UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CÂMPUS DE JABOTICABAL

RELAÇÃO IDEAL DOS AMINOÁCIDOS ESSENCIAIS PARA MANTENÇA, CRESCIMENTO E PRODUÇÃO DE AVES

Juliano Cesar De Paula Dorigam
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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 Doutor em Zootecnia.

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TÍTULO: RELAÇÃO IDEAL DOS AMINOÁCIDOS ESSENCIAIS PARA MANTENÇA, CRESCIMENTO E PRODUÇÃO DE AVES

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Jaboticabal, 26 de fevereiro de 2016.

DADOS CURRICULARES DO AUTOR

JULIANO CESAR DE PAULA DORIGAM – filho de Jose Carlos Dorigan e Apparecida Francisca de Paula Dorigam, nasceu no dia 20 de setembro de 1984, na cidade de Jaboticabal, São Paulo. Em fevereiro de 2005 ingressou no curso de Zootecnia na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista – Campus de Jaboticabal, São Paulo, graduando-se em abril de 2010. Durante o período de agosto de 2006 a julho de 2009 foi bolsista de iniciação científica pelo CNPq, sob orientação da Profª. Drª. Nilva Kazue Sakomura. De maio de 2010 a março de 2011 foi bolsista de treinamento Técnico nível III pela FAPESP, sob orientação da Profª. Drª. Nilva Kazue Sakomura. Em março de 2011 iniciou o curso de Mestrado em Zootecnia na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista – Campus de Jaboticabal, São Paulo, onde obteve bolsa pela FAPESP, sob orientação da Profª. Drª. Nilva Kazue Sakomura, defendendo a sua disseração em setembro de 2012. Em março de 2013 iniciou o curso de Doutorado em Zootecnia na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista – Campus de Jaboticabal, São Paulo, onde obteve bolsa pela FAPESP, sob orientação da Profª. Drª. Nilva Kazue Sakomura. De agosto a novembro de 2015, realizou parte do doutorado na universidade Georg-August em Göttingen, Alemanha, sob orientação do Prof. Frank Liebert. Em 2016 defendeu sua tese de doutorado como requisito para obtenção do título.

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"Se você encontrar um caminho sem obstáculos,
ele provavelmente não leva a lugar nenhum."
(Frank A. Clark)

"Uma vida sem desafios não vale a pena ser vivida."
(Sócrates)

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CERTIFICADO

Certificamos que o Protocolo nº 009999/14 do trabalho de pesquisa intitulado **"Modelagem da produção e das exigências nutricionais de aves e peixes – Determinação relação ideal dos aminoácidos para aves de corte e postura de peixes"**, 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 06 de junho de 2014.

Jaboticabal, 06 de junho de 2014.

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RELAÇÃO IDEAL DOS AMINOÁCIDOS ESSENCIAIS PARA MANTENÇA, CRESCIMENTO E PRODUÇÃO DE AVES

RESUMO – O tradicional método dose-resposta usado para determinar a relação ideal dos aminoácidos essenciais (IAAR) tem sido considerado muito oneroso, principalmente quando reprodutores são usados, pois é necessário um ensaio para cada aminoácido essencial (EAA). Por outro lado, o método da deleção tem sido considerado um meio rápido e prático para determinar a IAAR, pois apenas um ensaio é necessário. Dessa forma, o objetivo desta tese foi determinar a relação ideal dos aminoácidos essenciais para reprodutores (matrizes e galos) e frangos de corte utilizando o método da deleção. O primeiro estudo teve como objetivo estimar o perfil de aminoácidos essenciais e a relação ideal para a mantença de aves pelo método da deleção. Um ensaio de balanço de nitrogênio foi realizado utilizando 198 galos adultos, alojados individualmente em gaiolas metabólicas. Os tratamentos foram 33 dietas purificadas sendo 11 dietas com uma mistura de aminoácidos que fornecem alto consumo de proteínas de 500 mg N /$BW_{kg}^{0.75}$ por dia, 11 dietas que fornecem a ingestão média de proteína de 250 mg N /$BW_{kg}^{0.75}$ por dia (em cada dieta um aminoácido testado foi reduzido a 50%) e 11 dietas proporcionando baixa ingestão de proteína de 125 mg de N /$BW_{kg}^{0.75}$ por dia (formulada omitindo o aminoácido testado). Cada tratamento teve seis repetições. Após 48 h de jejum, recebendo água mais sacarose, os galos foram alimentados com 40 g das dietas por tubo, uma vez por dia, durante três dias. As excretas foram coletadas no prazo de 72 horas após a primeira ingestão. As dietas e as excretas foram analisadas quanto ao teor de nitrogênio. Para cada um dos aminoácidos estudados, uma regressão linear foi ajustada entre balanço de nitrogênio e ingestão do aminoácido. As exigências de mantença foram estimadas como a ingestão de aminoácidos para manter o equilíbrio de nitrogênio igual a zero. As exigências diárias dos aminoácidos para mantença foram estimadas em: Lys 11, Met 29, Thr 23, Trp 5, Arg 50, Val 29, His 6, Gly 54, Phe 49, Leu 78 e Ile 21 mg /$BW_{kg}^{0.75}$ por dia. Portanto, concluiu-se que a proporção de aminoácidos para mantença seria Lys 100, Met 276, Thr 220, Trp 48, Arg 467, Val 275, His 60, Gly 511, Phe 467, Leu 735 e Ile 198% independente da unidade usada. O perfil de aminoácidos essenciais e a relação ideal para mantença das aves, estimado neste estudo, contribui para a melhoria do modelo fatorial para estimar exigências de aminoácidos essenciais para aves. No segundo estudo, duas abordagens utilizando o método da deleção dos aminoácidos (AA), um com abate comparativo e outro com balanço de nitrogênio, foram utilizados para reavaliar as pressupostas relações ideais entre os aminoácidos essenciais (EAA): lisina (Lys), metionina+cistina (Met+Cys), treonina (Thr), triptofano (Trp), arginina (Arg), valina (Val), isoleucina (Ile), leucina (Leu), fenilalanina + tirosina (Phe+Tyr), glicina + serina (Gly+Ser), e histidina (His) para o crescimento de frangos de corte do genótipo Cobb 500 durante três períodos (I: 6 a 21, II: 22 a 37, e III: 38-53 d). Por ensaio, 120 frangos, machos, foram alojados em gaiolas metabólicas para avaliação dos dados de balanço de nitrogênio e eficiência de utilização de cada AA. Uma dieta balanceada em AA (BD) foi formulada de acordo com as recomendações das tabelas brasileiras (Rostagno et al., 2011) para a proteína ideal de frangos de corte em crescimento. As dietas com diferentes AAs limitantes foram criadas pela

deleção da BD com amido de milho para alcançar 0,70 do nível de AA como BD e resuplementado com AA cristalinos, exceto o AA em estudo. As dietas com a deleção de cada AA levaram a uma piora significativa na utilização da proteína e indicou posição limitante válida destes AA. Além disso, no início e no final do ensaio, um grupo de aves com peso médio de cada repetição foi abatida sem perda de sangue para determinar a deposição de nitrogênio pela técnica do abate comparativo. O valor médio das relações ideais dos onze EAA testados, determinadas pelo abate comparativo, nos três períodos foram: Lys 100, Met+Cys 65, Thr 66, Trp 17, Arg 108, Val 79, Ile 61, Leu 122, Phe+Tyr 128, Gly+Ser 155, e His 41. Com base nos dados de eficiência dos AA observados, as relações ideais determinadas pelo balanço de nitrogênio foram: Lys 100, Met+Cys 72, Thr 65, Trp 17, Arg 106, Val 76, Ile 67, Leu 107, Phe + Tyr 115, Gly+Ser 137, e His 35. Existem algumas diferenças entre os resultados obtidos pelos dois métodos (Louvain e Goettingen), mas o método de Goettingen apresentou resultados mais condizentes com a literatura e menor variação nos resultados. No terceiro estudo, o objetivo foi determinar os parâmetros do modelo para a máxima retenção de nitrogênio ($NR_{max}T$), a exigência de mantença de nitrogênio (NMR) e a eficiência de utilização de lisina (bc^{-1}) para determinar a exigência de lisina (Lys) de aves reprodutoras pesadas. Os ensaios de balanço de nitrogênio foram realizados em dois períodos (I: 31-35 semanas e II: 46-50 semanas). Foram utilizados sete tratamentos com oito repetições e uma ave por gaiola; os tratamentos consistiram de sete dietas com níveis de proteína variando de 58,8-311,9 g/kg de ração, com a Lys sendo limitante na proteína dietética (c = 3,91 g de Lys em 100 g de CP). Para cada período, os dados de nitrogênio ingerido (NI), nitrogênio excretado (NEX), nitrogênio na massa de ovos (NEM), nitrogênio depositado (ND, ND = NI-NEX) e nitrogênio retido (NR, NR = ND + NEM + NMR) foram obtidos num ensaio de balanço de nitrogênio de 25 dias. A NMR foi calculada pela relação exponencial entre NEX e NI. O $NR_{max}T$ e o b (inclinação relacionadas com a qualidade da proteína) foram estimados pelo ajuste exponencial entre NR e NI. Foi obtido o bc^{-1} dividindo b por c. Com base no teste da razão de verossimilhança para os parâmetros do modelo, os valores obtidos foram 255 mg /$BW_{kg}^{0.67}$ para NMR, 0,000117 para b e 1684 mg/$BW_{kg}^{0.67}$ (período I) e 1484 mg/$BW_{kg}^{0.67}$ (período II) para $NR_{max}T$. As ingestões de Lys foram estimadas pela função $Lys = (\ln (NR_{max}T) - \ln (NR_{max}T - NR)):(16 \times bc^{-1}))$ função, que resultou nas ingestões de Lys de 915 e 876 mg/d para matrizes nos períodos I e II, respectivamente. O estudo conclui que a exigência Lys ideal esta de acordo com os dados da literatura, mas as recomendações podem ser adaptadas de acordo com o consumo de ração, a deposição de proteína desejada e a eficiência de utilização do AA na dieta. No quarto estudo, o objetivo foi aplicar os dados de eficiência (bc^{-1}) dos AAs lisina (Lys), metionina+cistina (Met+Cys), treonina (Thr), triptofano (trp), arginina (Arg), valina (Val), isoleucina (Ile), leucina (Leu), fenilalanina+tirosina (Phe+Tyr), glicina + serina (Gli+Ser) e histidina (His) para obter uma relação ideal de AA (IAAR) para matrizes. Os ensaios de balanço de nitrogênio foram realizados de 31 a 35 semanas e de 46 a 50 semanas. Foram utilizados doze tratamentos com oito repetições e uma ave por gaiola. Uma dieta balanceada (BD) foi formulada para atender a IAAR e a exigência de outros nutrientes para matrizes. As dietas limitantes foram formuladas diluindo BD com amido de milho e resuplementados com AAs cristalinos e outros ingredientes para alimentação animal, com exceção do AA em estudo. Em cada período, os dados de nitrogênio ingerido (NI), nitrogênio excretado (NEX), nitrogênio na massa de ovo (NEM), nitrogênio depositado (ND, ND = NI-NEX)

