas é o *per mil* (‰) por convenções de escala (DUCATTI, 2007).

Com o uso da técnica de isótopos estáveis, as análises isotópicas tomaram proporções atômicas. Uma de suas principais vantagens são suas características de traçadores biológicas, as quais permitem participar das reações bioquímicas diferentemente de marcadores. Além disso, através da análise do isótopo ou então pela análise isotópica do composto específico (Compound Specific Isotope Analysis - CSIA), há a garantia de que os resultados são referentes ao composto marcado adicionado, mesmo que o composto marcado seja metabolizado ou não. As análises de composição aminoacídica não são capazes de fazer uma diferenciação entre aminoácidos de origem dietética e endógena.

Portanto, a evolução das análises permitiram muitas possibilidade e um grande avanço nos estudos relacionados a nutrição e fisiologia. A associação destas análises poderia fornecer ainda mais possibilidades, sendo possível atingir uma gama ainda maior de objetivos.

2.2. Metabolismo proteico

2.2.1 Amino ácidos sulfurados (Metionina + Cistina)

Aminoácidos sulfurados são compostos pela: metionina (Met), cisteína, homocisteína e taurina, porém, somente as duas primeiras são incorporadas as proteínas (BROSNAN e BROSNAN, 2006), de modo que a exigência de

aminoácidos sulfurados nas formulações de rações é considerado somente a soma das exigências de metionina e cist(e)ina (Cys). Seu metabolismo é complexo envolvendo diversos compostos biológicos e reações de transaminação, desaminação, transsulfuração e remetilação (figura 3).

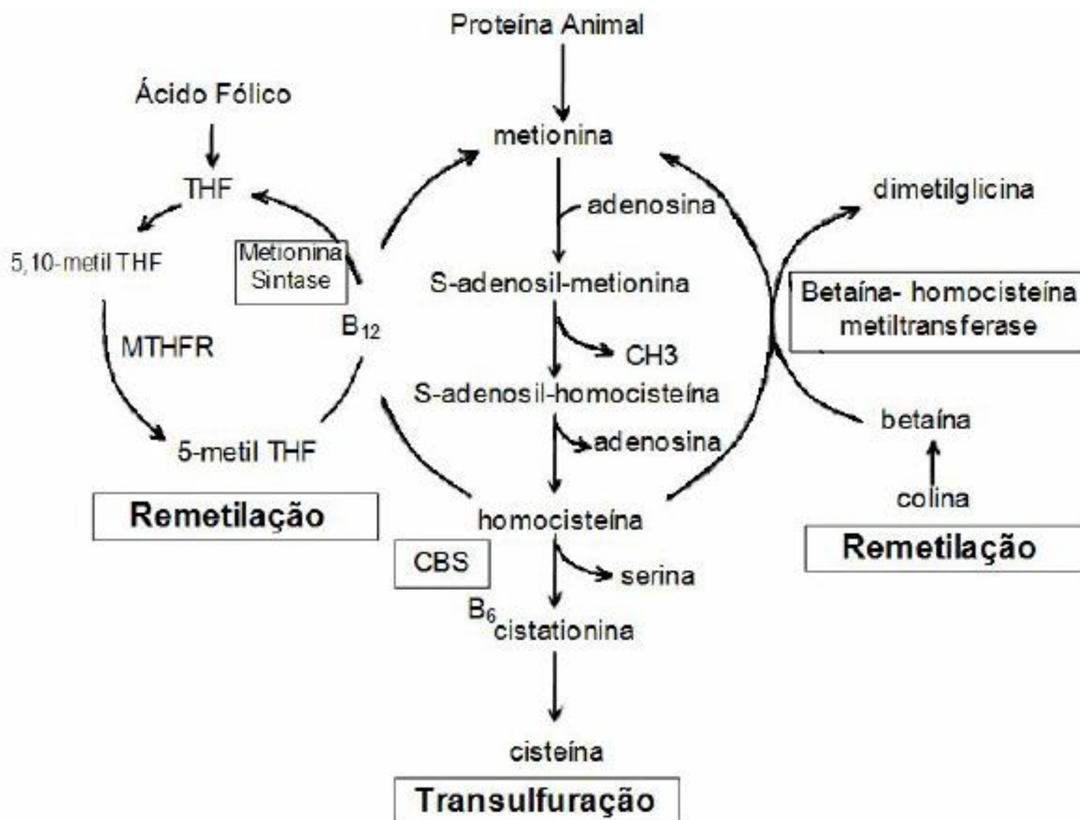

Figura 3. Metabolismo sulfurados (**Fonte:** AMORIM *et al.* (2011))

Dentre todos aminoácidos, os sulfurados considerados como Met + Cys são considerados os mais divergentes quanto às suas exigências aminoacídicas (NRC, 1994). Grande parte desta divergência se deve as inúmeras funções biológicas que esses aminoácidos desempenham, uma vez que a demanda nutricional depende das condições fisiológicas do animal (LE FLOC'H *et al.*, 2004). Suas funções além da síntese proteíca, incluem: doadores de grupos metilícos na produção de colina, creatina, carnitina e poliamina; produção de glutationa para regulação do estado oxidativo; e também são utilizados como precursores na produção de moléculas que atuam na regulação do metabolismo e funções celulares (TESSERAUD *et al.* 2009; BAKER e DILGER, 2008; HEGER *et al.*, 2002).

A associação dos requerimentos de Met e Cys, prática que é comumente adotada nas formulações de dietas, somente é possível devido a interconversão entre estes aminoácidos, na qual em uma reação irreversível de transsulfuração ocorre, de modo que a metionina é convertida a homocisteína, cistationa, e por fim cist(e)ína (TESSERAUD *et al.* 2009). Contudo, atender adequadamente aos requerimentos de aminoácidos sulfurados não é garantia de que as exigências individuais de Met e Cys também serão atendidas (HUYGHEBAERT e PACK, 1996), sendo que desta forma, a proporção entre Met e Cys irá afetar o desempenho animal, mesmo que seja fornecido uma mesma quantidade de aminoácidos sulfurados. Vários estudos objetivaram determinar qual a proporção máxima que a Cys seria capaz de ser inclusa em relação a exigência total de aminoácidos sulfurados sem que houvesse a perda de desempenho em diversas espécies (SHANNON *et al.*, 1972; CHUNG e BAKER, 1992; BAKER *et al.*, 1996). No geral, a cistina é capaz de contribuir com cerca de 50% das exigências totais de aminoácidos sulfurados de animais jovens, mas essa proporção aumenta com a idade (GRABER e BAKER, 1971). Contudo, a maior parte desses estudos são empíricos e tem como resultados o desempenho do animal, sem respostas fisiológicas que poderiam justificar tais resultados. Além disso, um grande fator que poderia contribuir para a otimização das exigências de aminoácidos sulfurados seria a determinação da taxa de interconversão entre esses aminoácidos.

Outra questão acerca dos aminoácidos sulfurados envolve as diversas possibilidades de suplementação da metionina. Considerados como primeiros aminoácidos limitantes em dietas convencionais a base de milho e soja para frangos de corte (SHEN *et al.*, 2015), a suplementação de Met é muito comum, pois a deficiência desses aminoácidos resulta em queda de ganho de peso, eficiência alimentar, além de aumento do consumo de ração, resultando em um aumento de deposição de gordura corporal (SUMMERS *et al.*, 1992; MORAN JR., 1994). As principais formas de suplementação de Met são: DL-Met, L-Met e DL-Met líquida hidróxi análoga (KALBANDE, 2009). Sabe-se que a forma isômera L é a forma ativa e utilizada na maioria dos tecidos das aves, e comparando especificamente a forma racêmica DL-Met (50% D-Met e 50% L-Met) e L-Met, é necessário a transformação da D-Met em L-Met (KALBANDE, 2009; ESTEVE-GARCIA e KHAN, 2018). Apesar de ser comprovada que a suplementação da DL-Met é tão eficaz quanto a L-Met,

ainda há divergências quanto essa eficácia em relação a diferentes idades (SHEN *et al.*, 2015).

2.2.2. Turnover proteico e treonina

O *turnover* proteico é definido como o processo dinâmico responsável pela renovação contínua das proteínas/aminoácidos, pois integra tanto a síntese e degradação dos mesmos, refletindo a velocidade com que ocorre o metabolismo do aminoácido. Todas as substâncias sejam elas orgânicas ou inorgânicos estão sujeitas ao *turnover* (WATERLOW, 2006).

Este processo depende não somente dos fatores intrínsecos referentes ao animal mas também de fatores externos (ZUANON, 2003). Deste modo, alterações na dieta, sanidade, ambiência ou idade podem ser refletidas no *turnover* proteico até que ocorra adaptação do organismo nesta condição atual. Assim, é esperado que o *turnover* proteico possa fornecer respostas inerentes à produção associadas a condição fisiológica do animal, ou então uma análise do direcionamento do aminoácido entre os diferentes tecidos e em diferentes tempos, refletindo sua utilização pela ave. Dentre as possíveis alterações fisiológicas, podemos ressaltar a diferença de idade, pois inúmeros estudos voltados a nutrição são de natureza empírica na qual se busca determinar, por exemplo, qual a inclusão de um determinado nutriente por meio de uma melhor performance animal, sem fazer alusão de explicações fisiológicas que favorecem aquela performance. Além disso, é muito comum o uso da técnica abate comparativo, na qual é analisado somente a deposição de um certo nutriente, sendo que o comportamento do metabolismo deste nutriente conforme a idade não é desconsiderada.

Em teoria, qualquer aminoácido poderia representar o *turnover* proteico, uma vez que proteínas são constituídos de aminoácidos, de forma diferentes aminoácidos gerariam resultados distintos porém proporcionalmente idênticos, pois as concentrações aminoacídicas se alteram conforme o aminoácido e o tecido (HAMM, 1981). Contudo, ainda há controvérsias quanto a questão: diferentes aminoácidos marcados representariam um mesmo *turnover* proteico? (HOFFER *et al.*, 1997).

A treonina é o terceiro aminoácido limitante em dietas convencionais a base de milho e soja para frangos de corte (DOZIER *et al.*, 2015) e participa de processos biológicos deposição de massa magra, *turnover* proteico, desenvolvimento

intestinal e das penas, formação de enzimas, colágeno, mucina, elastina e anticorpos (KIDD e KERR, 1996; KIDD, 1999; DOZIER *et al*., 2001; HORN *et al*., 2009). Sua formula molecular é $C_4H_9NO_3$ e sua degradação envolve três possíveis rotas metabólicas, sendo que as enzimas responsáveis por sua catabolização são: treonina desidratase, treonina aldolase e treonina desidrogenase (Figura 4). Apesar de não ser transaminada, o nitrogênio contido na treonina pode ser transferido a uma mólecula de glicina e consequentemente a uma serina também, por meio das duas últimas rotas metabólicas citadas (KIDD e KERR, 1996).

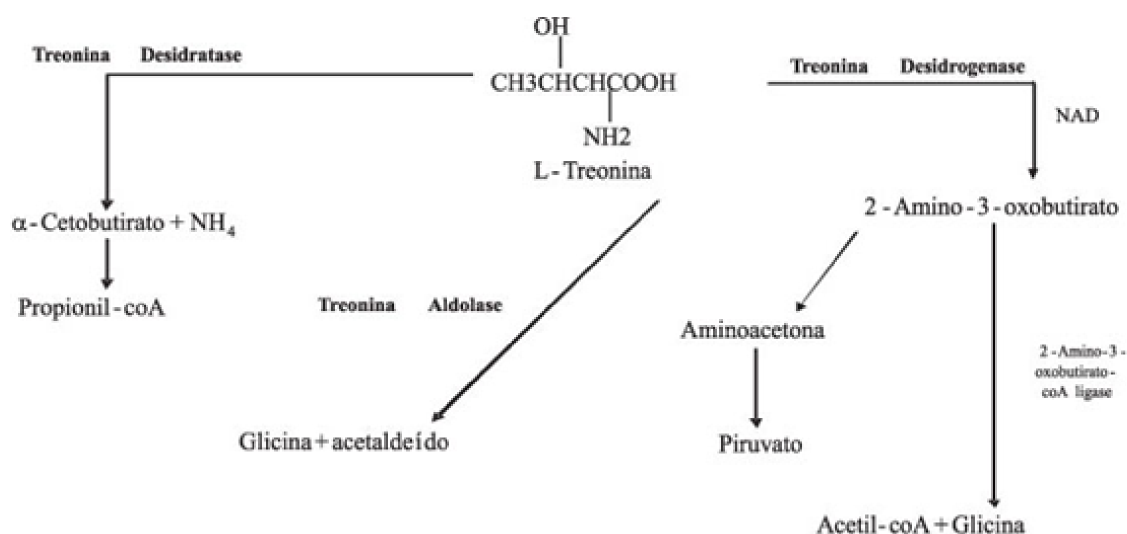

Figura 4. Metabolismo da treonina (**Fonte:** adaptado de DAVIS e AUSTIC, 1982)

2.3. Utilização de aminoácidos marcados

A técnica de isótopos estáveis tem contribuído significativamente para a expansão do conhecimento fisiológico e também para alcançar objetivos nutricionais, e tem sido empregada em diversas espécies, como ruminantes, frangos de corte, poedeiras, codornas, organismos aquáticos, dentre outros (DUCATTI *et al*., 2011; SAKOMURA e ROSTAGNO, 2016). Para que seja possível analisar o metabolismo por meio desta técnica, é necessário que haja uma alteração da composição isotópica tecidual de um determinado elemento, de modo que átomos da molécula de interesse são substituídos por átomos de um mesmo elemento químico, porém com massa diferente, ou seja, substituído por um isótopo. Estas perturbações podem ser realizadas por meio de uma troca de dietas com abundâncias isotópicas distintas ou então por meio da inclusão de compostos marcados (DUCATTI, 2007).