e nitrogênio retido (NR, NR = ND + NEM + NMR) foram obtidos em um ensaio de 25 dias. Os valores de qualidade de proteínas (b) foram estimadas por b = (ln ($NR_{max}T$) - ln ($NR_{max}T$-NR)) : (NI)), onde $NR_{max}T$ é o potencial para a retenção máxima de nitrogênio de matrizes. Os valores de bc^{-1} foram obtidos dividindo b pela concentração do AA na dieta (c, g AA / 16 g de N). A posição limitante de cada AA foi confirmada e os valores de bc^{-1} foram usados para obter um IAAR média: Lys (100), Met + Cys (83), Trp (24), Thr (81), Arg (114), Val (90), Ile (93), Leu (105), Phe + Tyr (109), Gly + Ser (95), e His (35). A IAAR estava de acordo com a recomendação da literatura, validando este procedimento alternativo para a determinação da IAAR para aves reprodutoras pesadas. Finalmente, o objetivo deste quinto estudo foi aplicar o método da deleção para obter uma IAAR para aves reprodutoras pesadas. Os ensaios de balanço de nitrogênio foram realizados de 31 a 35 semanas e de 46 a 50 semanas. Foram utilizados doze tratamentos com oito repetições e uma ave por gaiola. Uma dieta balanceada (BD) foi formulada para atender rigorosamente a IAAR e a exigência de outros nutrientes. As dietas limitantes foram formuladas diluindo BD com amido de milho e resuplementados com aminoácidos cristalinos (EAA) e outros ingredientes para alimentação animal, com exceção do EAA em estudo. Cada ensaio durou 25 dias. As perdas de penas, a produção de ovos e o peso do ovo foram registrados diariamente e as amostras foram armazenadas para determinar NEM e o nitrogênio nas perdas de penas (NDFL), respectivamente. No início e no final de cada período, um grupo de matrizes foi abatido para determinar o nitrogênio depositado no corpo (NDB) e penas (NDF). A NR foi calculada como a soma de NDB, NDF, NDFL, NEM, e a exigência de nitrogênio para mantença (NMR = 255 mg /$BW_{kg}^{0.67}$ por dia). A redução percentual no NR resultante da deleção de cada EAA em relação à BD e o percentual do AA para excluir a partir da BD foram utilizados para calcular a exigência ótima do AA na dieta. A IAAR média determinada foi: Lis (100), Met + Cys (86), Trp (23), Thr (80), Arg (113), Vai (90), Ile (91), Leu (133), Phe + Tyr (108), Gly+Ser (94), e His (35). A IAAR determinada neste estudo estava de acordo com a recomendação da literatura para frangos de corte, especialmente usando o método de Goettingen, validando o método da deleção para determinar o IAAR. Além disso, o método foi padronizado para matrizes reprodutoras e os resultados deste estudo permitiram atualizar a relação ideal para estas aves. Da mesma forma, o método da deleção para determinar a IAAR de mantença também foi padronizado e o perfil ideal atualizado. Por fim, a padronização destes métodos vai permitir que a pesquisa brasileira usufrua de um procedimento rápido e de baixo custo para estimar e avaliar a IAAR.

Palavras-chave: Aminoácidos, balanço de nitrogênio, método da deleção, relação ideal

THE IDEAL ESSENTIAL AMINO ACID RATIO FOR MAINTENANCE, GROWTH AND PRODUCTION OF POULTRY

ABSTRACT - The traditional dose-response method used to determine the ideal essential amino acid ratio (IAAR) has been considered too costly, especially when breeders are used, because an assay for each essential amino acid (EAA) is necessary. On the other hand, the deletion method has been considered a quick and practical way to determine the IAAR, because only one assay is required. Thus, the aim of this thesis was to determine the optimal ratio of essential amino acids for breeders (broiler breeder hens and roosters) and broilers using the deletion method. The first study aimed to estimate the essential amino acid profile and the ideal ratio for maintenance of poultry by deletion method. A nitrogen (N) balance trial was conducted using 198 adult roosters, housed individually in metabolic cages. The treatments were 33 purified diets being 11 diets with an EAA mixture providing high protein intake of 500 mg $N/BW_{kg}^{0.75}$ per day, 11 diets providing medium protein intake of 250 mg $N/BW_{kg}^{0.75}$ per day (in each diet one EAA tested was reduced 50%) and 11 diets providing low protein intake of 125 mg $N/BW_{kg}^{0.75}$ per day (made by omitting the EAA tested). Each treatment had six replicates. After 48 h of fasting receiving water plus sucrose, the roosters were fed 40 g of the diets by tube once a day for three days. The excreta were collected within 72 h after the first feeding. The diets and excreta were analyzed for nitrogen content. For each EAA studied, a linear regression was fitted by N balance and EAA intake. The maintenance requirements were estimated as the EAA intake to maintain the N balance equal to zero. The daily EAA requirements for maintenance were estimated to be: Lys 11, Met 29, Thr 23, Trp 5, Arg 50, Val 29, His 6, Gly 54, Phe 49, Leu 78 and Ile 21 $mg/BW_{kg}^{0.75}$ per day. Therefore, the EAA ratio for maintenance was concluded to be Lys 100, Met 276, Thr 220, Trp 48, Arg 467, Val 275, His 60, Gly 511, Phe 467, Leu 735 and Ile 198% independent of the scale. The EAA profile and the ideal ratio for maintenance of poultry estimated in this study contribute to improve factorial model for estimating EAA requirements for poultry. In the second study, two approaches using amino acid deletion method, one with comparative slaughter and another with N balance, were used to re-evaluate the actual assumptions of ideal ratios between the EAA: lysine (Lys), methionine+cystine (Met+Cys), threonine (Thr), tryptophan (Trp), arginine (Arg), valine (Val), isoleucine (Ile), leucine (Leu), phenylalanine+tyrosine (Phe+Tyr), glycine+serine (Gly+Ser), and histidine (His) for growing broilers of Cobb 500 genotype during three periods (I: 6 to 21, II: 22 to 37, and III: 38 to 53 d). Per trial, 120 male chickens were housed in metabolic cages for assessment of individual N-balance and AA efficiency data. An AA balanced diet (BD) was formulated according to recommendations of Brazilian tables (Rostagno et al., 2011) for the ideal protein in growing broilers. The diets with different limiting AAs were created by dilution of BD with corn starch to achieve 0.70 of the AA level in BD and supplemented with crystalline AAs, except the AA under study. The AA diluted diets led to significant impairment of protein utilization and indicated valid limiting position of these AAs. Also, at start and the end of the trial a group of birds with mean body weight of each replicate was killed with no blood loss to determine nitrogen deposition by comparative slaughter technique. The mean value of the optimum ratios

of the eleven tested EAAs determined by comparative slaughter in the three periods are: Lys100, Met+Cys 65, Thr 66, Trp 17, Arg 108, Val 79, Ile 61, Leu 122, Phe+Tyr 128, Gly+Ser 155, and His 41. Based on observed AA efficiency data, the optimum ratios determined by nitrogen balance are: Lys 100, Met+Cys 72, Thr 65, Trp 17, Arg 106, Val 76, Ile 67, Leu 107, Phe+Tyr 115, Gly+Ser 137, and His 35. There are some differences among the results obtained by the two methods (Louvain and Goettingen approach), but the Goettingen approach provided result in accordance with the literature and less variation in the results. The third study aimed to determine the model parameters for maximum nitrogen retention ($NR_{max}T$), nitrogen maintenance requirement (NMR) and the efficiency of lysine utilization (bc^{-1}) to determine the lysine (Lys) requirements of broiler breeder hens. The N balance trials were performed in two periods (I: 31-35 wks and II: 46-50 wks). Seven treatments were used with eight replicates and one hen per cage; the treatments consisted of seven diets with protein levels ranging from 58.8 to 311.9 g/kg of feed, with Lys being limiting in the dietary protein (c = 3.91 g of Lys in 100 g of CP). For each period, the data of nitrogen intake (NI), nitrogen excretion (NEX), nitrogen in egg mass (NEM), nitrogen deposition (ND, ND=NI-NEX) and nitrogen retention (NR, NR=ND+NEM+NMR) were obtained in a balance trial of 25 days. The NMR was calculated by the exponential relationship between NEX and NI. The NRmaxT and b (slope related to protein quality) were estimated by the exponential fit between NR and NI. The bc^{-1} was obtained dividing b by c. Based on the likelihood ratio test for the model parameters, the obtained values were 255 mg/$BW_{kg}^{0.67}$ for NMR, 0.000117 for b and 1684 mg/$BW_{kg}^{0.67}$ (period I) and 1484 mg/$BW_{kg}^{0.67}$ (period II) for NRmaxT. The Lys intakes were estimated by the function $Lys = (\ln(NRmaxT)-\ln(NRmaxT-NR)):(16\times bc^{-1}))$, which resulted in the Lys intakes of 915 and 876 mg/d for breeder hens in the periods I and II, respectively. The current study concludes that the optimal Lys requirement is in range with literature data, but the recommendations can be adapted according to feed intake, aimed protein deposition and dietary AA efficiency. The fourth study aimed to apply the individual AA efficiency data (bc^{-1}) for lysine (Lys), methionine+cystine (Met+Cys), threonine (Thr), tryptophan (Trp), arginine (Arg), valine (Val), isoleucine (Ile), leucine (Leu), phenylalanine+tyrosine (Phe+Tyr), glycine+serine (Gly+Ser) and histidine (His) to derive an ideal AA ratio (IAAR) for breeder hens. N-balance trials were performed from 31 to 35 wks and from 46 to 50 wks. Twelve treatments with eight replicates and one hen per cage were used. A balanced diet (BD) was formulated to meet the IAAR and the requirement of other nutrients for breeder hens. The limiting diets were formulated diluting BD with corn starch and refilled with crystalline AAs and other feed ingredients, except for the AA under study. In each period, the data of N-intake (NI), N-excretion (NEX), N in egg mass (NEM), N-deposition (ND, ND=NI-NEX) and N-retention (NR, NR=ND+NEM+NMR) were obtained in a balance trial of 25 days. The b values (protein quality) were estimated by $b = (\ln(NR_{max}T)-\ln(NR_{max}T-NR)):(NI))$, where NRmaxT is the potential for maximum nitrogen retention of breeder hens. The bc^{-1} values were obtained dividing b by the dietary AA concentration (c, g AA/16g N). The limiting position of each AA was confirmed and the bc^{-1} values were used to obtain an average IAAR: Lys (100), Met+Cys (83), Trp (24), Thr (81), Arg (114), Val (90), Ile (93), Leu (105), Phe+Tyr (109), Gly+Ser (95), and His (35). The IAAR was in the line with the recommendation from the literature, validating this alternative procedure for predicting dietary IAAR for broiler breeder hens. Finally, the aim of the fifith study was to apply the deletion method to derive an IAAR for broiler breeder hens. The nitrogen balance

trials were performed from 31 to 35 wks and from 46 to 50 wks. Twelve treatments with eight replicates and one hen per cage were used. A balanced diet (BD) was formulated to strictly meet the IAAR and the requirement of other nutrients. The limiting diets were formulated diluting BD with corn starch and refilled with crystalline amino acids (AA) and other feed ingredients, except for the AA under study. Each feeding trial lasted 25 days. The feather losses, egg production and egg weight were recorded daily and the samples were stored to further determine NEM and nitrogen in feather losses (NDFL), respectively. At the start and the end of each period, a group of breeder hens were slaughtered to further determine nitrogen deposition in the body (NDB) and feathers (NDF). The NR was calculated as the sum of NDB, NDF, NDFL, NEM, and the nitrogen maintenance requirement (NMR=255 mg/$BW_{kg}^{0.67}$ per day). The percent reduction in NR resulting from the individual AA deletions relative to BD and the percent of the AA to delete from the BD were used to calculate the optimum in-feed AA requirement. The average IAAR determined was: Lys (100), Met+Cys (86), Trp (23), Thr (80), Arg (113), Val (90), Ile (91), Leu (133), Phe+Tyr (108), Gly+Ser (94), and His (35). The IAAR determined in this study corroborate with the recommendations for broilers in the literature, particularly when using the Goettingen approach, validating the deletion method for determining the IAAR. In addition, the method has been standardized for broiler breeder hens and the results of this study allowed updating the ideal ratio for these birds. Likewise, the deletion method for determining the IAAR for maintenance was also standardized and the ideal profile updated. Finally, the standardization of these methods will allow Brazilian research enjoy a rapid and low-cost procedure to estimate and evaluate the IAARs.
Keywords: Amino Acids, deletion method, ideal ratio, nitrogen balance

LISTA DE ABREVIATURAS

AA – Amino acid (aminoácidos);

AAI – Amino acid intake (ingestão de aminoácidos);

AA_{test} – Amino acid test (aminoácido avaliado)

AA_{BD} – Amino acid in the balanced diet (Aminoácido na dieta balanceada)

Arg – Arginine (Arginina);

b – Slope of the exponential function that indicates the protein quality (inclinação da função exponencial que indica a qualidade da proteína);

bc^{-1} – Eficiency of amino acid utilization (eficiência de utilização do aminoácido);

BCAA – Branched chain amino acids (aminoácidos de cadeia ramificada);

BD – Balanced diet (dieta balanceada);

BP – Body protein (peso proteíco);

BP_m – Body protein at maturity (Peso proteíco à maturidade);

BW – Body weight (Peso corporal);

c – Concentration of the amino acid in the dietary protein (concentração do aminoácido na proteína dietética);

CP – Crude protein (proteína bruta);

Cys – Cystine (cistina);

DEL – Deletion rate (taxa de deleção);

e – Euler number (número de Euler);

EAA – Essential Amino acid (aminoácidos essenciais);

EAA_{BD} – Essential amino acid content in the balanced diet (Conteúdo do aminoácido essencial obtido com a dieta balanceada);

FI – Feed intake (consumo de ração);

Gly – Glycine (glicina);

His – Histidine (histidina);

HP – High protein diet (alto teor de proteína);

HPLC - high-performance liquid chromatography (cromatografia líquida de alto desempenho);

IAAR – Ideal amino acid ratio (Relação ideal de aminoácidos);

Ile – Isoleucine (isoleucina);

LAAI – Limiting amino acid intake (consumo do aminoácido limitante);

Leu – Leucine (leucina);

ln – Natural logarithm (logarítimo natural);

LP – Low protein diet (baixo teor de proteína);

Lys – Lysine (lisina);

ME – metabolizable energy (energia metabolizável);

Met – Methionine (metionina);

MP – Medium protein diet (médio teor de proteína);

N – Nitrogen (nitrogênio);

NB – nitrogen balance (balanço de nitrogênio);

ND – Nitrogen deposition (Nitrogênio depositado);

NDB – Nitrogen deposition in the body (Nitrogênio depositado no corpo);

NDF – Nitrogen deposition in the feathers (Nitrogênio depositado nas penas);

NDFL – Nitrogen deposition in the feather losses (Nitrogênio depositado nas perdas de penas);

$\mathbf{ND_{BD}}$ – Nitrogen deposition in birds feed balanced diet (Nitrogênio depositado em aves alimentadas com a dieta balanceada);

$\mathbf{ND_{EAA}}$ – Nitrogen deposition in birds feed limiting diets (Nitrogênio depositado em aves alimentadas com dietas limitantes);

NEM – Nitrogen in egg mass (nitrogênio na massa de ovo);

NEX – Nitrogen excretion (Nitrogênio excretado);

$\mathbf{N_f}$ – Body nitrogen content at the end of the experiment (teor de nitrogênio no corpo das aves no final do experimento);

NF – Nitrogen-free diet (dieta isenta de nitrogênio);

$\mathbf{N_i}$ – Body nitrogen content at the start of the experiment (teor de nitrogênio no corpo das aves no início do experimento);

NI – Nitrogen intake (nitrogênio ingerido);

NMR – Nitrogen maintenance requirement (exigência de nitrogênio para mantença);

NR – Nitrogen Retention (nitrogênio retido);

$\mathbf{NR_{max}T}$ – Maximum theoretical nitrogen retention (máxima retenção teórica de nitrogênio);

PER – Protein efficiency ratio (taxa de eficiência protéica);

Phe – Phenylalanine (fenilalanina);

R^2 - coefficient of determination (coeficiente de determinação);

RSME – Root square of the mean error (raiz quadrada do erro);

SD – Standard deviation (desvio padrão);

Ser – Serine (serina);

Thr – Threonine (treonina);

Trp – Tryptophan (triptofano);

TSAA – Total sulfur amino acids (aminoácidos sulfurados totais);

Tyr – Tyrosine (tirosina);

Val – Valine (valina);

W_f – Final body weight (peso corporal final);

W_i – Initial body weight (peso corporal inicial);

Δt – Duration of the feed period (duração no fornecimento da ração);

μ - Maturity rate (BP/BP_m) (taxa de maturidade);

Ω – Unrestricted model (modelos irrestritos);

ω_1, ω_2, ω_3 – Restricted models (modelos restritos);

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CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1.1. INTRODUÇÃO

Para a indústria avícola brasileira continuar em uma posição de destaque no competitivo mercado mundial, torna-se necessário o gerenciamento de pontos estratégicos dentro da cadeia produtiva avícola. Uma destas estrátegias é garantir que haja quantidade necessária de aves de corte para o abate a qual depende principalmente do desempenho reprodutivo e a qualidade de ovos das matrizes. Fatores relacionados à nutrição das matrizes afetam a produção e qualidade do ovo, a qual reflete no desenvolvimento inicial das aves e, consequentemente, em todo o crescimento do frango de corte (Pedroso et al., 2005).

A nutrição das matrizes é importante para produzir pintos de alta qualidade, entretanto, com o passar dos anos é possível observar que os níveis nutricionais propostos para as matrizes de corte permaneceram praticamente inalterados. Baião & Lúcio (2005) citam que isto se deve a uma pausa nas pesquisas com matrizes pesadas, sobretudo, em relação as exigências específicas de aminoácidos essenciais (EAA). A carência em estudos torna-se ainda maior quando nos referimos aos galos reprodutores, que no cenário atual, tem sido deixado em segundo plano (Danikowski et al., 2002). A escassez de informações sobre a exigência, sobretudo de EAA, constitui uma das principais razões da resistência por parte dos produtores em adotar um programa nutricional específico para galos reprodutores (Couto et al., 1998). As linhagens de frango de corte comercialmente disponíveis apresentam potenciais genéticos diferentes devido a pressão de seleção aplicada nas características de interesse econômico como o ganho de peso, por exemplo. Isto faz com que as exigências em EAA para atender o máximo desempenho se tornem específicas para cada genótipo e, uma vez que a alimentação representa cerca de 70% da cadeia produtiva avícola (Borges et al., 2006), devemos dar especial atenção a este segmento.

Frangos de corte têm sido criados de norte a sul do País e, diante da diversidade climática e produtiva do Brasil, torna-se um desafio para nutricionistas estabelecerem as exigências para maximizar o desempenho de frangos de corte. Uma

solução razoável para contornar essa situação tem sido o uso do conceito de proteína ideal na formulação de rações. Este conceito preconiza a existência de um perfil de EAA intimamente relacionados. Diante as adversidades nos diferentes ambientes de criação, o uso da relação ideal nas estimativas das exigências é mais estável em comparação a outros métodos usados para estabelecer as exigências. Dietas formuladas no conceito de proteína ideal, para frangos de corte, têm se tornado uma prática cada vez mais comum, com bons resultados de desempenho e rendimento das aves.

Embora o conceito da proteína ideal esteja difundido, alguns aspectos conceituais podem ser aperfeiçoados com base na abordagem fatorial. Um destes aspectos é a diferenciação do perfil de aminoácidos exigidos para crescimento (corpo e penas) e para mantença como sugerido por pesquisadores em épocas passadas (Hurwitz, 1985). Alguns pesquisadores concordam que a relação ideal entre os EAA sofre mudança durante a fase de crescimento e a principal causa da mudança é o aumento na proporção da exigência de mantença sobre o total exigido pela ave (Baker et al., 1996). Dessa forma, o perfil de EAA necessário para mantença é diferente do perfil necessário para crescimento ou produção (Jansen, 1974) e devem ser considerados separadamente.

A relação ideal dos EAA para crescimento/produção e mantença são importantes e complementares no entendimento sobre a utilização dos EAA pelas aves. Na presente pesquisa as relações para crescimento/produção e mantença serão determinadas em um único ensaio de curta duração pelo método da deleção. Este método se baseia na pressuposição que a remoção de um aminoácido não limitante não modifica a resposta das aves. Entretanto, a mudança na resposta da ave com a deleção de uma proporção específica do aminoácido teste é usada para calcular o perfil ideal dos EAA em uma dieta em que os EAA são igualmente limitantes (Wang & Fuller, 1989). Além disso, a praticidade e o baixo custo tornam esta metodologia digna de ser explorada, uma vez que pelos métodos tradicionais necessitaria realizar 33 ensaios de dose resposta, 11 para cada categoria (matrizes, galos e frangos) para obter o mesmo resultado conforme proposto nesta pesquisa com a realização de três ensaios. Dessa forma, este estudo teve o objetivo de determinar o perfil ideal de EAA para reprodutores e frangos de corte utilizando o método da deleção.

1.2. REVISÃO DE LITERATURA

1.2.1. Introdução do conceito da proteína ideal

O conceito de proteína ideal foi inicialmente definido por Mitchell (1964) como o balanço ideal de aminoácidos que atende às exigências dos animais tanto para mantença quanto para o crescimento. Tentativas já foram feitas para determinar um perfil ideal de aminoácidos através da composição da carcaça (Robel & Menge, 1973). Apesar da composição da carcaça ser utilizada como referência para uma relação ideal, ela não leva em consideração a dinâmica do crescimento animal como, por exemplo, o custo de mantença. Dean & Scott (1965) foram os primeiros a usar dietas experimentais nos estudos da determinação da relação ideal. Desde então vários estudos tentaram determinar a relação ideal de aminoácidos para aves e suínos (Fuller et al., 1989; Wang & Fuller, 1989; Baker & Han, 1994; Mack et al., 1999; Baker et al., 2002). A partir desse conceito foi possível estudar a deposição de proteína dos diferentes tecidos e também avaliar a mudança de proporção dos aminoácidos de acordo com o crescimento do animal.

O primeiro perfil de proteína ideal para frangos de corte foi publicado por Baker & Han (1994), baseados em estudos utilizando dietas purificadas, ou seja, com nitrogênio proveniente de aminoácidos sintéticos que são 100% digestíveis (Faria Filho & Torres, 2007). A relação entre estes aminoácidos é calculada como porcentagem da lisina, um aminoácido essencial que está entre os primeiros limitantes em dietas de animais monogástricos. Segundo Baker & Han (1994), a lisina é utilizada como aminoácido referência por três razões principais: (1) sua análise nos alimentos é relativamente simples, diferente do triptofano e dos aminoácidos sulfurados; (2) há uma grande quantidade de dados existentes sobre a digestibilidade da lisina em aves; (3) diferente de vários aminoácidos, a lisina é utilizada principalmente para acréscimo de proteína corporal. Dessa forma, a proteína ideal poder ser facilmente adaptada a uma variedade de situações já que as proporções ideais dos aminoácidos são as mesmas independente de alterações nos planos de nutrição dos aminoácidos.