Compostos marcados são constituídos de moléculas as quais possuem um isótopo de interesse, podendo variar desde um ^{13}C, ^{15}N, ^{18}O, ^{2}H, ^{34}S ou qualquer outro. Sua utilização viabiliza indiretamente o rastreamento das rotas metabólicas do composto de interesse, uma vez que o isótopo (traçador) acoplado ao composto pode ser analisado por meio das análises isotópicas, podendo ter sido metabolizado e se encontrar na forma de algum metabólito ou então se encontrar em sua forma intacta original acoplado a sua molécula de origem (MURAMATSU *et al*., 1987).

Apesar de se tratar uma ferramenta com um alto potencial para estudos fisiológicos, a tecnologia por trás da produção de compostos marcados é restrita dificultando a expansão da produção destes compostos e elevando o custo de importação destes produtos (STRADIOTTI, 2013). CERRATE e colaboradores (2017) enfatizam que algumas limitações do uso da técnica de isótopos estáveis são: o alto custo destes produtos e o acesso aos equipamentos de análises isotópicas que exigem uma manutenção com mão-de-obra especializada. No Brasil, o Centro de Energia Nuclear na Agricultura produz compostos marcados voltados principalmente para pesquisas agrícolas e saúde, porém a produção de aminoácidos marcados é crescente (BENDASSOLLI *et al*., 2002; MAXIMO *et al*., 2000).

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CAPÍTULO 2

Este artigo está nas normas da revista "Rapid Communication in Mass Spectometer"

Title: Utilization of labeled amino acids in the diet of broiler chickens to study protein metabolism

Short title: Labeled amino acids in the diet of broiler chickens

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RATIONALE: Many of studies that used metabolic tracers was designed for physiological studies. A methodology is proposed in some nutritional study, in which the labeled amino acid is added on the diet.

METHODS: We included L-^{15}N threonine in the diet of broiler chickens and analyzed the ^{15}N isotope abundance in plasma, liver and muscle of broiler chickens in an Thermo Fisher Flash EA elemental analyzer coupled to a Delta V Mass Spectrometer.

RESULTS: An equation is proposed to predict the ^{15}N isotope abundance according to the tracer dosage and time of tracer administration in diets. We determined that 3.52 mmol/kg.day^{-1} would be sufficient using L-[^{15}N] threonine to obtain a good enrichment analyzing the plasma of broiler chickens.

CONCLUSIONS: Tracer added on the diet was an effective procedure of administration in nutritional studies. In addition, it is possible to reduce the dosage of labeled amino acids determining a low effective enrichment and reducing costs.

Keywords: dietary tracer, isotope, labeled amino acid, nitrogen, nutrition.

INTRODUCTION

Since Schoenheimer (1939) who determined the dynamic state of body components, stable isotope labeled compounds have been used for many decades to study metabolism and metabolic routes in human and animal studies. In such studies, the tracer is usually administered through a vein or an artery over a short time period and taking account avian species, blood infusion requires training and skills to avoid undesirable results, mainly in young chicks due to the small and narrow blood vessels (Watson, 2012; Sheldon et al., 2008). In addition, cateter may not be adaptable to young chicks in order to facilitate constant infusions (Ivy et al., 1968). These experiments were designed for physiological studies with focus on protein metabolism and not for nutritional studies.

From this reasoning, the present methodology is proposed to use labeled amino acid on the diet and analyzing its incorporation by the tissue enrichment. Although many studies require non-invasive samples (e.g. plasma, exhaled carbon dioxide, excreta) which represents the whole body utilization or excretion of the nutrients, in some studies it is

necessary to collect the enriched tissues, e.g. *turnover* tissue-specific, kinetic models with many compartments, defining the metabolic route of a tracer. Moreover, labeled amino acid added on diet may be an interesting way of isotope administration, since this way would reflect more similar to the conditions of feeding trials and nutrients utilization study.

However, one limitation of stable isotope technique is its high cost (Cerrate et al., 2017). In addition, since the use of radioisotopes was more used in the past than in current tracer studies (Koletzko et al., 1997), worries about excessive tracer dosage, duration of tracer administration and toxic effects have been decrease and even regarding stable isotopes, although deuterium has a low systemic toxicity, concentrations of H_DO above 25% have been proved to be toxic for dogs and mice (Kusher et al., 1999; Jones and Leatherdale, 1991). Taking account specifically the L-[^{15}N] threonine use, there are a few studies in which varied the tracer dosage from 96 to 225 µmol/kg.day^{-1} (Rémond et al., 2009; Puiman et al., 2011). Labeled threonine has not been used so often in amino acid requirement trials as labeled glycine, leucine or phenylalanine, however some results are only possible to obtain using specific tracers regarding the labeled compound metabolism. Regarding the choose of the tracer, except when there is no need to measure the amino acid oxidation, ^{15}N has the advantage of being less diluted in the organism than ^{13}C, which contributes to a lower tracer cost. Therefore, to evaluate and determine a low effective dosage could reduce costs.

The aim of this study was to propose a stable isotope methodology providing the tracer on the diet of broiler chickens and to define a low effective L-[^{15}N] threonine dosage sufficient to be detected in different tissues.

EXPERIMENTAL

The study was conducted at the Poultry Science Laboratory (Lavinesp) of the Agricultural Sciences and Veterinary College, São Paulo State University (UNESP), Jaboticabal-Brazil. All experimental procedures were approved by the institutional Ethics Committee on Animal Research of the São Paulo State University, under protocol n° 018026/13. Two assays that were carried out to analyze the stable nitrogen isotope abundance in different tissues varying the L-[^{15}N] threonine dosage (Assay 1); and varying the time of isotope administration (Assay 2). In both assays, the birds were fed a corn and soybean meal based diet was formulated to meet or exceed the nutritional requirements recommended by Rostagno *et al*. (2011).

Dosage of L-[^{15}N] threonine (Assay 1)

In this assay was evaluated the stable nitrogen isotope abundances in the tissues according to the dosage of L-[^{15}N] threonine. A total of twenty-four male broilers (Cobb 500®) from seven to 14d old were used. The chicks were individually housed in cages (0.5 x 0.5m) equipped with feeders and nipple drinkers. The labeled threonine, initially in powder form, was diluted in distilled water using a proportion of 2.5 mg.mL^{-1} and stored in a reagent bottle kept at 5°C. The amount of labeled threonine-enriched solution added in the diet was calculated according to daily body weight of the chick. Labelled threonine was then homogeneously mixed to the feed. This procedure was repeated daily and additional freshly enriched feed was added on top of the remaining feed. Daily feed amounts were provided to birds according to the Broiler Management Guide (2011). The remaining enriched feed was measured in order to do a proportional tracer intake correction at the end of the trial. Five groups of four individual birds were randomly assigned to five dosage of L-[^{15}N] threonine (98% - Cambridge Isotope Laboratories) added in the diet: 17.4 µmol/kg.day^{-1}; 26.1 µmol/kg.day^{-1}; 34.8 µmol/kg.day^{-1} (Muramatsu et al., 1987); 52.2 µmol/kg.day^{-1}; 69.6 µmol/kg.day^{-1}.

On the first experimental day (seven days of age), a control group (n=4) was euthanized to determine natural isotope abundances (δ^{15}N) in broiler tissues, whereas the remaining twenty chicks fed with enriched feed for seven days, were euthanized. Samples of blood plasma, hepatic tissue and breast muscle were collected for isotopic analysis. All samples were dried using a freeze-dryer (Edwards 501, Thermo) under 800 mbar pressure for three days and allocated in polycarbonate flasks with metal balls and immersed in liquid nitrogen for a few minutes, powdered in a cryogenic mill (SPEX SamplePrep 2010 Geno / Grinder 2010) during approximately five minutes. Dried samples were weighed in tin capsules (500 to 600 µg) for δ^{15}N analysis. The stable nitrogen content was determined in a Thermo Fisher Flash EA elemental analyzer coupled to a Delta V Mass Spectrometer (Thermo Fisher Scientific Inc.). The isotopic enrichments were expressed in Delta notation (δ^{15}N) in relation to atmospheric nitrogen (universal standard), with error analysis of 0.2 ‰, as described below:

$$\delta^{15}N_{\text{(sample, standard)}} = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 1000 \quad \text{[Eq. 1]}$$

Where: δ^{15}N (‰) is the relative enrichment ratio of the sample compared to the atmospheric nitrogen standard; R_{sample} and $R_{standard}$ are the isotopic ratio between the heavy and light (^{15}N / ^{14}N) isotope fractions in the sample or the control, respectively.

The normality and homogeneity of variances of data were verified using Shapiro-Wilks and Levene tests ($p<0.05$). The ^{15}N isotope abundance data of breast, liver and plasma according to the tracer dosage obtained were fitted into linear regressions using the Proc REG procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC).

Time of isotope administration (Assay 2)

In the second assay, we evaluated the stable nitrogen isotope abundances in the tissues according to the time of tracer administration. Twenty-seven chicks from 18 to 25d old were individually housed in the same cages as in the first assay. Eight groups of three replicates were randomly assigned to nine treatments, defined by the different duration of isotope administration. Daily feed amounts were provided according to the Broiler Management Guide and the diet was daily enriched with L-[^{15}N] threonine using the same procedures as previously described using a dosage of 69.6 mmol/kg.day^{-1} and chicks were fed with enriched feed for five days. They were euthanized in order to collect breast, liver and plasma samples at following times after birds started to feed the enriched diet: zero, 6, 12, 24, 48, 72, 96, 120, and 168 hours.

The data normality and homogeneity of variances of the ^{15}N isotope abundance of breast, liver and plasma according to the age were also verified using the same tests used in the first assay ($p<0.05$). The ^{15}N isotope abundance data according to the age was fitted to quadratic polynomial regressions by Proc REG procedure of SAS program software.

The equation 2 was fitted and proposed to predict the isotope abundance for different broiler's tissues depending on the dosage and time of isotope administration, which was obtained using the constants obtained in both trials:

$$Y(X_1, X_2) = (A*X_1 + B) * (C*X_2^2 + D*X_2 + E) / (Max_{dos} - Min_{dos}) \quad \text{[Eq. 2]}$$

Where: Y is the isotope abundance depending on the dosage and duration of isotope administration; X_1 is the L-[^{15}N] threonine dosage; X_2 is the time of isotope administration; A and B are coefficients of the linear function obtained by the data of the first trial; C, D, and E are coefficients of the quadratic function obtained by the data of the second assay; Max_{dos} and Min_{dos} are the maximum and minimum L-[^{15}N] threonine dosages, respectively.

In order to determine a low effective dosage for L-[^{15}N] threonine, we calculated the dosage necessary to result in a minimum effective ^{15}N enrichment taking account into the ^{15}N trophic level difference used in ecology studies (Post, 2002) and five days for time of isotope administration, time necessary to reach an isotopic steady-steady in plasma (Cerrate

et al., 2017). It is worth to highlight that we choose the plasma since it is the sample that is most used in the isotope studies due to the non-invasiveness.

RESULTS AND DISCUSSION

In the first assay, we observed that the higher the L-[^{15}N] threonine dosage, the higher the nitrogen isotopic enrichment regardless of the tissue (Figure 1). Our results showed a linear increase in the ^{15}N isotopic enrichments of plasma (R^2 = 0.97), liver (R^2 = 0.98) and breast muscle (R^2 = 0.99) as the dietary L-[^{15}N] threonine increased in the diets.