A grande vantagem da aplicação do conceito da proteína ideal é que as relações dos aminoácidos essenciais com a lisina não são influenciadas por fatores

que normalmente afetam as exigências de aminoácidos como densidade energética, nível proteico, potencial genético e ambiente (Sakomura & Rostagno, 2007). Outro benefício do uso do conceito da proteína ideal é que ele permite reduzir a proteína dietética usando aminoácidos industriais para atender as exigências dos aminoácidos mais limitantes na dieta, consequentemente, reduz os custos da dieta uma vez que a proteína é o nutriente mais caro da ração após a energia (Suida, 2001).

Os níveis excessivos de proteína na ração não só geram custo adicional na formulação, como também levam ao aumento na excreção de nitrogênio, podendo aumentar a incidência de problemas sanitários e também a diminuição de desempenho. Sabe-se que 45% do nitrogênio consumido pelas aves são retidos como proteína animal e os demais 55% do nitrogênio ingerido são excretados, contribuindo para aumentar a poluição ambiental (Cauwenberghe & Burnham, 2001). A contribuição da proteína ideal neste aspecto possibilita, para cada 1% de Proteína bruta reduzida na dieta, uma redução de até 9% da excreção de nitrogênio nos dejetos (Ferket et al., 2002).

Para que a proteína ideal seja utilizada com sucesso, as exigências dos EAA e suas relações com a lisina digestível devem ser atualizadas constantemente em função dos avanços produtivos das linhagens modernas. Segundo Dozier et al. (2010), isto é necessário devido ao aumento na taxa de crescimento das linhagens modernas que é acompanhado do aumento na exigência de lisina e outros aminoácidos digestíveis. Na literatura, existe um volume considerável de informações sobre os níveis recomendados de metionina, lisina e treonina, considerados como o primeiro, segundo e terceiro aminoácidos limitantes e por isso são suplementados de maneira rotineira nas rações das aves (Campos et al., 2012). Entretanto, informações sobre a exigência ou a relação da lisina com os demais aminoácidos essenciais arginina, isoleucina, leucina, histidina valina, fenilalanina e triptofano são escassas principalmente para reprodutores e estes apresentam uma grande variação no perfil ideal.

1.2.2. Métodos para determinar a relação ideal dos aminoácidos

1.2.2.1. Método dose-resposta e fatorial

Ensaios de dose-resposta usando a técnica de formulação da “suplementação gradativa” têm sido tradicionalmente utilizados para determinar as relações aminoacídicas para aves (Sakomura & Rostagno, 2007). Os estudos de dose-resposta se baseiam na resposta da ave ao aumento gradativo da concentração do aminoácido teste nas dietas. Nestes ensaios é comum usar como critério de resposta o ganho de peso e a conversão alimentar em animais em crescimento, os quais devem ser conduzidos em períodos suficientemente longos para obter uma resposta (Van Milgen, 2015). Além disso, dependendo do critério usado como resposta, podem ser encontrados diferentes exigências para um mesmo aminoácido estudado, o que possívelmente gera uma variação entre as estimativas (Sakomura & Rostagno, 2007).

Existem diferentes modelos para descrever a resposta do animal para o aumento gradativo do EAA na dieta. O *broken-line* (linha quebrada) é um dos modelos mais frequentemente utilizado (Baker et al., 2002). Os modelos exponenciais e o polinomial quadrático também têm sido usados, mas estes modelos têm a característica de que a máxima resposta nunca é alcançada (modelo exponencial) ou que o modelo prediz um declínio a partir da resposta obtida com o nível mais altos do EAA estudado (Van Milgen, 2015). No modelo *broken-line* a resposta marginal abaixo da exigência de EAA é constante, proporcionando estimativas menores para as exigências em relação aos demais modelos citados. Entretanto, para Baker et al. (2002), o modelo broke-line é desejável para determinar a relação ideal dos aminoácidos essenciais, pois define a quantidade mínima dos EAA que proporciona o máximo desempenho do animal.

Independente do modelo usado para interpretar as respostas, vários estudos de dose-resposta são necessários para inferir sobre uma relação ideal entre os aminoácidos essenciais e dependem do número de aminoácidos avaliados, ou seja, faz-se necessário um ensaio para cada aminoácido estudado e um deles deve ser necessariamente a lisina. Como podemos observar na literatura, Baker et al. (2002) precisaram de seis ensaios para estabelecer a relação ideal entre lisina, triptofano, treonina, isoleucina e valina de frangos de corte de 14 a 21 dias de idade. Por outro lado, Mack et al. (1999) precisaram de nove ensaios para estabelecer uma relação ideal entre lisina, metionina, treonina, triptofano, arginina, valina e isoleucina. Consequentemente estes ensaios geram um custo elevado para a pesquisa e também

demanda um tempo maior para execução. Além disso, as exigências dos EAA mudam durante o crescimento e a fase reprodutiva do animal, sendo necessário uma abordagem fatorial para que se obtenha uma estimativa mais acurada.

O método fatorial baseia-se no conceito de que a exigência de um aminoácido pode ser divida em exigências para a deposição de proteína, para crescimento de penas e para mantença (Sakomura et al., 2015). As determinações das exigências de aminoácidos se baseiam nos dados obtidos nos ensaios de dose-resposta, levando em consideração a eficiência de utilização metabólica dos nutrientes para crescimento e para mantença (Sakomura et al., 2015). O método fatorial calcula a exigência para crescimento baseada na curva de crescimento e na deposição diária de proteína. Para os animais em crescimento, a maior parte das exigências dos aminoácidos é direcionada para deposição de proteína corporal, entretanto, a deposição dos aminoácidos na forma de proteína corporal não tem 100% de eficiência, ou seja, os aminoácidos não são totalmente retidos pois parte das perdas de aminoácidos são inevitáveis (Fuller et al., 1994). Como a exigência e a proporção de cada aminoácido para crescimento depende do tecido que está sendo prioritariamente depositado (Leveile et al., 1960), a composição de aminoácidos pode variar em relação à proteína corporal, penas e a exigência de mantença como mostra a Tabela 1.

Como pode ser observado na Tabela 1 o perfil de aminoácidos calculado para as penas se diferencia consideravelmente do perfil de aminoácidos da proteína do corpo, especificamente para alguns aminoácidos. A proporção de aminoácidos sulfurados totais (metionina+cistina) na pena é muito maior do que aquela encontrada na carcaça da ave, pois estão envolvidos em maior grau na síntese da queratina das penas, sendo que a cistina é o maior componente da queratina e a metionina está envolvida na sua conversão em cistina (Fisher et al. 1981). Por outro lado, o uso quase exclusivo da lisina para o acréscimo de proteína corporal (Pack, 1995) faz com que a quantidade deste aminoácido na carcaça seja muito maior em relação aos demais componentes do corpo. É interessante observar também que existe uma grande semelhança entre o perfil de aminoácidos exigidos para mantença e o perfil de aminoácidos encontrados na proteína das penas, o qual já foi observado anteriormente por outros autores (Leveille et al., 1960) e pode ser atribuído à maior

participação das penas nas perdas endógenas que faz parte da exigência de mantença (Hurwitz, 1985).

Tabela 1. Perfil de aminoácidos na carcaça, penas e mantença (% de N x 6,25)[1]

Amino acid	Carcaça	Penas	Mantença
Lisina	6,44 (100)	2,00 (100)	2,23 (100)
Histidine	2,44 (38)	0,61 (31)	0,76 (34)
Arginina	5,74 (84)	6,68 (334)	6,64 (298)
Treonina	3,63 (56)	4,90 (245)	5,51 (247)
Triptofano	0,90 (14)	0,67 (34)	0,65 (29)
Metionina+cistina	3,54 (55)	7,97 (399)	7,70 (345)
Metionina	2,47 (38)	0,51 (25)	1,15 (52)
Isoleucina	3,21 (50)	4,50 (225)	4,10 (184)
Leucina	6,81 (106)	8,29 (409)	9,13 (409)
Fenilalanina	3,41 (53)	5,00 (250)	4,84 (217)
Fenilalanina+tirosina	6,12 (95)	7,77 (389)	8,69 (390)
Valina	3,71 (58)	6,23 (312)	6,29 (282)

[1]Adaptado de Li et al. (2003)

Em termos relativos, a exigência de mantença varia entre 30% e 100% da exigência total de proteína ou aminoácidos para animais em crescimento e adultos, respectivamente (Hurwitz, 1985). Em relação a proteína total corporal, a proteína das penas corresponde de 20 a 25% e, portanto, é quantitativamente importante (Hurwitz, 1985). Uma vez que a taxa de crescimento e empenamento mudam em função da genética, dieta e ambiente, a proporção destes diferentes componentes faz com que as exigências de aminoácidos mudem conforme estes fatores e, portanto, devem ser calculados separadamente (Hurwitz, 1985).

1.2.2.2. Método da deleção

1.2.2.2.1. Introdução ao método da deleção

A qualidade da proteína da dieta é determinada pelo seu conteúdo em aminoácidos, pela sua digestibilidade e disponibilidade (Wang & Fuller, 1989). Embora a quantidade de proteína consumida pelos animais seja semelhante, nem sempre o desempenho pode ser o mesmo, devido a influência do perfil de aminoácidos na proteína dietética, o qual influencia expressivamente o valor nutritivo da proteína. Na

literatura, encontramos diversos métodos usados na avaliação da qualidade da proteína dietética, cujo critério pode ser uma comparação entre taxas de crescimento, retenção de nitrogênio corporal ou outras medidas fisiológicas do desempenho animal consumindo diferentes proteínas dietéticas (Wu, 2013).

O valor biológico tem sido usado como um método para avaliar a qualidade da proteína e foi definido por Mitchell (1924) como sendo “a porcentagem do nitrogênio ingerido que foi retido no corpo”. Este valor é obtido em ensaios de balanço de nitrogênio, no qual uma proteína teste é fornecida e a quantidade excretada é corrigida pelas perdas endógenas quando uma dieta isenta de nitrogênio é fornecida. Segundo Block e Mitchell (1946/47) todos os aminoácidos devem estar presentes em quantidade adequada no ribossomo para que ocorra a síntese proteíca, sendo que o déficit de qualquer aminoácido poderia limitar a síntese proteica. Estes pesquisadores sugeriram que uma proteína “ideal” com composição de aminoácidos conhecida e em quantidade suficiente para atender as exigências, poderia ser usado como referência para determinar o valor nutricional de uma proteína. Assim, o déficit de cada aminoácido essencial seria obtido ao dividir-se a concentração dos aminoácidos na proteína teste em relação à concentração dos aminoácidos na proteína “ideal”. Neste caso, o aminoácido mais limitante determinaria o valor nutritivo desta proteína pelo conceito da pontuação química. Na prática, estes pesquisadores sugeriram usar a proteína do ovo como referência (proteína “ideal”) uma vez que seu valor biológico é próximo de 100.

Desde então, a pontuação química tem sido bastante utilizada para avaliar a qualidade de proteínas. Entretanto, Block e Mitchell (1946/47) reconheceram que a proteína do ovo pode conter alguns aminoácidos que excedem as exigências e, assim, a FAO (1957) sugeriu usar como referência o perfil de aminoácidos baseado nas exigências dos aminoácidos e não na proteína do ovo. Anos depois, Mitchel (1964) propôs que a proteína considerada ideal seria aquela na qual a composição de aminoácidos do alimento correspondesse estritamente à exigência do animal, para otimizar a utilização da proteína (relação entre retenção e consumo de proteína) e minimizar a excreção de nitrogênio. Naquele momento foi um conceito mais teórico do que prático. Um ano depois, foi proposto correlacionar o valor da pontuação química com o valor biológico para uma melhor predição da qualidade da proteína,

usando os conceitos do valor biológico e da pontuação química, ou seja, a qualidade da proteína dietética estaria relacionada tanto à qualidade do alimento quanto ao desempenho animal (Bender, 1965). Em estudo publicado com ratos, Bender (1965) correlacionou o valor nutritivo (biologicamente determinado) de fontes proteicas com a pontuação química de uma mistura de aminoácidos de composição conhecida e determinou que a pontuação química de uma mistura incompleta de aminoácidos (em que um dos aminoácidos é limitante) corresponde numericamente ao valor biológico determinado, sendo que a suplementação do aminoácido limitante na dieta aumenta o valor biológico desta proteína até atingir o balanço perfeito (100%).

Baseado no estudo de Bender (1965) e levando em consideração os conceitos apresentados anteriormente (Wang e Fuller, 1989) adaptaram este método para determinar a relação ideal dos aminoácidos essenciais exigido pelos animais, ou seja, a proteína “ideal” tomada como referência seria uma dieta balanceada que atende as exigências de aminoácidos do animal sem falta ou excesso destes na dieta. Neste método, o nitrogênio retido como percentual do total ingerido passa a ser avaliado como resposta ao invés de usar o valor biológico e a pontuação química que é substituída pela porcentagem do aminoácido avaliado no alimento em relação à quantidade do aminoácido em um alimento referência. A mudança na retenção de nitrogênio (como porcentagem do consumo de nitrogênio) devido ao fornecimento proporcionalmente reduzido de cada um dos aminoácidos, é usada para determinar um perfil dietético de aminoácidos, no qual todos os aminoácidos testados são igualmente limitantes a uma proteína ideal. Este método foi nomeado como “método da deleção” e foi inicialmente aplicado para suínos.

O princípio do método da deleção se baseia no conceito de que a remoção de um aminoácido não-limitante não tem efeito na retenção de nitrogênio, sendo que as mudanças que ocorrem na retenção de nitrogênio devido à remoção de uma proporçãode cada aminoácido é usado para calcular o perfil ideal de aminoácidos em uma dieta na qual todos os aminoácidos são igualmente limitantes (Wang & Fuller, 1989). Este método foi originalmente estabelecido em pesquisas com suínos (Wang e Fuller, 1989) e usado em aves (Gruber et al., 2000; Roth et al., 2001; Dorigam et al., 2015) para definir a proteína ideal para frangos de corte. Mais recentemente, este

método vem sendo muito utilizado para peixes (Green & Hardy, 2002; Rollin, et al. 2003; Abboudi et al., 2006; Peres e Oliva-Teles, 2009; Diogenes et al., 2015).

Os dados são obtidos através de um único ensaio de balanço de nitrogênio para determinar a retenção de nitrogênio em função da ingestão do aminoácido avaliado. A abordagem usada por Wang & Fuller (1989) e demais autores, consiste em formular inicialmente uma dieta balanceada para atender às relações ideais dos aminoácidos essenciais para a espécie ou linhagem estudada. A dieta balanceada é suplementada com aminoácidos industriais para atender 100% das exigências dos aminoácidos estudados. Em seguida os aminoácidos da dieta controle são parcialmente reduzidos com amido de milho ou trigo e novamente suplementadas para 100% com aminoácidos industriais, exceto o aminoácido a ser avaliado. A proporção do aminoácido teste a ser reduzida da dieta controle ou balanceada varia de 15 a 45% nos estudos encontrados na literatura (Wang e Fuller, 1989; Gruber et al., 2000; Roth et al., 2001; Green & Hardy, 2002; Rollin, et al. 2003; Abboudi et al., 2006; Peres e Oliva-Teles, 2009; Diogenes et al., 2015; Dorigam et al., 2015), porém, não existe uma regra geral para redução. A redução não deve ser drástica e deve apenas garantir que o animal apresente uma resposta ao aminoácido limitante, ou seja, a retenção de nitrogênio com o fornecimento das dietas limitantes deve ser estatisticamente diferente da retenção de nitrogênio proporcionada pela dieta balanceada. Muitas vezes um ensaio piloto torna-se necessário para definir a necessidade de uma redução maior.

A relação entre a retenção de nitrogênio e a ingestão do aminoácido avaliado obtidos nos ensaios de balanço de nitrogênio é testada na análise de regressão com os dados obtidos em dietas especificamente deficientes em cada aminoácido e com a dieta balanceada. Para avaliar as respostas de retenção de nitrogênio, Wang & Fuller (1989) propuseram um modelo linear simples como mostrado na Figura 1.

A partir da Figura 1 são feitas algumas pressuposições: (A) a remoção do primeiro aminoácido limitante causaria a maior redução na retenção de nitrogênio; (C) se a remoção de um aminoácido não causar redução na retenção de nitrogênio, então a quantidade removida está em excesso relativo ao primeiro aminoácido limitante; (B) Se a remoção de um aminoácido resultar em uma moderada redução na retenção de nitrogênio, então a proporção que pode ser removida sem reduzir a retenção pode ser

interpolada proporcionalmente. Se todas as condições iniciais foram atendidas, então o aminoácido reduzido se tornará primeiro limitante na dieta. Desta forma, a equação da reta proporcionada por A é usada como referência para determinar a proporção que cada aminoácido (B) poderia ser reduzido da dieta controle até atingir a máxima resposta obtida no Platô formado por C.

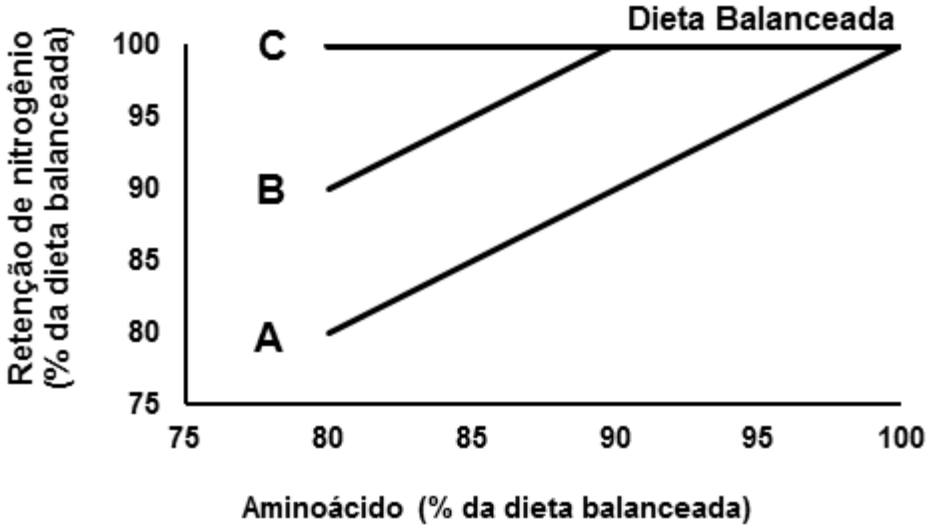

Figura 1. Princípio do método para determinar a exigência de aminoácidos por dedução. **A** é o primeiro aminoácido limitante; **B** e **C** são aminoácidos que estão, respectivamente, 10 e 20% (ou mais) em excesso relativo à **A** (Wang & Fuller, 1989).

A grande vantagem do método da deleção do aminoácido é a possibilidade de determinar a relação ideal de todos os aminoácidos essenciais através de um único ensaio. Como mencionado no item 1.2.2.1., Baker et al. (2002) e Mack et al. (1999) precisaram de uma quantidade considerável de ensaios para obter a relação ideal de alguns EAA, ao passo que, pelo método da deleção, Roth et al. (2001) determinaram a relação ideal entre de lisina, metionina, metionina+cistina, treonina, triptofano, isoleucina, leucina, valina, fenilalanina, arginina e histidina para frangos em um único ensaio. Outra vantagem do método é que todas as relações de aminoácidos são determinadas simultaneamente usando o mesmo grupo de animais e a mesma dieta controle (Rollin et al., 2003; Dorigam et al., 2015). Isto permite um maior grau de uniformidade e consistência nos resultados, que facilita a precisão ao determinar as relações ideais dos aminoácidos.

Baseado nos princípios apresentados, serão apresentadas a seguir algumas abordagens de diferentes universidades usando o método da deleção. Embora as metodologias apresentadas na revisão tenham um enfoque diferente, conceitualmente, todas elas se baseiam no método da deleção.

1.2.2.2.2. Uso do método da deleção em ensaios de mantença

As exigências de aminoácidos para animais em crescimento incluem dois componentes, a exigência para o crescimento e a exigência para mantença. Entretanto, as estimativas da relação ideal dos aminoácidos essenciais determinado por Wang & Fuller (1989) pelo método da deleção se refere a soma destes componentes e é apenas aplicável nas condições experimentais em que foi determinado. Dessa forma, a abordagem adotada na Universidade de Rowett, Escócia, foi incorporada nos estudos de Fuller et al. (1989) para determinar separadamente um perfil ideal de aminoácidos para deposição de proteína e mantença em suínos. Assim como nos tradicionais ensaios de mantença usando a técnica do balanço de nitrogênio, neste método também é ajustada uma regressão linear entre retenção de nitrogênio e ingestão diária de cada aminoácido. A exigência de mantença é determinada pela ingestão do aminoácido quando o balanço de nitrogênio é zero. A estimativa da exigência de mantença dos aminoácidos essenciais pelo procedimento usado por Fuller et al. (1989) foi feita considerando uma faixa maior de ingestão de nitrogênio (250 a 1350 mg $N/BW_{kg}^{0,75}$ por dia), contudo, estes pesquisadores comprovaram que é possível determinar a exigência de mantença dos aminoácidos com um faixa mais estreita de ingestão de nitrogênio (250 a 500 mg $N/BW_{kg}^{0,75}$ por dia).

No primeiro ensaio de mantença realizado por Fuller et al. (1989), a composição de aminoácidos das dietas controle com alta (1350 mg $N/BW_{kg}^{0,75}$ por dia) e baixa ingestão (250 mg $N/BW_{kg}^{0,75}$ por dia) de nitrogênio foram baseadas no perfil de aminoácidos essenciais estabelecido para suínos por Wang & Fuller (1989). As outras séries de dietas semi-purificadas com alta e baixa concentração de aminoácidos foram formuladas de tal forma que o aminoácido teste foi totalmente removido da dieta controle e, com a adição de uma mistura de aminoácidos não essenciais, manteve-se

a mesma concentração de nitrogênio nas dietas teste em comparação com as dietas controle. Além disso, foi fornecido uma dieta isenta de nitrogênio para quantificar as perdas endógenas de nitrogênio. Regredindo as retenções de nitrogênio obtidas neste ensaio com as ingestões dos aminoácidos teste, foi possível determinar inicialmente um perfil ideal de aminoácidos para mantença. Além disso, estes autores concluíram que a diferença entre a perda de nitrogênio encontrada em suínos alimentados com dietas de baixa ingestão de nitrogênio forneciam exatamente a quantidade de aminoácido exigido para mantença (18 mg $N/BW_{kg}^{0,75}$ por dia) e a perda de nitrogênio em suínos que receberam uma dieta isenta (268 mg $N/BW_{kg}^{0,75}$ por dia) foi de exatamente 250 mg $N/BW_{kg}^{0,75}$ por dia, ou seja, a quantidade de nitrogênio foi utilizada com 100% de eficiência.