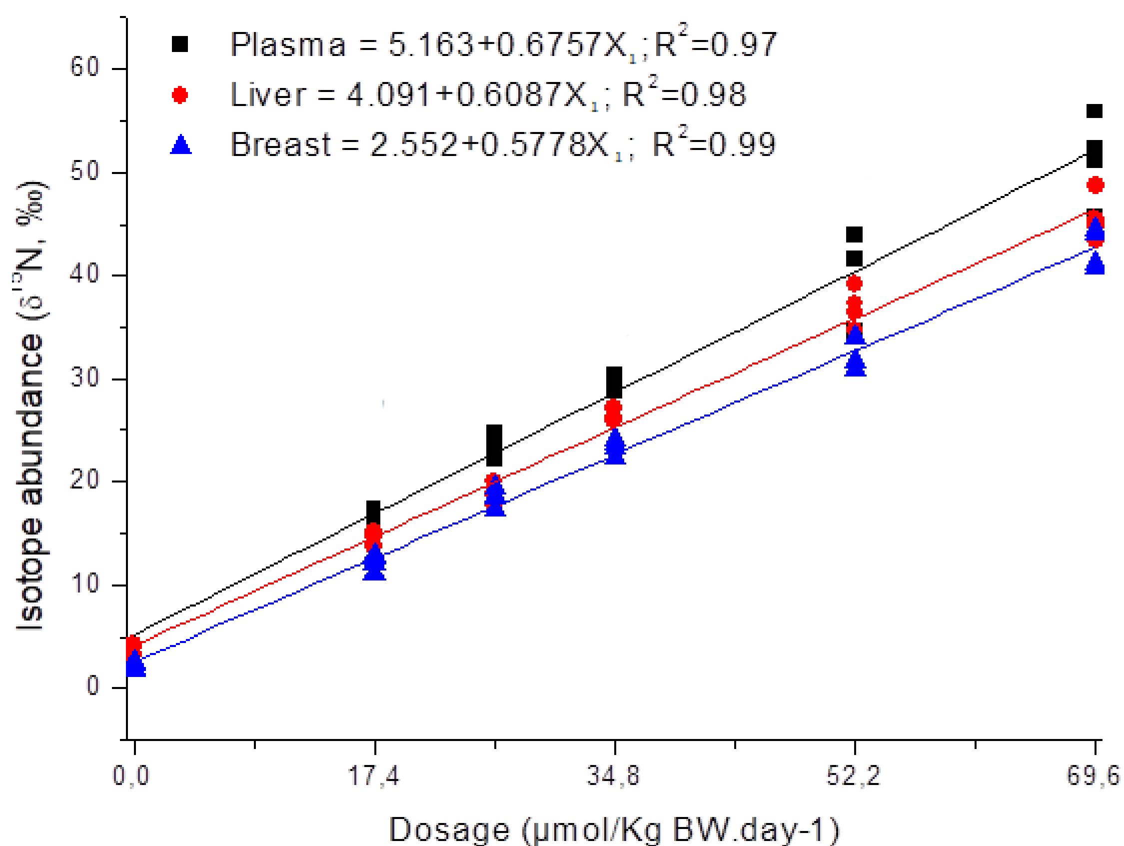

Figure 1 – Linear regression equations for isotopic enrichment of plasma (□), liver (●) and breast muscle (▲) of broiler chickens after seven days of tracer addition in diet using different L-[^{15}N] threonine dosages.

The high correlation between dosages and tissue isotopic enrichments (figure 1) suggests a same threonine incorporation of the L-[^{15}N] threonine regardless of the dosage since different tracer dosages resulted in proportional enrichments. In addition, we fed the birds formulated balanced diets and total threonine intake (dietary threonine + L-[^{15}N] threonine) did not exceed the threonine requirements, because the additions of L-[^{15}N]

threonine were so small that did not affect threonine metabolism proved by the proportional ^{15}N isotope abundances. It is known that suboptimum intakes result in a linear increase utilization, and when exceed the amino acid requirements, a decrease in efficiency of amino acid utilization is observed (Heger, 2002).

The lowest L-[^{15}N] threonine dosage (17.4 mmol/kg.day^{-1}) resulted in a significant difference from basal isotopic abundances, whereas the differences between the ^{15}N isotope abundances were 13.46; 10.75 and 9.95 ‰ for plasma, liver and breast muscle, respectively. Assuming that there is an increase of 3.4±1.0 ‰ per trophic level for $\delta^{15}N$ (Post, 2002), the lowest dosage of L-[^{15}N] threonine used in the first assay could assure that this difference in the isotope abundances was provided by the labeled threonine, since the ^{15}N isotope abundances of 13.46; 10.75 and 9.95‰ were obtained for plasma, liver and breast muscle, respectively, were higher than 4.4 ‰. Therefore, a dosage of 17.4 mmol/kg.day^{-1} during seven days could be sufficient to obtain a low effective enrichment regardless of the tissue.

Figure 2 illustrates the ^{15}N isotope enrichments according to the time of tracer administration. The isotope abundances of the plasma and liver had quadratic responses, while breast muscle had a linear increase.

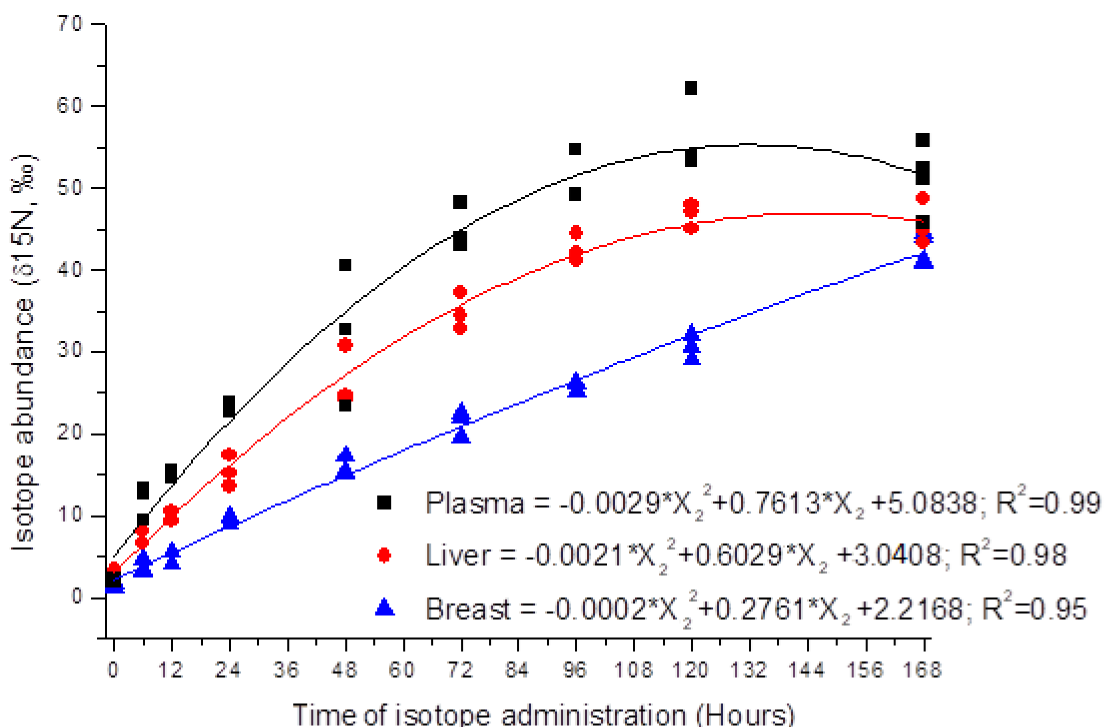

Figure 2 – Regression equations for isotope abundance of plasma (□), liver (●) and breast muscle (▲) of broiler chickens fed enriched diet with L-[^{15}N] threonine according to time of isotope administration. The tracer dosage was 69.6 mmol/kg.day^{-1}.

Once was possible to verify that dosage and time of administration of tracer are influential factors for isotope abundances of the tissues, we combined both factors in equations to predict the isotope abundance in the tissues. The equations (Equations 3, 4 and 5) determined herein were developed to predict the isotope abundances according to the L-[^{15}N] threonine dosage and time of isotope administration for breast, liver and plasma, respectively, are presented as follows:

$Y_B = (0.5778*X_1+2.552) * (-0.0002*X_2^2+0.2761*X_2+2.2168) / (69.6-17.4)$, $R^2 = 0.68$ Eq3

$Y_L = (0.6087*X_1+4.091) * (-0.0021*X_2^2+0.6029*X_2+3.0408) / (69.6-17.4)$, $R^2 = 0.75$ Eq4

$Y_P = (0.6757*X_1+5.163) * (-0.0029*X_2^2+0.7613*X_2+5.0838) / (69.6-17.4)$, $R^2 = 0.93$ Eq5

Where: Y_B, Y_L, and Y_P are the isotope abundances of breast, liver and plasma, respectively, depending on the dosage and time of isotope administration; X_1 is the L-[^{15}N] threonine dosage; and X_2 is the time of isotope administration.

Plasma enrichment presented a better fitting ($R^2 = 0.93$), followed by the liver ($R^2 = 0.75$) and breast ($R^2 = 0.68$) when comparing the predicted and observed data of both assays considering the dosage and time of administration. Taking account the equation 5 obtained for plasma and considering a ^{15}N isotope abundance of 7.9‰ (3.5‰ from basal isotope abudance + 4.4‰ from the difference of trophic level according to Post (2002)) and 120 hours to obtain a steady-state in the plasma according to Cerrate et al. (2017), we determined that 3.52 mmol/kg.day^{-1} could be the minimum L-[^{15}N] threonine dosage in tracer studies. In other studies, Rémond et al. (2009) used 96 µmol/kg.day^{-1} for minipigs, while Cerrate et al. (2017) used about 100 µmol/kg.day^{-1} for broiler chicks. Tuvdendorj et al. (2013) using rabbits, chose 170 µmol/kg.day^{-1} and Puiman et al. (2011) defined a 600 µmol/kg.day^{-1} for preterm pigs. It is worth to highlight that the decision of determining a tracer dosage is influenced by different factors inherent to the methodology applied (e.g. bolus injection, constant infusion, time of tracer administration and tissue sample) and tracer itself (e.g. tracer abundance and tracer element due to different proportions of isotope dilution). Therefore, assessing a tracer dosage in long studies or with a high number of animals could reduce the costs carrying out some pilot-trials. In addition, the price of labeled amino acids and analysis of L-[^{15}N] threonine could limit the use in the studies.

In many studies in which were used labeled amino acids have been administered by the intravenous procedure. Although oral and intravenous tracer could provide the same results in some situations (Kriengsinyos et al., 2002), in nutritional studies, it is important to consider biological processes such as digestion and absorption of the amino acids and thus,

we believe that the labeled amino acid on diet would represent more similar the behavior of the dietary nutrients metabolism. Moreover, specifically for broiler chickens, the caliber of their blood vessels is too thin depending on their age, which could difficult the intravenous way in this specie without specific training for blood sampling. The results of these assays were sucessful to prove that it is possible to analyze the incorportion of a tracer in tissues and plasma when providing labeled amino acids on the diets.

However, the main problem of providing labeled amino acid on diet might be regarding tracer wastes. We are aware that could have some feed wastes in the feeders, hence tracer wastes, even when providing the amount of daily feed recommended by the Broiler Management Guide in an attempt to avoid such wastes. However, in a previous study of our group, high recovery rates were obtained following such procedures, varying 96 to 100% using L-[^{15}N] Methionine and 86 to 92% to L-[$^{15}N_2$] Cystine (Pacheco et al., 2019). Another possibility would be to separate a group of chicks (*pair-feeding* technique) out of the assay to measure their daily feed intake and in the next day, provide this measured feed amount to chicks in the trial.

The outcomes of these assays demonstrate the possibility of applying the procedures evaluated herein in nutritional studies using labeled amino acids. The base assumption for these studies is that tissue isotopic enrichment represents the dietary nutrient (DeNiro and Epstein, 1980). The effectiveness of the methodology evaluated indicate that the tracer added on the diet could be used to understand the metabolism in nutritional studies. In addition, the equations could be useful to predict the stable nitrogen isotope abundance in breast, liver and plasma, according to the L-[^{15}N] threonine dosage and time of isotope administration in further studies using L-[^{15}N] threonine. A L-[^{15}N] threonine dosage of 3.52 mmol/kg.day^{-1} would be sufficient to result in a low effective enrichment when plasma is collected and considering a 5-d assay.

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CAPÍTULO 3

Este artigo está submetido e nas normas da revista científica “Animal”

Use of stable isotopes to study the sulfur amino acid utilization in dose response trial with broiler from 42 to 56 days

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Short title: Sulfur amino acids metabolism in finishing broilers

Abstract

Over the years, nutritionists have been discussing if the dietary supplementation of **cystine (Cys)** can cover for part of the dietary **methionine (Met)** required in the **total sulfur amino acid requirement (TSAA)** to achieve optimum performance. Assuming that Met can be converted to cysteine to meet the metabolic needs for Cys, TSAA requirement must be defined as the dietary Met intake in the absence of Cys that provides optimal growth

performance. It is also argued that the Met to Cys ratio changes with broiler age and even with different Met sources. Thus, the objective of this study was to evaluate two sources of Met while determining the adequate proportion of Met and Cys in the TSAA for optimal performance of broilers from 42 to 56 d and to use stable isotopes to evaluate the utilization of these amino acids. A performance assay was carried out in a factorial arrangement (4x2+1) using 1,080 broilers from 42 to 56 d fed diets containing four proportions between Met and Cys maintaining the same dietary TSAA (Met:Cys/TSAA) in all diets (46:54, 48:52, 50:50 or 52:48) supplemented with two synthetic methionine sources (DL-Met or L-Met) or fed a control diet without Met supplementation (44:56). In addition, a total of twenty-one broilers with the same age were fed the diets 44:56, 48:52, and 52:48 with the addition of L-(^{15}N) Met or L-($^{15}N_2$) Cys on the diet in order to analyze TSAA sulfur amino acid metabolism. No differences between Met sources were observed **for feed intake (FI)**, **body weight gain (BWG)**, and **feed conversion ratio (FCR)** ($P>0.05$) however, FCR was improved at 50% of Met/TSAA. Regarding TSAA utilization, it was observed that the higher the Met:Cys/TSAA, the higher the Met conversion, but Met intermediates decreases.
Keywords: chick, cystine, labeled amino acid, methionine, transsulfuration.