No segundo ensaio de Fuller et al. (1989), o perfil de aminoácidos para mantença determinado no primeiro estudo foi usado como base para a formulação das dietas. Neste ensaio, uma série de dietas com baixa ingestão de proteína (250 mg $N/BW_{kg}^{0,75}$ por dia) é fornecida, cada uma contendo metade da exigência de mantença do aminoácido avaliado e os demais aminoácidos na quantidade total estimada para mantença no ensaio anterior. Essa ingestão de proteína baseia-se na exigência de mantença de nitrogênio para repor as perdas endógenas que é de 250 mg $N/BW_{kg}^{0,75}$ por dia como determinada no ensaio anterior. Este valor também se aproxima dos valores observados na literatura para aves (Samadi & Liebert, 2007, Dorigam et al., 2015), sendo um dos motivos para adotar este mesmo critério em nosso estudo. No mesmo ensaio de Fuller et al. (1989), outra série de dietas com alto teor de proteína (500 mg $N/BW_{kg}^{0,75}$ por dia) é fornecida, cada uma contendo um dos aminoácidos avaliado na quantidade necessária para mantença e com o dobro da quantidade dos outros aminoácidos exigidos para mantença determinados no ensaio anterior. Essa ingestão de nitrogênio corresponde ao dobro da exigência de nitrogênio para mantença, a qual é fornecida para garantir um balanço positivo de nitrogênio.

Este experimento segue dois princípios: (1) Se a exigência de mantença do aminoácido estiver em excesso, o fornecimento de um excesso relativo dos demais aminoácidos (incluindo os aminoácidos não essenciais) irá proporcionar um balanço positivo de nitrogênio e (2) Nas dietas com baixo valor proteico, a redução da exigência de mantença pela metade, em relação aos demais, é suficiente para tornar

o aminoácido limitante e proporcionar um balanço negativo de nitrogênio (Fuller et al., 1989). A partir das regressões relacionando o balanço de nitrogênio com a ingestão de cada aminoácido são feitas as estimativas de cada aminoácido exigido para manter o balanço de nitrogênio igual a zero (aminoácido ingerido quando NB=0).

Este tipo de ensaio também foi realizado com peixes, mas apenas para determinar a exigência de mantença de lisina. No estudo de Abboudi et al. (2006), dois grupos de dieta foram formulados com a mesma composição de nitrogênio (sendo um com deleção e outro sem deleção de lisina). A composição de aminoácido nas dietas com baixa (2,9% N), média (6,2% N) e alta concentração de nitrogênio (8,5% N) foram baseadas no perfil de aminoácidos do corpo do peixe. Nas outras três dietas a lisina foi especificamente removida, i.e., removeu-se completamente a lisina na dieta com menor nível de nitrogênio e 50% de lisina nas outras duas dietas com médio e alto teor de nitrogênio. Adicionalmente, também foi fornecida uma dieta isenta de nitrogênio. Como somente houve uma redução significativa na retenção de nitrogênio nos tratamentos formados pela dieta isenta, dieta com baixa concentração de nitrogênio (com deleção e sem deleção de lisina) e a dieta com média concentração de nitrogênio (com deleção da lisina), foram usados apenas estes níveis para fazer uma regressão linear. Assim, como no ensaio de Fuller et al. (1989) a exigência de lisina para mantença determinada por Abboudi et al. (2006) também foi obtida pelo intercepto da linha de regressão no eixo X.

Algumas considerações importantes são feitas por estes autores para esta metodologia. Uma delas é que a confiança do modelo linear usado para estimar a exigência de mantença se baseia principalmente no balanço positivo de nitrogênio (Fuller, et al. 1989; Abboudi et al., 2006) uma vez que estes valores são usados como referência para confirmar a limitação do aminoácido teste. Além disso, o período experimental deve ser suficientemente longo para medir as diferenças no crescimento proteíco e também ser curto o bastante para assegurar que as exigências de mantença não irão variar durante este período (Abboudi et al., 2006). De acordo com estes autores, a duração de experimentos usando abate comparativo não deve ser maior do que um mês para a determinação das exigências de aminoácidos para mantença de animais em crescimento. Pelo balanço de nitrogênio, o período experimental é mais curto, sendo geralmente usado cinco dias para aves em

crescimento (Samadi & Liebert, 2006a,b; Samadi & Liebert, 2007a,b; Samadi & Liebert, 2008) e sete dias para suínos em crescimento (Wang & Fuller, 1989).

Para aves, as estimativas das exigências dos aminoácidos para mantença têm sido realizadas usando galos adultos, uma vez que nesta idade a ave já estabilizou seu crescimento proteíco e também porque a maior parte da exigência total de aminoácidos é destinada para a mantença destas aves (Nonis & Gous, 2008). O perfil de aminoácidos para mantença foi determinado anteriormente por alguns pesquisadores (Leveille & Fisher, 1958; 1959a, 1959b; 1960; Leveille et al. 1960) em ensaios individuais de balanço de nitrogênio. Entretanto, não foram encontradas na literatura pesquisas que estimassem simultaneamente as exigências de todos aminoácidos essenciais para mantença.

1.2.2.2.3. Uso do método da deleção para estimar a relação ideal de aminoácidos para crescimento

Originalmente, nos cálculos pelo método da deleção preconizado por Wang & Fuller (1989), pressupõe-se que a inclinação das retas individuais proporcionadas pela resposta dos animais com as dietas limitantes e com a dieta balanceada (controle) são idênticas para todos os aminoácidos essenciais. Isto porque a proporção do aminoácido estudado que poderia ser removida da dieta controle é determinada por interpolação matemática, ou seja, toma-se como referência a equação da reta proporcionada pelo aminoácido mais limitante (Figura 1, A) e determina-se a porcentagem do aminoácido a ser reduzido (eixo X) para a retenção de nitrogênio proporcionada por B, como mostra a Figura 1 no item 1.2.2.2.1. Segundo essa pressuposição, pode ser calculado um ponto no eixo X para cada aminoácido até o qual uma redução do aminoácido não teria efeito na retenção de nitrogênio (Figura 1, **C**). Por outro lado, a pressuposição de que as inclinações das relações dose-resposta são idênticas para todos os aminoácidos está sujeita a críticas, uma vez que, os três compartimentos (mantença, crescimento e produção) que definem a exigência total contribuem em proporções diferentes, dependendo do aminoácido (Sakomura & Rostagno, 2007). Semelhante metodologia também foi empregada para frangos de corte (Gruber et al., 2000; Roth et al., 2001).

Anos depois esta metodologia foi adaptada por Green & Hardy (2002) para estabelecer uma relação ideal de aminoácidos essenciais para peixes. A diferença principal entre as duas técnicas é que Green & Hardy (2002) padroniza os valores de retenção de nitrogênio dividindo os valores obtidos pela taxa de deleção (DEL) de cada aminoácido. Como sugerido por estes autores, esta correção contribui para a melhoria dos resultados obtidos por diminuir a variação entre as respostas determinadas. De acordo com Green & Hardy (2002), a exigência de cada aminoácido usada como referência para determinar a relação ideal dos aminoácidos essenciais é calculada em quatro passos. Apesar de ser uma técnica rápida para avaliar as relações ideal dos aminoácidos essenciais para diferentes espécies de peixes, ainda assim, apresenta um certo grau de complexidade em seus cálculos.

Por outro lado, Rollin et al. (2003) simplificam estes cálculos com uma única equação baseada no modelo *broken-line* (Figura 1) para calcular de forma prática a "exigência" dos aminoácidos usados como referência na determinação da relação ideal. A equação sugerida por estes pesquisadores tem a seguinte representação:

$$\text{Exigência} = EAA_{BD} \times (2 - DEL - (ND_{EAA} / ND_{BD})), \text{ Eq. 1.1}$$

Onde (EAA_{BD}) é a concetração do aminoácido na dieta controle, DEL é a taxa de deleção do aminoácido obtida ao dividir a concentração do aminoácido na dieta teste (EAA_{teste}) pela EAA_{BD}, ND_{EAA} é o nitrogênio depositado obtido com a dieta teste, e ND_{BD} é o nitrogênio depositado obtido com a dieta balanceada. A relação ideal entre os aminoácidos essenciais é obtida ao dividir a exigência estimada de cada aminoácido pela exigência estimada de lisina. É importante considerar que a "exigência" determinada pelo método da deleção não é usada para fazer recomendações de níveis nutricionais na dieta, pois somente deve ser usada como referência para o cálculo da relação ideal dos aminoácidos. Conforme Rollin et al. (2003), esta abordagem viabiliza uma metodologia rápida e prática para determinar a relação ideal dos aminoácidos.

1.2.2.2.4. Abordagem metodológica de Goettingen para determinar a relação ideal

Esta abordagem desenvolvida na universidade de Goettingen, Alemanha, necessita que sejam conduzidos dois ensaios. No primeiro ensaio são determinados os parâmetros do modelo do metabolismo protéico das aves. Este grupo de pesquisadores preconiza que cada animal possui uma característica genética própria para sua máxima retenção de nitrogênio ($NR_{max}T$) e que está associada à exigência de nitrogênio para mantença (NMR). Estes parâmetros são determinados em ensaios de balanço de nitrogênio com o uso de dietas com níveis crescentes de proteína e as respostas são avaliadas usando modelos exponenciais (Samadi & Liebert, 2006a,b, 2007a,b, 2008; Pastor et al., 2013; Dorigam et al. 2014; Khan et al. 2015). Neste caso, o $NR_{max}T$ é representado pela resposta assintótica da função exponencial entre nitrogênio retido e ingerido. Como $NR_{max}T$ é determinado pelo valor assintótico da função exponencial, atribui-se o conceito de "teórico" a este parâmentro, pois este valor não é observado em condições práticas de criação (Samadi & Liebert, 2007a). Por outro lado, o NMR é obtido pelo valor que intercepta o eixo-y de uma função exponencial entre nitrogênio excretado e ingerido quando se considera a ingestão de nitrogênio igual a zero. Uma vez que estes dois parâmetros tenham sido estimados para um dado genótipo, ele pode ser utilizado em modelos de crescimento, na determinação da eficiência de utilização e exigência dos aminoácidos.

De posse destas informações, o próximo passo é determinar as eficiências de utilização dos aminoácidos. Para isso é necessário outro ensaio de balanço de nitrogênio em que uma dieta controle (balanceada) é formulada contendo todos os aminoácidos em níveis adequados. Para avaliar os efeitos da deleção do aminoácido na utilização da proteína, a dieta controle é diluída com amido para atender 80% dos aminoácidos e novamente suplementada com aminoácidos industriais para 100%, exceto o aminoácido a ser estudado (Pastor et al., 2013). As respostas das aves à ingestão destas dietas em que um dos aminoácidos está deficiente é utilizada para determinar a qualidade da proteína. A qualidade da proteína nestas dietas é dada pela inclinação da função exponencial entre nitrogênio retido e ingerido (b). De acordo com Samadi & Liebert (2008), a função linear entre a concentração do aminoácido limitante na dieta (c) e a qualidade da proteína (b) indica a eficiência de utilização do aminoácido (bc^{-1}).

Para uma dada deposição diária de proteína, a exigência do aminoácido torna-se dependente somente da eficiência de utilização do aminoácido estudado. Consequentemente, é possível comparar as eficiências de cada aminoácido diretamente (Samadi & Liebert, 2008). Estas eficiências são relacionadas com a eficiência de utilização da lisina para traçar um perfil ideal de aminoácidos (Samadi & Liebert, 2008; Wecke & Liebert, 2013; Dorigam et al., 2015). É importante ressaltar que este procedimento para determinar a relação ideal dos EAA somente deve ser feito para a mesma idade em que foi determinado o $NR_{max}T$, pois a máxima deposição de proteína depende deste fator e afeta o valor da eficiência de utilização do aminoácido (Samadi & Liebert, 2008).

Embora a relação ideal entre a lisina e alguns aminoácidos tenha sido determinada para frangos de corte (Samadi & Liebert, 2008; Wecke & Liebert, 2013; Pastor et al., 2013), as aplicações deste procedimento para obter a relação ideal dos aminoácidos através de um único experimento ainda estão sob avaliação por esses pesquisadores. Até o momento não foram desenvolvidos estudos com matrizes reprodutoras e foram realizados pouquíssimos estudos com poedeiras comerciais. Assim, aproveitando os dados do máximo potencial de retenção de nitrogênio da linhagem Cobb 500 obtidos em estudos realizados (Dorigam et al., 2014), será aplicado este procedimento para determinar a relação ideal dos aminoácidos essenciais para frangos de corte através de um único ensaio. Após a padronização desta metodologia com frangos de corte, serão desenvolvidos estudos com as matrizes reprodutoras.

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CAPÍTULO 2 - Establishing an essential amino acid profile for maintenance in poultry using deletion method

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TITLE PAGE

Short title: Amino acid maintenance requirements in poultry

Title: Establishing an essential amino acid profile for maintenance in poultry using deletion method

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ABSTRACT

This study aimed to estimate the essential amino acid profile and the ideal ratio for maintenance of poultry by deletion method. A nitrogen balance trial was conducted using 198 adult roosters, housed individually in metabolic cages. The treatments were 33 purified diets being 11 diets with an amino acid mixture providing high protein intake (500 mg $N/BW_{kg}^{0.75}$ per day), 11 diets with medium protein intake (250 mg $N/BW_{kg}^{0.75}$ per day) (in each diet one amino acid tested was deleted 50%) and 11 diets providing low protein intake (125 mg $N/BW_{kg}^{0.75}$ per day) (made by omitting the amino acid tested). Each treatment had six replicates. After 48 h of fasting receiving water plus sucrose, the roosters were fed 40 g of the diets by tube once a day for three days. The excreta were collected within 72 h after the first feeding. The diets and excreta were analyzed for nitrogen content. For each amino acid studied, a linear regression was fitted by nitrogen balance and amino acid intake. The maintenance requirements were estimated as the amino acid intake to maintain the nitrogen balance equal to zero. The amino acid requirements for maintenance were estimated to be: Lys 10.60, Met 29.28, Thr 23.36, Trp 5.06, Arg 49.53, Val 29.15, His 6.40, Gly 54.16, Phe 49.45, Leu 77.87 and Ile 20.98 mg/$BW_{kg}^{0.75}$ per day. Therefore, the amino acid ratio for maintenance was concluded to be Lys 100, Met 276, Thr 220, Trp 48, Arg 467, Val 275, His 60, Gly 511, Phe 467, Leu 735 and Ile 198%. The essential amino acid profile and the ideal ratio for maintenance of poultry estimated in this study contribute to improve factorial model for estimating essential amino acid requirements for poultry.

Key words: deletion method, ideal ratio, maintenance, nitrogen balance, rooster

2.1. INTRODUCTION

Factorial models have been used to determine the optimum amino acid intake for poultry by considering separately the requirements for maintenance, growth or egg production. A good assessment of the requirements for these components is prerequisite to develop an accurate model to optimize amino acid intake and, consequently, to improve the performance of poultry. Moreover, an estimation of the optimal amino acid intake allows the reduction of feed costs and environmental pollution caused by excess nitrogen excretion in poultry production (Firman and Boling, 1998).

Maintenance requirement is an important component in factorial models because in young animals the maintenance requirement is lower in relation to the total requirement and assumes greater importance as the poultry reaches maturity due to the lower need for amino acids for growth (Owens and Pettigrew, 1989). In previous studies was shown that the amino acid pattern required for optimum growth is quite different than that required for maintenance (Smith and Johnson, 1967). The growth pattern is greatly influenced by the amino acid composition of the new tissue formed, primarily muscle, while the maintenance pattern depends on the turnover rates of the individual essential amino acids (Jansen, 1974). Therefore, it is important not only to consider an ideal ratio for growth but also an ideal ratio for maintenance, because this clear affects the estimation of the essential amino acid intake by factorial models.

The amino acid requirements for maintenance in poultry have been studied (Leveille and Fisher, 1958, 1959; Leveille et al. 1960), using the conventional dose-response method in the nitrogen balance trials. Although the validity of this method, multiple assays are needed to determine the maintenance requirements for each essential amino acid and so far, not all essential amino acids for maintenance were determined. Moreover, estimates may vary widely,

e.g. lysine vary from zero (Leveille et al. 1960) to 114 mg/$BW_{kg}^{0.75}$ per day (Edwards et al., 1999). This variation is associate to differences in genotype, diet composition and environmental conditions, since these latter factors have a direct influence on performance.

One alternative approach used to determine the maintenance requirement for each essential amino acid is the deletion method, based on monitoring the nitrogen balance that arises as the amino acids are reduced from the diets. Different from the conventional procedure, this approach allows determining the requirements for all essential amino acids in one set of experiments. The deletion method is generally accepted as an efficient and rapid tool to estimate the ideal amino acid profile and has been applied to determine maintenance requirements in rats (Said and Hegsted, 1970; Gahl et al., 1991) and pigs (Fuller et al., 1989), but not in poultry. Thus, in the present study, the deletion method was used to update and obtain new coefficients for maintenance requirements for poultry to derive an ideal essential amino acid ratio for maintenance.

2.2. MATERIAL AND METHODS

2.2.1. Ethics approval

This study was approved by the Ethics Committee on Animal Use of the Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal (nº 9999/14).

2.2.2. Experimental design

A nitrogen balance trial was conducted at the Laboratory of Poultry Sciences of the University of Agrarian and Veterinary Sciences – FCAV / UNESP, Jaboticabal – SP. In this trial, 198 adult roosters of Cobb500® genotype were used with an average initial weight of 6.09 ± 0.16 kg. Adult roosters are ideal animal models to estimate maintenance requirements for poultry, as these birds are most likely to be in a steady state and theoretically are not using amino acids for growth (Nonis and Gous, 2008). The roosters were housed individually in metabolic cages, equipped with nipple-type drinkers and individual feeders. The treatments consisted of three protein levels (low, medium and high) where 11 essential amino acids were evaluated (lysine, methionine, threonine, tryptophan, arginine, valine, phenylalanine, histidine, glycine, isoleucine, and leucine), totalling 33 treatments with six replicates each. The treatments were arranged in a completely randomized design.

2.2.3. Experimental diets

In this trial, 33 purified diets were formulated based on the mixture of corn starch, cellulose, sucrose, vegetable oil, vitamins, and minerals to meet the requirements for energy and other nutrients according to the recommendations for roosters (Rostagno et al., 2011), except protein and amino acids. The amino acid composition of the diets with low (LP), medium (MP) and high protein concentration (HP) was based on the amino acid profile for maintenance established by Leveille et al. (1960) corrected by the metabolic body weight of the rooster in this study. The nutritional composition of the diets were calculated based on the daily consumption of 40 g of feed.

Following the procedure proposed by Fuller et al. (1989), the series of HP diets were made, each providing one of the essential amino acids tested in the quantity required for

maintenance but with twice the other amino acids required for maintenance, resulting in a total nitrogen intake of 500 mg/$BW_{kg}^{0.75}$ per day. In this case, the provision of a relative excess of every other amino acid with additional non-essential amino acid would permit a positive nitrogen balance. Since energy and other nutrients (except protein and amino acids) wasn´t limiting in the study, nitrogen balance depends on the capacity of the animal for nitrogen retention, which would be very small for adult birds, so nitrogen balance don't have improvements by given more amino acids than their requirements in the HP diet (Rizzo et al., 2004). The series of MP diets were made, each providing half of the amino acid tested in the quantity required for maintenance and the other amino acids in adequate quantities to meet their requirements for maintenance, totalling the daily nitrogen intake of 250 mg/$BW_{kg}^{0.75}$ that meet the nitrogen requirement for maintenance. Therefore, the series of MP diets were formulated to give an intake of amino acids close to a nitrogen balance equal to zero. The series of LP diets were made by omitting the amino acid tested and providing half of the other amino acids required for maintenance, totalling the nitrogen intake of 125 mg /$BW_{kg}^{0.75}$ per day. The series of LP diets were formulated to give an intake of amino acids that results in negative nitrogen balance. The nitrogen content in these diets were corrected with the addition of L-glutamic acid to meet the proposed nitrogen intakes. In addition, a nitrogen-free diet (NF) was formulated simply by omitting the L-amino acids and provided *ad libitum* in the feeders only to avoid that energy would be a limiting factor for nitrogen retention. The composition of these diets are presented in Table 1.

2.2.4. Management and data collection

At the beginning of the trial, all birds were weighed individually, enabling the formation of homogeneous experimental units. Afterwards the birds underwent a fasting period of 48 hours, receiving 60 ml of water with sucrose (50% each), once a day, by intubation. In the subsequent 72 hours the birds received 40 g of the experimental diet by intubation. Simultaneously the NF was made available ad libitum during the balance period, to ensure that the birds remained in positive energy balance (Burnham and Gous, 1992; Nonis and Gous, 2008). To delimit the beginning and end of the period of excreta collection, iron oxide was used as a marker added in the experimental diets at a concentration of 1%. The excreta that were not marked in the first collection day and the excreta marked in the last day of collection were discarded. The excreta were collected twice a day (at 8:00 and 16:00 hours), using trays coated with plastic sheet arranged under each metabolic cages. The excreta produced by each experimental unit during each collection period were placed in plastic pots properly identified and frozen (- 20^{o}C) for later analysis. At the end of the trial period, leftover feed was quantified to determine the daily feed intake (test diet plus NF diet provided ad libitum in the feeder). At the end of the experiment, six roosters were slaughtered with CO_2 to further quantify the protein and amino acid content in feathers and carcass free of feathers.

2.2.5. Chemical analysis

The excreta produced by each experimental unit were homogenised using a blender (Philipis Walita® RI2008, Varginha, Minas Gerais, Brazil) adding a known volume of distilled water for suitable consistency when required, as described by Burnham and Gous (1992). The weight corresponding to the volume of distilled water was added to the total excreta produced by the experimental unit.