Implications

Providing an adequate total sulfur amino acid level to the broilers does not assure that methionine and cystine requirements will be met individually. Such controversy exists due to a common practice to formulate diets assuming the sum of both **amino acids (AA)** represents the same as total sulfur amino acid requirements once methionine can be converted to cystine. The outcomes of the present study provide the ideal proportion between these amino acids, which was determined to be 50%, in order to maximize the performance of broilers from 42 to 56d old, and thus minimizing environmental impacts and maximizing economic return.

Introduction

Over the years, nutritionists have been discussing if the dietary supplementation of cystine (Cys) can cover for part of the dietary methionine (Met) required in the total sulfur amino acid requirement (TSAA) to achieve optimum performance. TSAA requirement has been considered as the requirements of methionine (Met) plus cystine (Cys) in feed formulations due to its interconversion of methionine to cystine, however, meeting TSAA requirements does not necessarily assure that both sulfur amino acid requirements will be met individually (Huyghebaert and Pack, 1996).

Hence, numerous studies aimed to determine the optimum proportion for Cys in relation to TSAA, also called by Cys replacement in different species (Shannon *et al*., 1972; Chung and Baker, 1992; Baker *et al*., 1996). In overall, dietary cystine can contribute approximately 50% of the TSAA requirement for young animal and this proportion would increase with aging (Graber and Baker, 1971). However, there is a few physiological information about how aging affects sulfur amino acid metabolism in broilers and most of these studies evaluated sulfur amino acid metabolism indirectly from the animal responses instead of obtaining physiological findings.

Given this background, our hypothesis is that sulfur amino acid metabolism would change according to different **proportion of methionine and cystine to total sulfur amino acids (Met:Cys/TSAA)** with increasing age in order to balance TSAA. Therefore, the current study aimed to determine an optimum proportion between Met and Cys maintaining the same dietary TSAA level supplying different Met sources in practical-type diets and analyze how sulfur amino acid utilization is affected using labeled amino acids in broilers from 42 to 56 d of age.

Material and methods

Performance assay

Experimental design, animals and housing. A total of 1080 one-d-old male Cobb 500 broilers were weighed at 42d and separated into groups with similar weights (2.742 ± 0.016 kg) and assigned randomly to nine treatments with six replicate pens of 20 broilers each. The factorial arrangement (4x2+1) consisted of four Met:Cys/TSAA ratios (46:54, 48:52, 50:50 or 52:48), two synthetic methionine sources (DL-Met or L-Met) and a control which consisted in a diet with 44:56 Met:Cys/TSAA attained without Met supplementation. Broilers were housed in pens (1.4m × 3.0m) in a controlled environment facility with free access to the diet and drinking water provided by tubular feeders and nipple drinkers. The environmental conditions during the feeding trial, thermo-hygrometers recorded an average temperature of 23 ± 6ºC and average humidity of 58 ± 16%, whilst a timer was set to provide 18 hours of light per day, as recommended by the strain guidelines (Cobb-Vantress, 2015).

Experimental diets. All experimental diets consisted mainly of corn and soybean meal (Supplementary Table S1) and were formulated to meet or exceed the nutrientional requirements of the broilers (Rostagno *et al*., 2017) except for dietary total sulfur amino acid that was supplied at 0.63 to ensure that TSAA will be limiting in all treatments. To obtain the different proportions between Met and Cys, the diets were supplemented either with DL and L-Met (99%); and/or L-Cystine (100%). All ingredients were analyzed prior to diet formulation to determine total AA concentrations. Standardized ileal digestible AA were calculated based on coefficient of digestibility described by Rostagno *et al*. (2017) and crystalline AA were assumed to be 100% standardized ileal digestible (SID).

[Insert Table 1]

Data collection. The FI and body weight were measured at 56d to calculate BWG and FCR. Mortatily was recorded and FCR was adjusted according to the mortality.

Statistical analysis of results. Performance data were analyzed as two-way ANOVA using the GLM procedure of SAS 9.0 (SAS Institute Inc., Cary, NC). The main effects were Met sources (DL-Met and L-Met) and proportions of Met and Cys in relation to total sulfur amino acids (Met:Cys/TSAA), as well as the interaction between both factors. Only main effects were considered in the absence of interactions.

Labeled stable isotope assay

Experimental design and diets. Twenty-one male broilers were weighed (2.585 ± 0.105 kg) kg and allocated in individual metabolic cages at the beginning of the trial (42d old). Initially, three broilers were euthanized in order to obtain basal natural isotope enrichments of samples. After that, the remaining 18 birds were divided into two groups, in which a half of them received L-(^{15}N) Methionine and the other half, L-($^{15}N_2$) Cystine. Each group of nine birds that received the same labeled AA were randomly assigned to three groups (n=3) fed diets containing different Met:Cys/TSAA (44:56, 48:52, and 52:48) daily enriched with either L-Met (45 mmol.kg^{-1}) or L-($^{15}N_2$) Cystine (25 mmol.kg^{-1}). During a 14-d feeding assay broilers were housed into metabolic cages (0.5 x 0.5 m) in a controlled environmental room with feed and water provided *ad libitum*. The environmental conditions were maintained similar to the first assay.

Enrichment with labeled amino acids. Three diets used in the dose-response assay were also used in this assay, but with the addition of labeled AA to track the utilization of Met and Cys. The addition of labeled AA were performed daily by feed with a fixed-dose according to the body weight (45 and 25 mmol.kg^{-1}, respectively). The L-(^{15}N) Met (150.21 g.mol^{-1}, 96-98 atm% abundance, Cambridge Isotope Laboratories®) was diluted in deionized water (100 mL) in a graduated reagent bottle resulting in a ^{15}N-Met-enriched compound and was stored under refrigeration at 5ºC. As the L-($^{15}N_2$) Cys (242.29g.mol^{-1}, 98 atm% abundance, Cambridge Isotope Laboratories®) is insoluble in water, the ^{15}N-Cys amount was weighed

daily on a precision analytical balance and supplemented in the crystalline form on the feed as well. The dose (in μL.kg^{-1}) of ^{15}N-Met-enriched compound for each bird (Qtd_{aa}) was calculated according to the the following equation 1:

$$Qtd_{aa} = (D_{aa} * BW * MW * Wast) / (1000 * C) \qquad \text{Eq.1}$$

Where D_{aa} is the labeled AA dose relative to the addition of ^{15}N of the AA (μmol); BW is the weight of the animal (kg); MW is the molecular weight of the labeled AA (g.mol^{-1}); Wast is an increase of 3% assuming a waste of feed by broilers (Pacheco *et al.*, 2019); the value 1000 is used as the conversion factor from micrograms to milligrams; C is the concentration of labeled amino acid (mg.mL^{-1}). It is possible to transform mL into μL after this calculation by multiplying the Qtd_{aa} value by 1000 and, thus determining the dose (μL.kg^{-1}). Initially, the birds were weighed to obtain the BW value and calculate the amount of labeled AA based on Eq. 1. Labeled AA was added homogeneously on the feed prior to mixing with an individual plastic spoon. The labeled amino acid addition was repeated daily and the additional enriched feed was always added on over the remaining feed of the last day. At the end of the trial, feed wastes were measured to do a proportional tracer intake correction.

Data collection. At 42d of age, three birds were euthanized with carbon dioxide as reference to determine the natural enrichment at the beginning of the experiment. Labeled AA addition was performed daily until the end of the experimental period (56d of age). At 56d of age, all broilers were euthanized. Excreta were daily collected and at the end of the assay, a pool for each cage was homogenized to estimate losses. All the digesta content present in the gastrointestinal tract was manually removed. All the samples were weighed and conserved for further analysis.

Processing sample and chemical analysis. All whole body and excreta samples were freeze-dried (Edwards 501, Thermo) under 800 mbar pressure for 72 hours. Then, samples were frozen by nitrogen liquid under -196^{0}C and grounded in cryogenic mill (SPEX

SamplePrep 2010 Geno/Grinder 2010) for three minutes. The samples, weighed and packed in tin capsules, were burned in a combustion furnace of the elemental analyzer coupled to the mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) to analyze the isotope abundance and percentage of nitrogen. The isotope concentrations were expressed in atm%^{15}N, with permissible analytical error of 0.1‰. The determination of labeled Met was performed by the derivatization and normalization procedures in the compound-specific stable isotope analysis of nitrogen in amino acids as described by Yarnes and Herszage (2017).

Isotope/Amino acid recoveries and sulfur amino acid utilization. All the calculations of sulfur amino acid metabolism presented herein were also presented in the study of Pacheco *et al.* (2018). ^{15}N retentions ($^{15}N_R$) in the samples were calculated based on isotope dilution between two sources: enriched feed and whole body as follows (Trivelin *et al.*, 1995):

$$^{15}N_R = DM \times \%N \times [(Ab_p - Ab_{nat})/(Ab_{aa} - Ab_{nat})] \quad \text{Eq. 2}$$

Where $^{15}N_R$ was the ^{15}N retention (mg) from labeled amino acid; DM was the dry mass of the sample (mg); %N was the nitrogen percentage (%); Ab_p was the isotope abundance of whole body; Ab_{nat} was the natural isotope abundance of whole body (determined at 42d of age); Ab_{aa} was the isotope abundance of the L-(^{15}N) amino acid.

The same equations were applied for the compound specific isotope analysis (CSIA) results, only replacing $\delta^{15}N$ by methionine-specific $\delta^{15}N$, as presents in equation 3:

$$^{15}N\text{-}Met_R = DM \times \%Met \times [\,(Ab_{pCSIA} - Ab_{natCSIA}) / (Ab_{aa} - Ab_{natCSIA})\,] \quad \text{Eq. 3}$$

Where ^{15}N-Met_R was the ^{15}N-Met retention (mg) from L-(^{15}N) Met; DM was the dry mass of the sample (mg); %Met was the Met concentration (%); Ab_{pCSIA} was the ^{15}N-Met abundance of enriched tissue; $Ab_{natCSIA}$ was the natural ^{15}N-Met abundance of tissue; Ab_{aa} was the isotope abundance of the L-(^{15}N) Met.

The whole body AA retention was calculated using the aminogram results (CBO lab, Brazil), according to the equation 4:

$$AA_R = (DM \times \%AA) - AA_{Ri} \quad \text{Eq.4}$$

Where AA_R was the whole body AA retention; DM was the dry mass of the sample (mg); %AA was the amino acid concentration (%) and AA_{Ri} was the mean amino acid retention of the reference group.

In following, it was necessary to determine the recovery rates of the unlabeled and labeled Met and Cys after calculated all the unlabeled and labeled Met and Cys retentions. The isotope/AA recoveries rates were determined by the relation between the total isotope/AA retention by the total isotope/AA intake.

$$Rec = (X_R / X_I) \times 100 \quad \text{Eq.5}$$

Where Rec is the recovery rate; X_R is the retention of Met or Cys calculated based on the equation 2, 3, or 4 and X_I is the intake of ^{15}N, methionine-specific ^{15}N, or Met and Cys.

Regarding sulfur amino acid utilization, the conversion of Met into intermediates and Cys ($Met_{Cys+interm}$) was calculated based on the difference between the recovery rates of ^{15}N ($^{15}N_{Rec}$ (Met)) and methionine-specific ^{15}N ($^{15}N\text{-}Met_{Rec}$) as follows:

$$Met_{Cys+interm} = {}^{15}N_{Rec}(Met) - ({}^{15}N\text{-}Met_{Rec}) \qquad \text{Eq.6}$$

The amount of Met converted into Cys (Cys_{Met}) were calculated based on the difference between the recovery of Cys using the aminogram analysis (AA_{Rec} (Cys)) and the recovery of ^{15}N from Cys ($^{15}N_{Rec}$ (Cys)):

$$Cys_{Met} = AA_{Rec}(Cys) - {}^{15}N_{Rec}(Cys) \quad \text{Eq.7}$$

The amount of Met converted into intermediates of Met cycle ($Met_{interm.}$) was calculated based on the difference between the conversion of Met into intermediates and Cys ($Met_{Cys+interm}$) detailed in Eq. 6 and the amount Met converted into Cys (Cys_{Met}) as detailed in Eq.7:

$$Met_{interm.} = Met_{Cys+interm.} - Cys_{Met} \quad \text{Eq.8}$$

Results

[Insert Table 1]

Taking into account the performance trial, no interactions were observed between Met:Cys/TSAA ratios and Met sources effects regardless of the performance parameter

evaluated and so, the means are presented in table 1. Between L and DL-Met sources, no differences were detected in broiler performance (P>0.05). Considering the Met:Cys/TSAA effects, the broilers fed 44:56 diet presented a higher FCR than 50:50 treatment.

AA intake and deposition of Met and Cys in whole body and excreta for each treatment are detailed in table 2. In overall, lower recovery rates were observed for Met (101 ± 11, 87 ± 3, and 88 ± 31) than Cys (99 ± 6, 101 ± 3, and 108 ± 2).

[Insert Table 2]

^{15}N intake and deposition provided from L-(^{15}N) Met and L-($^{15}N_2$) Cys in whole body and excreta for each treatment are detailed in table 3. We obtained high recoveries rates from nitrogen stable isotope analysis varied from 91 ± 7 to 103 ± 9% and were expected to be close to 100%.

[Insert Table 3]

The L-(^{15}N) Met intake and deposition provided from L-(^{15}N) Met in whole body and excreta for each treatment are detailed in table 4. The higher L-(^{15}N) Met, the higher the L-(^{15}N) Met recovery rate (53 ± 9 for 44:56; 45 ± 1 for 48:52; and 43 ± 1 for 52:48). The recoveries of metabolic tracers were expressed as percentage in order to standardize the results.

[Insert Table 4]

The data presented in tables 2, 3 and 4 were used to calculate the outcomes showed in table 5 regarding the sulfur amino acid metabolism. It was observed the higher the Met proportion, the higher Met conversion (16 ± 10, 24 ± 3, and 38 ± 7). On the other hand, Met intermediates (32 ± 15, 31 ± 6, and 13 ± 1) was inversely proportional to increasing Met:Cys/TSAA.

[Insert Table 5]

Discussion

In a previous study of our group, Pacheco *et al.* (2018) determined an optimum proportion of Met:Cys/TSAA for broilers from 14 to 28 days of age, in which they combined a performance assay with labeled amino acid assay to analyse the biological changes

regarding sulfur amino acids metabolism in order to justify their performance findings of the first study. However, one question that would arise is how these biological changes would behave in face of sulfur AA requirements for older birds, being necessary to repeat these studies at several stages (Wheeler and Latshaw, 1981). Regarding sulfur AA requirements, Met requirements would decrease as the broilers grows and although Met has an essential role for protein deposition, growth rate also decreases with aging (Wheeler and Latwhan, 1981; López *et al.*, 2000; Tesseraud *et al.*, 2009). Indeed, the recommendation (%) of most amino acids would decrease linearly with aging, but Cys could be an exception (Heger *et al.*, 2002). Cys contribute significantly for feather protein since keratin synthesis requires high amounts of Cys, confering the strength and stiffness of feather struture due to the dissulfite bonds that are formed (Kalinowski *et al.*, 2003). The feathering in the period from 14 to 28 days would be greater than the present study which comprehends 42 to 56 days and thus Cys requirment would reduce (Wheeler and Latshaw, 1981; Baker *et al.*, 1996). However, many studies reported that keratoid tissue growth plus the synthesis of essential body metabolites such as glutathione and taurine would demand more cysteine with increasing age when maintenance increases proportionally in relation to the growth (Hartsook and Mitchell, 1956; Baker and Han, 1993). Because AA requirements depend on the animal physiological conditions, although a lower amount of both sulfur AA is expected for older birds than younger, a constant or slightly high proportion of Cys has been recomended between Met and Cys in relation to SAA for older birds (Graber and Baker, 1971; Chung and Baker, 1992; Le Floc'h *et al.*, 2004).

It is important to highlight that although our definition of Cys/TSAA is the same as Cys replacement value as defined in many studies, there is a difference between their determination. We are aware that both determine the fraction of TSAA that could be supplied as Cys in order to optimize the animal performance, however, TSAA level influence on the transsulfuration pathway and therefore, our outcome would optimize animal performance only for this TSAA level (Shannon *et al.*, 1972; Chung and Baker, 1992; Ohta and Ishibashi, 1994). On the other hand, Cys replacement studies first determine an

optimum TSAA requirement before determining an optimum relation between sulfur amino acids, and commonly consider a range of either SAA and Met levels. Although such results could be more interesting, in order to obtain a physiological responses, the SAA level must be at or slightly bellow its requirements (Baker *et al*, 1996). In the current study, FCR was improved when Cys corresponded to 50% of TSAA and although it was possible to determine an optimum proportion of Met and Cys, broiler performance varied a little among the treatments due to a low TSAA limitation and/or narrow range of dietary Met:Cys/TSAA. Purified diets would enable to obtain diets with a wide range of proportions between Met and Cys, however, since we looked for practical results, we opted to use conventional corn and soybean meal diets with a lower range of Met:Cys/TSAA. Moreover, the excess of both sulfur amino acids is toxic to many species, including for broilers (Baker, 2006).

Regarding Met:Cys/TSAA recommendations, in the National Counceil Research (1994) is proposed a slight increase in this ratio comparing 52.7% for broilers from 3 to 5 weeks and 53.3% from 6 to 8 weeks of age, whilst Rostagno *et al.* (2017) recommended a constant ratio of 55.3% for males with a medium superior performance irrespective of age. A slighty lower Cys replacement value was obtained comparing 1-to-3-wk-old to 3-to-6-wk-old broilers (50% *vs.* 52%, respectively) and these authors atributted this difference to the higher Cys maintenance for older birds (Baker *et al.*, 1996). All those afromentioned studies corroborates with our findings, being 52% of Met/TSAA for broilers from 2-to-4-wk-old (Pacheco *et al.*, 2018) and a slighty higher in older birds estimated herein in 50% to improve FCR. In contrast, Graber and Baker (1971) suggested that dietary cystine can contribute to around 50% of TSAA requirements in younger pigs and up to 90% for non-reproducing animals, whilst Fuller *et al.* (1989) concluded this value would be around 79 to 82% of TSAA for growing pigs. Such high values are explained by the difference of species (broilers *vs.* pigs) used in these studies but mainly by the maturity of animals in these experiments.

Irrespective of performing parameter, no difference was observed between DL and L-Met in the current study. Theis finding is supported by Baker (2006), who affirmed that DL-Met is well utilized as L-form in most species, including broilers. It is known that the only

isomer that is incorporated into protein deposition is L-Met, thus D-Met must be converted before used to the birds (Dibner and Ivey, 1992; Hasegawa *et al.*, 2005). In addition, the liver of growing broilers produces sevenfold excess of enzyme to convert any Met sources to L-Met, assuring that DL-Met would be convereted L-form and this capacity increases with aging (Dibner and Ivey, 1992). Pacheco *et al.* (2018) founded no differences for Met sources using younger chicks aging 14 to 28d.

The proposal of the stable isotope methodology presented herein is to investigate sulfur amino acid metabolism, such as the utilization of sulfur amino acids into different biological processes, e.g. transsulfuration and Met intermediates. For this, different proportions of Met:Cys/TSAA were given fixing the same limiting-TSAA intake, in order to analyze how the organism deal with and balance this sulfur amino acid deficiency by biological reactions. Taking account the transsulfuration process, it was calculated the proportion of Cys which was provided from Met (Cys_{Met}) by the difference between Cys unlabeled (Cys_R) and labeled ($^{15}NCys_R$) depositions, obtained by aminogram and nitrogen stable isotope analyses. As aminogram analysis does not distinguish if Cys was provided by Met or Cys, whilst nitrogen stable analysis ensure that the ^{15}N analyzed was provided by ^{15}N-Cys assuming that cysteine remains as cysteine due to its stability. It was observed the higher the Met proportion, the higher the transsulfuration utilization, since in a Met deficiency condition transsulfuration rate increases in an attempt to meet the Cys demand (38 ± 7 for 52:48 diet in the present study *vs.* 43 ± 4 for 56:44 as reported by Pacheco *et al.*, (2018)). Moreover, the higher recovery rates equal or more than 100% for Cys in aminogram analysis might be explained by the transsulfuration process. The CSIA determines the Met that had remained with the same structure and the difference between Met unlabeled (Met_R) and labeled ($^{15}NMet_R$) would the portion of Met to transsulfuration and intermediates ($Met_{cys+int}$). Finally, it is possible to obtain the Met intermediates (Met_{int}) by the difference between portion of Met to transsulfuration and intermediates and transsulfuration. The fraction of Met intermediates represent biological compounds such as S-adenosylmethionine, S-adenosylhomocysteine, homocysteine and also related to the

remethylation process and this fraction decreases as Met proportions increases, thus the highest Met intermediates was obtained in the 44:56 diet, and this behavior was also observed in Pacheco *et al.* (2018) (32 ± 15 *vs.* 24 ± 8). Therefore, a 50% proportion of Met:Cys/TSAA improves FCR for broilers with 42 to 56 d old. In addition, the higher the Met:Cys/TSAA, the higher the Met conversion but Met intermediates decreases. Further studies could compare the outcomes using a more limiting TSAA or even using roosters in advanced age.

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Declaration of interest

The authors declare that they have no conflict of interest.

Ethics committee

All animal care procedures were approved by the institutional Animal Care and Use Committee under protocol n.0 9999/14, prior to the beginning of the trials.

Software and data repository resources

The data will be available from the corresponding author on reasonable request. For more information, please, contact the correspondent author.

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Table 1. *Daily body weight gain, feed intake and feed conversion rate (mean ± SD) for broilers fed diets containg different Met sources (DL and L-Met) and Met:Cys ratios (44:56, 46:54, 46:54, 48:52, 50:50, and 52:48) from 42 to 56 d old*

Met:Cys/TSAA	Body weight gain (g)		Feed intake (g)		Feed conversion rate (g/g)	
	DL-Met	L-Met	DL-Met	L-Met	DL-Met	L-Met
44:56	1 610 ± 67	1 610 ± 67	3 427 ± 80	3 427 ± 80	2.12 ± 0.06	2.12 ± 0.06
46:54	1 644 ± 81	1 659 ± 47	3 413 ± 175	3 501 ± 74	2.07 ± 0.05	2.11 ± 0.06
48:52	1 649 ± 73	1 693 ± 82	3 397 ± 125	3 472 ± 109	2.06 ± 0.07	2.05 ± 0.04
50:50	1 666 ± 104	1 705 ± 109	3 419 ± 158	3 423 ± 165	2.05 ± 0.03	2.01 ± 0.10
52:48	1 658 ± 81	1 679 ± 75	3 448 ± 105	3 418 ± 148	2.08 ± 0.08	2.03 ± 0.09
Main effects						
Ratios						
44:56	1 610 ± 64		3 427 ± 76		2.12 ± 0.06 b	
46:54	1 651 ± 64		3 457 ± 136		2.09 ± 0.06 ab	
48:52	1 671 ± 77		3 434 ± 118		2.05 ± 0.06 ab	
50:50	1 686 ± 104		3 421 ± 154		2.03 ± 0.07 a	
52:48	1 669 ± 75		3 433 ± 123		2.06 ± 0.08 ab	
Met Sources						
DL-Met	1 645 ± 79		3 421 ± 125		2.08 ± 0.06	
L-Met	1 669 ± 81		3 448 ± 117		2.06 ± 0.08	
Statistics			Probability			
Proportion	P = 0.20		P = 0.96		P = 0.02	
Source	P = 0.26		P = 0.40		P = 0.51	
Proportion x Source	P = 0.96		P = 0.75		P = 0.64	

Means in columns followed by distinct letters are different by the Tukey test ($P < 0.05$)

Table 2. *Amino acid recovery rates from aminogram analysis of broilers fed diets containing different Met:Cys ratios (44:56, 48:52, and 52:48)*

Aminogram	AA intake (g)	Whole body (g)	Excreta (g)	Total (g)	Rec (%)
			Met retention		
44:56	9.06 ± 0.17	7.80 ± 0.79	1.36 ± 0.03	9.16 ± 0.82	101 ± 11
48:52	9.45 ± 0.58	6.96 ± 0.55	1.27 ± 0.17	8.24 ± 0.68	87 ± 3
52:48	9.34 ± 1.51	7.10 ± 1.40	1.10 ± 0.12	8.20 ± 1.52	88 ± 31
			Cys retention		
44:56	10.35 ± 0.76	7.83 ± 1.11	2.50 ± 0.45	10.33 ± 1.34	99 ± 6
48:52	10.04 ± 0.44	7.87 ± 0.46	2.27 ± 0.32	10.14 ± 0.69	101 ± 3
52:48	9.61 ± 0.30	8.08 ± 0.60	2.33 ± 0.12	10.41 ± 0.52	108 ± 2

Table 3. *The ^{15}N recovery rates from aminogram analysis of broilers fed diets containing different Met:Cys ratios (44:56, 48:52, and 52:48)*

	L-(^{15}N)-Met				
$\delta^{15}N$	^{15}N intake (g)	Whole body (g)	Excreta (g)	Total (g)	Rec (%)
44:56	35.25 ± 0.54	25.40 ± 1.28	8.11 ± 1.09	33.51 ±2.18	95 ± 6
48:52	34.58 ± 0.25	25.23 ± 0.66	8.18 ± 0.76	33.41 ± 0.91	97 ± 3
52:48	33.09 ± 0.53	23.96 ± 1.37	6.35 ± 1.26	30.31 ± 0.11	92 ± 1
			L-($^{15}N_2$)-Cys		
44:56	36.74 ± 1.44	27.03 ± 2.77	7.94 ± 0.47	37.93 ± 3.77	103 ± 9
48:52	37.14 ± 0.85	28.38 ± 0.79	5.31 ± 0.99	33.69 ± 0.97	91 ± 3
52:48	38.57 ± 1.54	30.53 ± 2.81	7.39 ± 109	34.96 ± 2.79	91 ± 7

Table 4. ^{15}N-Met recoveries from compound-specific stable isotope (CSIA) analysis of broilers fed diets containing different Met:Cys ratios (44:56, 48:52, and 52:48)

		^{15}N-Met retention			
^{15}N-Met[1]	^{15}N-Met intake (g)	Whole body (g)	Excreta (g)	Total (g)	Rec (%)
44:56	0.353 ± 0.005	0.166 ± 0.027	0.020 ± 0.002	0.185 ± 0.028	53 ± 9
48:52	0.346 ± 0.002	0.144 ± 0.006	0.012 ± 0.001	0.156 ± 0.005	45 ± 1
52:48	0.331 ± 0.005	0.135 ± 0.001	0.008 ± 0.002	0.143 ± 0.001	43 ± 1

[1] Results from compound-specific stable isotope analysis using labelled methionine (CSIA)

Table 5. Sulfur amino acid utilization in broilers fed different Met:Cys/TSAA diets

	Sulfur amino acids utilization						
	AA_R (Cys)[1]	$^{15}N_R$ (Cys)[2]	Cys_{Met}[3]	$^{15}N_R$ (Met)[4]	$^{15}N\text{-}Met_R$[5]	$Met_{Cys+interm}$[6]	Met_{interm}[7]
Met:Cys/TSAA	%						
44:56	99 ± 6	83 ± 7	16 ± 10	95 ± 6	47 ± 8	48 ± 7	32 ± 15
48:52	101 ± 2	76 ± 1	24 ± 3	97 ± 3	42 ± 2	55 ± 4	31 ± 6
52:48	108 ± 3	70 ± 6	38 ± 7	92 ± 1	41 ± 1	51 ± 1	13 ± 1

[1] AA_{Rec} (Table 2) of Cys
[2] $^{15}N_{Rec}$ (Table 3) from Cys, excluding excreta
[3] $Cys_{Met} = AA_{Rec}$ (Cys) - $^{15}N_{Rec}$ (Cys)
[4] $^{15}N_{Rec}$ (Table 3) from Met
[5] $^{15}N\text{-}Met_{Rec}$ (Table 4) excluding excreta
[6] $Met_{Cys+interm} = {}^{15}N_{Rec}$ (Met) - ($^{15}N\text{-}Met_{Rec}$)
[7] $Met_{interm} = Met_{Cys+interm} - Cys_{Met}$

Supplementary Table S1. *Composition of experimental diets and nutrient composition of experimental diets as-fed basis*

Met source	[1]No Met	DL-Met				L-Met			
Proportion (Met:Cys/SAA)	44:56	46:54	48:52	50:50	52:48	46:54	48:52	50:50	52:48
Ingredients (g/Kg)									
Corn (7.86%)	674.16	676.51	676.56	676.61	675.68	676.36	676.27	676.17	675.68
Soybean meal (45%)	253.52	253.46	253.45	253.45	253.56	253.48	253.49	253.50	253.56
Soy oil	44.02	43.11	43.07	43.02	43.32	43.24	43.32	43.41	43.63
Dicalcium phosphate	9.44	9.44	9.44	9.44	9.44	9.44	9.44	9.44	9.44
Limestone	7.12	7.12	7.12	7.12	7.12	7.12	7.12	7.12	7.12
Sodium bicarbonate	4.26	-	-	-	1.59	-	-	-	0.64
Salt	1.47	4.37	4.37	4.37	3.29	4.37	4.37	4.37	3.93
Choline chloride (60%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
BHT	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Coccidiostat	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-methionine (98%)	-	0.13	0.27	0.41	0.55	-	-	-	-
L-methionine (99%)	-	-	-	-	-	0.13	0.27	0.41	0.55
L-cystine (100%)	0.55	0.41	0.27	0.13	-	0.41	0.27	0.13	-
L-lysine HCl (78%)	2.21	2.21	2.21	2.21	2.21	2.21	2.21	2.21	2.21
L-threonine (98%)	0.52	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
L-valine (95.6%)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Premix[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Nutriental content									
Met. Energy (MJ/Kg)	13.59	13.59	13.59	13.59	13.59	13.59	13.59	13.59	13.59
Crude protein (g/Kg)	170 (184)	170 (182)	170 (180)	170 (172)	170 (185)	170 (181)	170 (183)	170 (186)	170 (179)
Dig. Met+Cys (g/Kg)	6.3 (6.3)	6.3 (6.2)	6.3 (6.1)	6.3 (6.2)	6.3 (6.5)	6.3 (6.3)	6.3 (6.4)	6.3 (6.3)	6.3 (6.1)
Dig. Met (g/Kg)	2.7 (2.8)	3.1 (2.8)	3.5 (3.0)	3.9 (3.1)	4.3 (3.4)	3.1 (2.9)	3.5 (3.1)	3.9 (3.1)	4.3 (3.1)
Dig. Cys (g/Kg)	4.5 (3.5)	4.1 (3.4)	3.7 (3.2)	3.4 (3.1)	3.0 (3.1)	4.1 (3.4)	3.7 (3.3)	3.4 (3.2)	3.0 (2.9)
Dig. Lys (g/Kg)	10.0 (11.1)	10.0 (10.8)	10.0 (10.6)	10.0 (10.4)	10.0 (11.0)	10.0 (10.9)	10.0 (11.0)	10.0 (11.4)	10.0 (9.8)

Dig. Thr (g/Kg)	6.5 (7.2)	6.5 (7.1)	6.5 (6.9)	6.5 (6.9)	6.5 (7.2)	6.5 (7.1)	6.5 (7.1)	6.5 (7.2)	6.5 (6.7)

[1]No Met synthetic supplementation.

[2]Content (per kg of diet) – vit. A = 10,575 UI; vit. D3 = 2554 UI; vit. K = 1.8 mg; vit. E = 14.87 mg; vit. B1 = 2.00 mg; vit. B2 = 4.5 mg; vit. B6 = 2.50 mg; vit. B12 = 2.00 mg; niacin = 30.00 mg; folic acid = 0.75 mg; calcium pantothenate = 11.74 mg; biotin = 0.01; iron = 43.44 mg; zinc = 43.35 mg; copper = 8.56 mg; manganese= 56.00 mg; iodine = 0,56 mg; selenium = 0.34 mg; antioxidant 4.20 mg; Salinomycin sodium 12%; Butil hidroxy toluene BHT. Values in parentheses indicate analyzed dietary concentration of the amino acids

CAPÍTULO 4

Este artigo está de acordo com as normas da revista "Journal of Animal Physiology and Animal Nutrition"

Title: Threonine incorporation in tissues of growing broiler chickens using L-[^{15}N] threonine

Short running title: L-[^{15}N] threonine incorporation of broiler tissues

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Abstract: Most of amino acid requirement trials look for whole body responses but there is a few information concern amino acid incorporation in individual tissues, which may vary according to the age. In this study the L-[^{15}N] threonine was used to evaluate its incorporation rate and distribution among broiler tissues in different ages. Seventy-two male broiler chickens were distributed into three different phases: starter phase (4 to 9d old); grower phase (18 to 23d old); and finisher phase (32 to 37 old). The broilers were housed into metabolic cages, L-[^{15}N] threonine was added on balanced diets and the birds were fed for five days. The broilers were slaughtered and enriched samples of feather-free body, breast muscle, feathers, liver, jejunum, and plasma were collected at at 0; 6; 12; 24; 48; 72; 96; and 120 hours after fed birds with tracer in each phase. In the tissues were analyzed dry matter, nitrogen and stable nitrogen. The ^{15}N isotope abundance according to the time were fitted into exponential or linear equations using a same intercept. The ratio of the stepness or slope coefficients were determined to compare the L-[^{15}N] threonine incorporation according to the age. In addition, L-[^{15}N] threonine mass balances were performed to assess the L-[^{15}N] threonine distribution among the tissues. Except for feathers, the L-[^{15}N] threonine incorporation rate decreased with aging. Taking account into the L-[^{15}N] threonine distribution to the tissues, only in the jejunum was not observed an increase as the broiler grew. The L-[^{15}N] incorporation varied in each tissue and according to the age of the broiler chickens and these outcomes could be useful to comprehend changes in amino acid requirements tissue-specific according to the age.

Key-words: amino acid, isotope, metabolism, nitrogen, protein.

1. Introduction

Since Schoenheimer (1939) started to use labeled stable isotopes as biological tracers, this tool has been extensively applied to elucidate physiologial issues. Differently from markers, labeled amino acids participate in biological reactions, being possible to define its metabolic pathways through indirect measures (e.g. excreta, indicating the excretion of the labeled compound) or direct measures (e.g. plasma or tissues, assessing the incorporation or deposition of the labeled compound). Although any heavy atom of a labeled amino acid is able to be tracked in order to evaluate its incorporation, the ^{15}N is a good stable isotope tracer to study amino acid metabolism, since nitrogen is an essential component in amino acids (Wolfe & Chinkes, 2004).

Threonine is an essential amino acid utilized for many biological processes as protein accretion, feather growth, synthesis of enzymes, immune function, and gut health (Wu, 2003). However, the utilization of the amino acids on these biological functions may change according to the age of the broilers and thus changing the threonine requirements as well. It is known that threonine does not transaminate and as an essential amino acid, this amino acid will be spared of being catabolized as an energy source, thus, L-[^{15}N] threonine may be a good tracer to represent the protein incorporation (Kidd & Kerr, 1996).

It is known that different tissues may differ from each other considering their metabolic activity, nutrient demands, and dynamism or even with aging (Sartori *et al.*, 2015; Pelícia *et al.*, 2018). In studies that evaluated threonine requirements were based on determining the threonine deposition in the feather-free body and feathers; or to the whole body (Donato *et al.*, 2016; Sklan & Noy, 2004). Therefore, threonine requirements changes in tissues according to age are poorly understood and such information is essential to understand the metabolism and utilization of this amino acid (Kidd, 2000).

Given this background, this study was proposed to assess the L-[^{15}N] threonine incorporation rate and its distribution in different broiler tissues at different ages.

2. Material and methods

2.1 Animals and housing

A total of 72 male broiler chickens, Cobb 500® strain were raised in floor pens until the beginning of each trial. The trials were divided in starter phase (4 to 9-d old), grower phase (18 to 23-d old) and finisher phase (32 to 37-d old). At 4-, 18- and 32-days-old, a group of twenty-four chicks were weighed and allocated randomly in individual metabolic cages (0.5 m x 0.5 m) equipped with feeders and nipple drinkers in an environmentally controlled room with an evaporative cooling system. Two thermohygrometer recorded the maximum and minimum temperatures (ºC) and humidity (%) to control the environmental conditions and maintain the birds in a thermal neutral conditions.

2.2 Experimental diets and tracer addition

Corn and soybean meal diets were formulated to meet the broiler nutritional requirements (Rostagno *et al.*, 2011) as shown in table 1. All three diets were balanced and thus the tracer would represent a balanced protein condition, instead of representing amino acid deficiency or excess condition. During five days of each phase, amounts of L-[^{15}N] threonine (Cambridge Isotope Laboratories, 98% abundance, dose = 69.6 mmol/day.kg^{-1} bird) was administered daily by feed. The tracer dosage was determined (Troni *et al.*, 2016) in a pilot trial to define an L-[^{15}N] threonine dosage without exceeding the threonine requirements. The labeled amino acid was diluted in distilled water and amounts of enriched compound were provided to each bird calculated according to the

body weight to obtain a similar isotope dilution for all animals. After that, the enriched compound was included on the diet.

Table 1. Ingredient and nutritional compositions of the experimental diets.

Inclusion (%)	1-16d	17-30d	31-44d
Corn (7.88%)	57.94	60.93	65.77
Soy oil	2.39	3.38	3.67
Soybean meal (45%)	35.67	32.08	27.35
DL-Methionine (99%)	0.35	0.31	0.28
L-Lysine HCL (54.6%)	0.43	0.41	0.44
L-Threonine (98.5%)	0.11	0.09	0.09
Dicalcium phosphate	1.78	1.43	1.15
Limestone	0.70	0.78	0.71
Salt	0.43	0.41	0.40
†Premix (0,2%)	0.20	0.18	0.14
Nutrients	1-16d	17-30d	31-44d
ME (Mcal/kg)	3010	3114	3196
Crude protein (%)	21.72	20.30	18.59
Dig. Met+Cys (%)	0.91	0.84	0.78
Dig. Met (%)	0.63	0.58	0.53
Dig. Lys (%)	1.26	1.16	1.07
Dig. Thr (%)	0.82	0.75	0.69
Dig. Gly+Ser (%)	1.47	1.40	1.34
‡$\delta^{15}N$ (‰)	6.02	4.65	6.02

†Content/kg: Folic acid 437.50 mg; pantothenic acid 6250 mg; biotin 44mg; copper 6,250mg; iron 31.25 mg; iodine 625 mg; manganese 44g; niacin 18.75 g; selenium 187.5 mg; vit. A 4687500 UI; vit. B 1250 mg; vit B12 7500 mg; vit B2 3,125 mg; vit B6 1,750 mg; vit. D3 1187500 UI; vit. E 17500 UI; vit. K3 940 mg; zinc 40.65 g.

‡Stable nitrogen isotope abundance analyzed of the diet.

2.3 Sample of tissues

At the start of each phase (4, 18 and 32d-old of age), a group of three birds was euthanized to obtain a baseline nitrogen isotope abundance of the samples. After feeding birds with enriched diet, groups of three birds were slaughtered in each sampling points to collect enriched tissues at 6; 12; 24; 48; 72; 96; and 120 hours after feeding birds with tracer to assess the L-[^{15}N] threonine incorporation in the tissues. The sampling

collections were closer at the beginning because a more pronounced enrichment response was expected. Five tissues with different biological roles and metabolic activities were collected: samples of breast meat (*Pectoralis major*), feathers, jejunum, liver and plasma. The samples were weighed and stored in a freezer (-20^{0}C). Because differences in body weight could result in different isotope dilutions among the broilers, free-feather bodies were also collected at 0; 3; and 5 days after feeding birds with tracer in each phase to analyze the isotope dilution.

2.4 Sample processing and chemical analyses

All samples were dried using a freeze-dryer (Edwards 501, Thermo) under 800 mbar pressure for three days. The samples were put in polycarbonate flasks with metal balls and immersed in liquid nitrogen for a few minutes. The samples were powdered in a cryogenic mill (SPEX SamplePrep 2010 Geno / Grinder 2010) during three to five minutes. Dried samples were weighed in tin capsules (500 to 600 μg) for $\delta^{15}N$ analysis. Nitrogen content and stable nitrogen isotope ratios were determined on Delta S Isotope Ratio Mass Spectrometer equipped with an elemental analyzer (EA-IRMS, Finningan MAT, Bremen, Germany).

2.5 ^{15}N isotope abundances and L-[^{15}N] threonine deposition

The isotope abundances were expressed in δ notation (‰) relative to atmospheric nitrogen as follows (equation 1):

$$\delta^{15}N_{\,(sample,\ standard)} = [\ (\ R_{sample} / R_{standard}) - 1]\ \text{x}\ 10^3 \quad \text{(Equation 1)}$$

Where: $\delta^{15}N_{\,(sample,\ standard)}$ is the relative enrichment of $^{15}N/^{14}N$ ratio of the sample when compared to atmospheric nitrogen standard; R_{sample} and $R_{standard}$ are isotopic ratios ($^{15}N/^{14}N$) of the sample and standard, respectively.

The L-[^{15}N] threonine deposition for each tissue were calculated considering the isotope dilution principles (Trivelin, Victory, & Rodrigues, 1995), and as described in the equation 3:

$$^{15}NDep = DM_p \text{ x } N\%_p \text{ x } [(AB_p - AB_{nat}) / (AB_{thr} - AB_{nat})] \quad \text{(Equation 3)}$$

Where: $^{15}NDep$ is ^{15}N deposition from L-[^{15}N] threonine; DM_p is the dried mass of the enriched tissue; $N\%_p$ is nitrogen content; AB_p is isotope abundance of the enriched tissue; AB_{nat} is natural isotope abundance of non-enriched tissue; and AB_{thr} is isotope abundance of the L-[^{15}N] threonine.

2.6 Statistical analysis

All statistical analysis were performed using SAS software. In order to test if the isotope dilution was similar among the animals, means of isotope abundances were compared by Tukey HSD test at 5% of probability.

Isotope enrichment data were standardized by transforming into log 10 values to reduce the variance heterogeneity, because the tissue enrichment behave differently among them. In order to compare the L-[^{15}N] threonine incorporation of a given tissue changed at different phases, the transformed isotope enrichment data were fitted to regression analysis using GLM and NLIN procedures. The transformed data of stable nitrogen isotope abundance were fitted into exponential (equation 2) or linear (equation 3) models using a same intercept was described by Littell, Lewis, & Henry (1995) and Littell, Henry, Lewis, & Ammerman (1997). The best model (linear or exponential) was chosen according to the lower values of the Akaike information criterion (Kenny, 2015). The L-[^{15}N] threonine incorporation was assessed by the relation of the stepness of coefficients or by the slope coefficients of different phases.

$$Y = b0 + \text{b1} * (1 - \exp(X_S * b_S + X_G * b_G + X_F * b_F) + e \text{ (Equation 2)}$$

Where: Y is the stable nitrogen isotope abundance, in ‰; $b0$ is the basal enrichment; $b1$ is the enrichment difference between the highest and basal enrichments, in ‰; X_S, X_G, and X_F are the time in days of the enrichment measurement in starter (4 to 9-d old), grower (18 to 23-d old) and finisher (32 to 37-d old) phases respectively; b_S, b_G, and b_F are the steepness coefficients of L-[^{15}N] threonine incorporation in starter, grower and finisher phases, respectively; e is the random error.

$$Y = b0 + (X_S * b_S + X_G * b_G + X_F * b_F) + e \text{ (Equation 3)}$$

Where: Y is the stable nitrogen isotope abundance in ‰; $b0$ is the basal enrichment; X_S, X_G, and X_F are the time in days of the enrichment measurement in starter (4 to 9-d old), grower (18 to 23-d old) and finisher (32 to 37-d old) phases respectively; b_S, b_G, and b_F are the slopes of L-[^{15}N] threonine incorporation in starter, grower and finisher phases, respectively; e is the random error.

In this method, the outcomes of interest would be the ratio values ($b_{S/G}$, $b_{S/F}$, $b_{G/F}$), which was obtained by the ratio of the stepness of coefficients or by the slope coefficients and it indicates the difference in the L-[^{15}N] threonine incorporation between two phases of a given tissue. In doing so, it is necessary to choose a phase as a reference. Taking account the L-[^{15}N] threonine mass balances among the three phases and expressed as recovery rates in each tissues, the means of these percentage values were also compared by Tukey HSD test at 5% of probability to evaluate if the L-[^{15}N] threonine delivery changed with aging.

3. Results

Figure 1 illustrates the isotope abundances in $\delta^{15}N$ (‰) in the free-feather body at the beginning, in the middle and in the peak enrichment of each phase. Similar isotope dilutions were observed in all three phases even after three days of tracer addition. The isotope abundances in the free-feather body differed only after five days of tracer addition when a more pronounced isotope dilution was observed in starter phase in relation to the other phases.

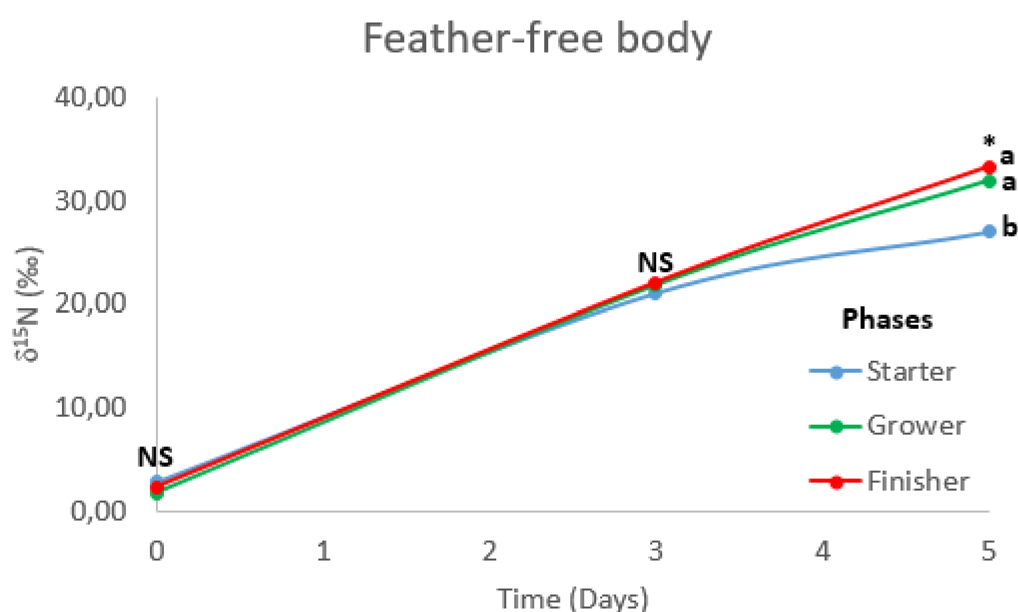

Figure 1. Means of free-feather body isotope abundances ($\delta^{15}N$, ‰) at different phases. *Different letters are significantly different ($P<0.05$, Tukey's HSD test).

†n=3.

The isotope abundances in different broiler tissues according to the age are presented in table 2. Except for feathers in the starter phase, which the ^{15}N isotope abundances oscilated, all tissues were enriched with the L-[^{15}N] threonine addition on the diet. Higher enrichments were obtained in the finisher phase and concomitant lower

enrichments in the starter phase for plasma, liver, and jejunum. On the other hand, breast muscle presented higher values in the starter phase and lower values in finisher. Taking account the feather enrichments, grower enrichments were superior than finisher, which were higher than starter.

Table 2. Means (±SD) of isotope abundances ($\delta^{15}N$, ‰) in different broiler tissues (n=3) enriched with L-[^{15}N] threonine according to the phases.

Age (Days)	Time of administration (Days)	Breast	Feathers	Jejunum	Liver	Plasma
		Starter phase				
4	0	1.91 ± 0.01	5.56 ± 1.22	1.91 ± 0.01	4.65 ± 0.01	3.74 ± 1.22
4.25	0.25	4.65 ± 0.01	5.56 ± 1.22	4.65 ± 0.01	6.48 ± 1.22	8.30 ± 1.22
4.5	0.5	9.22 ± 1.22	6.48 ± 1.22	9.22 ± 1.22	7.39 ± 0.01	13.78 ± 1.22
5	1	11.04 ± 1.22	6.48 ± 1.22	12.87 ± 0.01	10.13 ± 0.01	15.61 ± 1.83
6	2	18.35 ± 0.01	9.22 ± 1.22	20.18 ± 4.26	16.52 ± 3.05	21.09 ± 2.74
7	3	26.57 ± 1.83	7.39 ± 0.01	24.74 ± 1.22	22.92 ± 1.22	28.40 ± 1.22
8	4	32.97 ± 1.22	16.52 ± 4.87	29.31 ± 1.83	27.49 ± 3.05	32.05 ± 0.01
9	5	35.71 ± 1.22	9.22 ± 3.04	31.14 ± 1.22	31.14 ± 1.22	32.97 ± 1.22
		Grower phase				
18	0	1.43 ± 0.17	1.93 ± 0.09	1.90 ± 0.11	2.99 ± 0.36	2.15 ± 0.11
18.25	0.25	3.79 ± 0.64	4.04 ± 0.26	7.50 ± 0.53	7.63 ± 0.61	11.83 ± 1.59
18.5	0.5	5.11 ± 0.62	5.08 ± 0.18	11.48 ± 1.06	10.09 ± 0.53	15.03 ± 0.28
19	1	9.64 ± 1.22	9.59 ± 1.22	19.34 ± 1.08	15.41 ± 1.31	23.16 ± 0.43
20	2	16.04 ± 0.88	16.40 ± 2.07	31.69 ± 1.49	26.64 ± 2.79	36.62 ± 3.95
21	3	21.49 ± 1.21	24.37 ± 4.57	40.31 ± 2.91	34.85 ± 1.62	45.08 ± 2.13
22	4	25.93 ± 0.45	29.48 ± 1.24	45.24 ± 2.27	42.60 ± 1.26	51.07 ± 2.44
23	5	30.63 ± 1.00	37.17 ± 0.50	47.80 ± 1.57	46.71 ± 1.12	56.47 ± 3.79
		Finisher phase				
30	0	1.87 ± 0.06	1.82 ± 0.17	2.56 ± 0.24	3.65 ± 0.23	1.00 ± 1.22
30.25	0.25	3.83 ± 0.24	2.93 ± 0.16	10.07 ± 0.82	8.97 ± 0.18	11.96 ± 1.22
30.5	0.5	4.80 ± 0.48	3.93 ± 0.58	14.90 ± 2.12	12.09 ± 1.35	13.78 ± 2.44
31	1	7.20 ± 0.36	6.16 ± 1.07	23.16 ± 1.04	18.97 ± 0.63	23.83 ± 1.83
32	2	13.97 ± 0.29	12.13 ± 2.26	39.01 ± 2.27	32.71 ± 1.62	43.02 ± 0.01
33	3	18.32 ± 1.29	18.33 ± 3.24	50.32 ± 1.82	39.37 ± 1.09	55.81 ± 6.70
34	4	24.39 ± 0.79	21.96 ± 0.62	57.89 ±2.03	45.30 ± 3.24	72.26 ± 6.09
35	5	30.82 ± 1.60	23.98 ± 5.05	65.41 ± 0.76	60.33 ± 2.47	80.48 ± 1.22

Except for feathers, all tissues were better fitted in an exponential model. The L-[15N] threonine incorporation of jejunum, liver, and plasma in the starter phase were approximately 45% and about 60% higher than grower and finisher phases, respectively (table 3). In addition, we observed a reduction in the L-[15N] threonine incorporation rate for breast muscle as the age increased, being about 30 and 35% reduction respectively. The isotope abundances of feathers had some oscillations in the starter phase and its L-[15N] threonine incorporation rate increased 2.23 and 1.89 times comparing the starter phase to the grower and finisher phases, respectively.

Table 3. Paramenters (±SD) of L-[15N] threonine incorporation of broiler tissues in three different phases.

Tissues	Parameters					
	†b_0	‡b1	§b_S	§b_G	§b_F	R^2
Breast	0.44 ± 0.02	1.15 ± 0.03	-0.68 ± 0.07	-0.48 ± 0.05	-0.45 ± 0.04	0.96
Feathers	0.64 ± 0.03	-	0.09 ± 0.01	0.21 ± 0.01	0.17 ± 0.01	0.80
Jejunum	0.62 ± 0.04	1.11 ± 0.04	-0.40 ± 0.04	-0.74 ± 0.07	-1.27 ± 0.14	0.94
Liver	0.73 ± 0.03	1.01 ± 0.03	-0.28 ± 0.03	-0.50 ± 0.05	-0.74 ± 0.09	0.94
Plasma	0.85 ± 0.04	1.00 ± 0.05	-0.30 ± 0.04	-0.53 ± 0.08	-0.76 ± 0.13	0.90

Tissues	¶$b_{S/G}$	¶$b_{S/F}$	¶$b_{G/F}$
Breast	0.70	0.65	0.93
Feathers	2.23	1.89	0.80
Jejunum	0.54	0.31	0.58
Liver	0.56	0.38	0.68
Plasma	0.57	0.40	0.69

†*b0* is the basal enrichment transformed into log 10 values

‡*b1* is the difference between the highest and basal enrichments transformed into log 10 values

§b_S, b_G, and b_F are the stepness or slopes coefficients of the L-[15N] threonine incorporation in the starter, grower phases and finisher phases, respectively.

¶ $b_{S/G}$, $b_{S/F}$, and $b_{G/F}$ are the ratios of the stepness or slopes coefficients of the L-[15N] threonine incorporation in different phases.

The mass balances of L-[15N] threonine for different phases is presented in table 4. The recovery rate of all tissues increased with aging, except for free-feathers body and

jejunum. A difference in the recovery rate was detected for starter phase to finisher phase, while in feathers, the recovery rate in the starter phase differed from the other phases. A higher recovery rate was observed in liver in the starter phase in relation to the grower and finisher phase.

Table 4. Means (±SD) of L-[^{15}N] threonine deposition among in the tissues in mass (µg ^{15}N) and the recovery rate (in parenthesis, as %) of tracer intake in the peak enrichment (9, 23 and 35-d old).

Phase	Body	Breast	Feathers	Jejunum	Liver	Plasma
Starter	447±49 (67.7±7.4 a)	101±10 (15.3±1.5b)	21 ± 4 (3.5±1.3b)	9 ± 1 (1.4±0.2a)	16 ± 2 (2.4±0.3b)	6 ± 1 (1.8±0.3c)
Grower	2,685±171 (64.7±4.1a)	826±34 (19.9±0.8ab)	277±10 (6.7±0.2a)	58±5 (1.4±0.1a)	111±11 (2.7±0.3b)	69±4 (3.3±0.2b)
Finisher	6,390±293 (61.6±2.8a)	2,453±199 (23.7±1.9a)	837±72 (8.1±0.7a)	106±16 (1.0±0.2a)	494±8 (4.8±0.1a)	228±4 (4.4±0.1a)

†Means in columns with different letters are significantly different (P<0.05, Tukey's HSD test).

‡L-[^{15}N] threonine intake: starter phase (658±11µg); grower phase (4,145±40µg); and finisher phase (10,369±143µg).

§n=3.

4. Discussion

This study was designed in order to assess the L-[^{15}N] threonine incorporation in broiler tissues at different ages under two perspectives: comparing different phases of a given tissue, and according to the L-[^{15}N] threonine delievery among the tissues in a given time. We performed a course-time trial with the addition of L-[^{15}N] threonine on the diet during five days to represent the threonine metabolism with a focus on amino acid

deposition. Since threonine is an essential amino acid, it was expected that most of ^{15}N analyzed was still a threonine molecule in the deposition tissues and part of ^{15}N in metabolic tissues would represent products of threonine catabolism as well (e.g. glycine, serine, α-amino-β-ketobutyrate, NH_3) (Wu, 2003).

In the present study, we measured how much the L-[^{15}N] threonine incorporation changes with time by analyzing the isotope abundance of different broiler tissues in differnt ages. The incorporation ratio represents herein a comparison of how much lower or higher is the L-[^{15}N] threonine incorporation comparing two different phases and choosing a phase as a standard. Higher concentrations implies in a higher incorporation rate due to a higher ^{15}N deposition and *vice versa* considering a deposition tissues. However, when a metabolic tissue is assessed, higher concentrations implies in a slower incorporation due to a ^{15}N accumulation and *vice-versa*. The second perspective considers the distribution of L-[^{15}N] threonine among the tissues and how this distribution changes with aging.

It is worth to highlight that the dosage of tracer was based on the daily weight of the broilers in an attempt to obtain similar isotope dilution among them. Our attempt was succeed (Figure 1), since even after three days of L-[^{15}N] threonine addition the isotope abundance of feather-free body still remained similar among all phases. However, after five days adding the tracer in the diet, lower isotope abundances in whole body were obtained in the starter phase due to the higher metabolism compared to the older broilers, because there is an deacrease in the whole body protein synthesis and deposition and the difference between these rates decreases with aging as well (Millward, Bates, & Rosochacki, 1981; Muramatsu, Muramatsu, Okumura, & Tasaki, 1987; Holloszy & Nair, 1995). Differently from body, the isotope abundances of individual tissues presented different incorporations for each phase (Table 2). Studies that aimed the effect of age on

protein *turnover* in broilers mainly focused on whole body responses, while developmental changes in individual tissues are still not well known (Tesseraud, Peresson, & Chagneau, 1996).

A decreasing L-[^{15}N] threonine incorporation ratio was observed in the breast muscle as the broiler grows (Table 3), which means a lower L-[^{15}N] threonine incorporation rate in breast muscle of older broilers. Kang, Sund, & Swick (1985) also reported a decreasing protein *turnover* in the breast muscle according to the age for broiler from 1 to 42d of age and justified this fall by the substantial decrease of fractional protein synthesis rate (48.4 *vs.* 15.7%/d, i.e. about 300% lower) concomitantly with a lower decrease in fractional protein breakdown rate (15.6 *vs.* 12.1%/d, i.e. approximately 30% lower). Acar, Moran, & Mulvaney (1993) and Scheuermann, Bilgili, Hess, & Mulvaney (2003), evaluating the development of the breast muscle in broiler chickens, concluded that higher relative weight changes were observed in younger chicks than olders, which agrees with the 30% and 35% reduction observed in this study in the grower and finisher phases, respectively. Taking into account into the mass balance of L-[^{15}N] threonine, it was possible to observe that the L-[^{15}N] threonine distribution into the breast muscle increased about 4% in each phase. This is no surprising, since the weight of *Pectoralis major* is one of the most economically important traits in the poultry industry being improved by genectic selection and represented about 15 to 24% of L-[^{15}N] threonine deposition (Scheuermann *et al.*, 2003). This increase is justified by the hyperplasia and hypertrophy, and the last is responsible to the development post-hatch of muscle when there is a protein accretion from proliferation and fusion of muscle satellite cells, in which would demand more amino acids (Moss, 1968). Biologically, it may be interesting for broiler chickens to have a well-developed breast, since this tissue is the major endogenous

protein source in cases of amino acid deficiency or imbalance conditions (Waterlow, Garlick, & Mill, 1978; Macari, Furlan, & Gonzales, 2002).

Although it was not possible to fit the data of the starter phase for feathers, the slope of the isotope abundance would be clearly lower than others phases, meaning that the L-[^{15}N] threonine incorporation rate for feathers would be higher in the grower phase (18 to 30d) and lower in the initial phase (4 to 16d). Wheeler & Latshaw (1981) evaluated sulfur amino acid requeriments and reported a rapid feather growth between the second and third weeks in chicks, agreeing with the outcomes of the presente study. Moreover, the oscilations of the isotope abundances according to the time of feathers in the starter phase occurred probably due to the different patterns of development and growth of neoptile and teleoptile feathers, which corresponds to feathers formed in embryonic development that begin to grow at about day 5 of incubation and may reflect the isotopic composition of broiler breeder diet; and those produced after this first generation of feathers that would assimilate the isotopic composition of broiler diet, respectively (Leeson & Walsh, 2004; Yu *et al*., 2004). Considering the L-[^{15}N] threonine mass balance, feathers represented 3.5; 6.7; and 8.1% in starter, grower and finisher phases, and since the recovery rate in the started phase differed from others, it also indicate an increase in the delivery of amino acids for the feathers in the grower phase. The findings of Wecke, Khan, Sünder, & Liebert (2017) corroborates with our outcomes because these authors reported an increasing relative weight of feathers in relation to the body weight as the broiler chickens grew, which necessary means a higher amino acid demand and feathers are composed basically by proteins, about 90% or even more. (Fisher, Leeson, Morrison, & Summers, 1981).

Analyzing the patterns of the isotope abundances among jejunum, plasma, and liver, it was observed some similarities among these tissues, since they presented

proportional results comparing the isotope abundances in different phases and similar maximum ^{15}N enrichments. This is agreement with Bergström, Fürst, Noree, & Vinnars (1974) who claimed that the concentrations of essential amino acids in the liver are approximately the same in plasma. The connection among these tissues in to metabolize and transport amino acids before depositing for protein accretion, in tissues such as breast muscle or feathers, may explain these similar patterns of these metabolic tissues.

The L-[^{15}N] threonine incorporation in jejunum reduced 46% and 69% in grower and finisher phases respectively and there was no increase in the L-[^{15}N] threonine distribution for jejunum with aging, which could suggest an earlier development of this tissue. Palo, Sell, Piquer, Soto-Salanova, & Vilaseca (1995) found that the relative weight of all gastrointestinal segments, including jejunum, descreases with aging in broiler chickens. Moreover, Troni (2016) determined that among the tissues evaluated in this study, jejunum would be the first tissue to reach the maturity by determining the Gompertz parameters (P=28.48g; t*=16.58d; B=0.1035d^{-1}). Indeed, jejunum needs an early development since the small intestine is the gastrointestinal segment responsible for the major absorption of amino acids (Ribeiro & Moraes, 2017).

It is known that plasma and liver present a high metabolic activity (Pelicia *et al.*, 2018). The plasma presented the highest L-[^{15}N] incorporation reduction, being 43 and 60% lower than starter phase, however, its delivery increased with aging. Moreover, the liver had 44 and 62% reduction considering the same phases and finisher phase presented the highest L-[^{15}N] threonine incorporation distribution. These results suggest that although there was a lower incorporation, a higher delivery is also demanded in older broiler chickens. The increase in the whole body protein breakdown in older animals may be explain these results since liver and plasma play essential roles to catabolism the L-[^{15}N] threonine and excrete the ^{15}N (Millward, Bates, & Rosochacki, 1981; Muramatsu,

et al., 1987). In addition, although the small relative weight of the liver is responsible for about 25% of whole body catabolism in growing monogastric (Barraco, 2005). These outcomes help to understand the biological role, intrinsic feature, and even the dynamism of each tissue according to the age in relation to the threonine metabolism. In addition, it could be useful to comprehend changes in amino acid requirements tissue-specific according to the age.

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6. Conflict of interest:

The authors declare that they have no conflict of interest.

7. Animal welfare statement:

The authors confirm that the ethical policies of the journal have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes. All animal care procedures were approved by the institutional Animal Care and Use Committee under protocol nº 9999/14.

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CAPÍTULO 5

Implicações

O uso de aminoácidos marcados vem sendo empregado cada vez mais e nas mais diversas áreas, sendo que há muito tempo se provou o potencial desta ferramenta. Espera-se que com o aumento da produção nacional de compostos enriquecidos, novas aquisições de equipamentos como os espectrômetros de massas ou então avanços das análises isotópicas como o acoplamento de cromatrógrafo líquido gasoso em espectrômetro de massas de razão isotópica, possibilitem esclarecer questões bioquímicas e fisiológicas por trás de resultados até então empíricos em estudos relacionados a nutrição de frangos de corte.

Deste modo, no capítulo 2 é apresentado a padronização de uma metodologia na qual o traçador é incluído diluído em água e aplicado sob a ração. Esta via de administração seria mais representativa em relação a intravenosa (comumente utilizada em estudos isotópicos) para refletir o metabolismo do traçador, sendo também menos invasivo. Após a padronização, todos os experimentos seguintes foram realizados com esta metodologia. Ainda neste capítulo, vimos que um aumento proporcional das dosagens resultaram em enriquecimentos isotópicos proporcionais, o que permitiria reavaliar as dosagens padrões utilizadas nestes tipos de estudos com a finalidade de redução de custos.

A seguir, no capítulo 3, é utilizado metionina e cistina marcadas com o objetivo de analisar a utilização dos aminoácidos sulfurados variando-se a sua proporção. Deste modo, foi determinado uma proporção ótima de metionina e cistina, enfocando-se aspectos fisiológicos que permitiram fracionar a utilização

dos aminoácidos sulfurados, incluindo uma taxa de conversão de metionina em cistina, questão que têm gerado muitas dúvidas e divergências há muitos anos.

Por fim, no capítulo 4, foi abordado um estudo capaz de se avaliar a incorporação da treonina marcada em diferentes tecidos e idades. Os resultados deste estudo permite um maior conhecimento a respeito da incorporação da treonina marcada assim como sua distribuição em diferentes tecidos de frangos de corte.

Portanto, os estudos que compõe esta tese permitiram a padronização de metodologias, possibilidades de redução de custos relacionados ao composto marcado e a descoberta de resultados fisiológicos que poderão ser aplicados a nutrição de frangos de corte.