Table 1. Composition of nitrogen-free (NF), low protein (LP), medium protein (MP) and high protein concentration (HP) diets used in the experiment[1]

Ingredients (g/kg)	NF	LP	MP	HP
Corn starch	609.54	623.53	528.95	339.78
Sucrose	200.00	100.00	100.00	100.00
Cellulose	110.00	110.00	110.00	110.00
Soybean oil	24.73	24.73	24.73	24.73
Dicalcium Phosphate	16.22	16.22	16.22	16.22
Potassium Chloride	14.30	14.30	14.30	14.30
Sodium Bicarbonate	8.52	8.52	8.52	8.52
Limestone	6.70	6.70	6.70	6.70
Vitamin-and mineral mix[2]	10.00	10.00	10.00	10.00
L-Glutamic acid (99%)	-	28.49	65.57	139.72
L-Leucine (98%)	-	9.49	18.98	37.96
DL-Methionine (99%)	-	6.87	13.75	27.49
Glycine (96%)	-	6.56	13.12	26.25
L-threonine (96%)	-	5.77	11.55	23.10
L-Isoleucine (99%)	-	5.45	10.91	21.82
L-Valine (98%)	-	4.67	9.34	18.67
L-Arginine (99%)	-	9.09	18.18	36.36
L-Phenylalanine (99%)	-	4.54	9.09	18.18
L-Lysine HCl (78%)	-	2.76	5.52	11.04
L-Tryptophan (98%)	-	1.45	2.91	5.82
L-Histidine (99%)	-	0.83	1.67	3.33
Analysed nutritional composition and digestible amino acids (g/kg)				
Crude Protein	0.25	75.15	151.97	302.63
Metabolizable energy (MJ/kg)	13.42	13.40	13.38	13.22
Phenylalanine	-	4.00	8.03	16.02
Glycine	-	5.61	11.21	22.43
Histidine	-	0.73	1.47	2.94
Isoleucine	-	4.81	9.61	19.22
Leucine	-	8.30	16.55	33.11
Lysine	-	1.93	3.87	7.74
Methionine	-	6.05	12.11	24.22
Arginine	-	8.01	16.02	32.05
Threonine	-	4.94	9.88	19.78
Tryptophan	-	1.26	2.55	5.10
Valine	-	4.07	8.14	16.30

[1]Content/kg: vit.A 12000000 IU, vit.D3 22000000 IU, vit.E 30000 mg, vit.B1 2200 mg, vit.B2 6000 mg, vit.B6 3300 mg, vit.B12 16000 mg, Niacin 53000 mg, pantothenic acid 13000 mg, vit.K 2500 mg, folic acid 1000 mg, Selenium 250mg, manganese 75000 mg, iron 50000 mg, zinc 70000 mg, copper 6500 mg, cobalt 200 mg, iodine 1500mg.

To determine body protein content, the carcasses were processed in a meat mill. Representative quantities of the samples (excreta and carcass) were weighed in Petri dishes, and frozen again (- 20ºC) for freeze-drying.The samples were freeze-dried for 72 h (Edwards® 501 Modulyo freeze drier, West Sussex, United kingdom), weighed, and subsequently processed in a ball mill (Marconi® MA-350, Piracicaba, São Paulo, Brazil) for 2 min. The diets, excreta and carcass samples were sent to the laboratory for the determination of dry matter and total nitrogen content. The definitive drying of the diets, excreta and carcass was performed in an oven (Quimis®, Diadema, SP, Brazil) at 105ºC for 16 hours. For the determination of total nitrogen content of the samples, the Kjeldahl method was used (AOAC, 1995). The total amino acid content of the ingredients, feathers and carcass free of feathers were analysed by Ajinomoto Ltd. using high performance liquid chromatography (HPLC), and the values obtained for the ingredients were corrected for digestible amino acids using the tabulated coefficients of digestibility (Rostagno et al., 2011). Nitrogen balance (NB) was calculated as the difference between nitrogen intake and excretion. The amino acid requirement for maintenance was considered as the quantity of ingested amino acid capable of providing nitrogen balance equal to zero, as described by Sakomura and Rostagno (2007).

2.2.6. Statistical analysis

Data from nitrogen balance trial were subjected to regression analyses, considering the amino acid intake (AAI) as the independent variable and the nitrogen balance (NB) as dependent, according to the model: $NB = \beta_0 + \beta_1 \times AAI + \varepsilon$; where β_0 and β_1 are the regression parameters and ε is the random error. The amino acid requirements for maintenance were obtained using the relationship β_0/β_1, i.e. when NB=0. The statistical analyses were performed

considering significance at 5% by the GLM procedure in the Statistical Analysis System statistical software package version 9.2 (SAS Institute, Cary, NC, USA). The maintenance requirements and the amino acid composition in feathers and body free of feathers were compared by Pearson´s correlation (R). The requirements for maintenance were expressed using four different scales that are usually found in the literature (mg/bird, mg/BW_{kg}, mg/$BW_{kg}^{0.75}$, and mg/$BP_m^{0.73} \times \mu$; were BW is the body weight, BP_m is the mature body protein content and μ is the degree of maturity or BP/BP_m).

2.3. RESULTS

The mean values and their respective standard deviations (±SD) obtained from nitrogen balance assay are presented in Table 2. The nitrogen balance data obtained from LP, MP and HP diets for each amino acid (Table 2) were used to establish a linear regression to estimate amino acid requirement for maintenance when NB was considered equal to zero, i.e. at nitrogen equilibrium. The standard deviation of the data used in the linear regression was low considering the small amount of data obtained for each amino acid. Negative nitrogen balance was observed for roosters fed the LP diets, in which the amino acids tested were omitted. A higher nitrogen excretion was observed for roosters fed LP diets, in which valine, histidine and Phenylalanine were omitted, consequently, the negative balance occurred in greater proportion. The roosters fed diets limiting in lysine and tryptophan in the MP diets presented higher NB than other amino acids indicating that these amino acids provided in the diet were above the maintenance requirement. However, roosters fed limiting diets in valine and histidine in MP diets had the NB close to zero, according to the initial assumption of an amino acid intake for

nitrogen equilibrium in the MP diets. Nevertheless, the linear equations obtained using the NB data and amino acid intake from LP, MP, and HP diets are presented in Table 3.

Table 2. Mean of daily values (±SD) obtained in the nitrogen balance assay resulting from individual amino acid (AA) deletions in the low (LP), medium (MP) and high protein concentration (HP) diets

Amino Acid	Diets[1]	AA intake (mg/kg)	Body Weight (kg)	Nitrogen		
				Intake (mg/kg)	Excretion (mg/kg)	Balance (mg/kg)
Lysine	LP	0.0±0.0	5.8±0.3	88.5±3.8	190.3±7.4	-101.9±5.8
	MP	9.9±0.6	6.0±0.3	174.1±9.9	130.5±6.0	43.6±6.0
	HP	20.1±0.8	6.0±0.2	313.1±15.4	112.5±6.9	200.6±15.0
Methionine	LP	0.00±0.0	5.9±0.2	78.6±2.0	141.9±18.7	-63.3±17.4
	MP	30.3±1.2	6.0±0.2	128.5±5.5	107.8±6.8	20.8±7.7
	HP	60.7±2.1	6.1±0.2	306.2±11.6	121.9±9.3	184.3±9.0
Threonine	LP	0.0±0.0	5.9±0.2	97.9±2.3	166.4±16.3	-68.5±15.2
	MP	25.1±0.7	5.9±0.2	166.4±4.7	129.0±7.9	37.5±7.6
	HP	49.8±1.4	6.1±0.2	301.6±8.9	121.4±12.2	184.2±15.3
Tryptophan	LP	0.0±0.0	6.0±0.1	93.4±5.3	150.3±23.9	-56.9±19.7
	MP	6.5±0.1	5.9±0.1	160.1±5.3	114.4±10.1	45.7±5.8
	HP	12.9±0.4	5.9±0.2	312.2±9.7	106.7±5.2	205.5±9.2
Valine	LP	0.0±0.0	5.9±0.1	100.2±5.5	202.6±25.1	-102.4±20.5
	MP	18.5±0.4	6.0±0.1	153.3±5.3	156.4±9.4	-3.0±4.4
	HP	36.0±1.1	6.1±0.2	243.4±7.1	127.2±7.0	116.1±5.3
Arginine	LP	0.0±0.0	6.0±0.1	99.1±6.4	186.0±20.6	-86.9±14.8
	MP	36.1±1.0	6.0±0.2	146.5±4.2	139.7±7.1	6.8±9.0
	HP	76.3±2.3	6.1±0.2	265.3±9.8	114.9±4.7	150.4±11.0
Isoleucine	LP	0.0±0.0	6.0±0.1	93.6±3.8	148.4±18.2	-54.8±15.7
	MP	22.5±0.4	6.0±0.1	143.6±3.3	110.5±6.4	33.2±4.6
	HP	42.7±1.3	6.1±0.2	245.4±6.9	98.9±18.4	146.5±7.1
Leucine	LP	0.0±0.0	6.0±0.1	95.9±4.4	175.6±13.5	-79.7±10.7
	MP	37.3±0.6	6.1±0.1	141.2±3.6	128.9±5.8	12.2±4.5
	HP	73.8±1.0	6.1±0.1	235.4±3.5	107.1±13.3	128.2±1.8
Histidine	LP	0.0±0.0	5.9±0.2	102.9±2.2	210.9±23.1	-108.0±21.2
	MP	2.4±0.0	6.0±0.1	165.7±2.6	164.1±5.4	1.5±6.4
	HP	4.7±0.1	6.1±0.2	235.2±5.6	135.0±7.4	100.2±5.1
Phenylalanine	LP	0.0±0.0	6.0±0.2	85.1±5.0	191.2±22.1	-106.1±19.0
	MP	18.2±0.3	6.1±0.1	130.8±1.6	142.4±6.9	-11.5±6.0
	HP	35.7±0.5	6.1±0.1	232.5±3.5	128.9±5.2	103.7±5.7
Glycine	LP	0.0±0.0	6.0±0.2	96.1±1.8	182.5±9.9	-86.3±8.4
	MP	25.5±0.5	6.0±0.1	150.8±2.8	137.3±3.7	13.5±4.3
	HP	51.2±1.2	6.0±0.2	268.4±3.8	132.2±6.6	136.2±4.7

[*]More details about the composition of the LP, MP and HP diets can be seen in item 2.2.3

The linear effect of the responses to the limiting amino acid intake is indicated by statistical analysis ($P < 0.001$). The high values of the R^2 observed indicate a good adjustment of the data to the linear models. Moreover, the mean variations of the coefficients estimated for the equations were lower than 4%.

Table 3. Equations resulting from the linear regression obtained with values of amino acid intake and nitrogen balance (NB), considering the values obtained with low (LP), medium (MP) and high protein concentration (HP) diets

Amino acid[1]	Equations from linear regression	R^2	Significance
Lysine	NB = -102.85 (±6.80) + 15.19 (±0.60) × Lys	0.989	< 0.001
Methionine	NB = -76.55 (±8.50) + 4.09 (±0.30) × Met	0.936	< 0.001
Tryptophan	NB = -66.18 (±8.30) + 20.46 (±1.20) × Trp	0.962	< 0.001
Threonine	NB = -74.49 (±7.70) + 4.99 (±0.30) × Thr	0.958	< 0.001
Arginine	NB = -96.53 (±7.10) + 3.05 (±0.20) × Arg	0.941	< 0.001
Valine	NB = -105.81 (±5.30) + 5.68 (±0.20) × Val	0.963	< 0.001
Isoleucine	NB = -61.26 (±8.10) + 4.57 (±0.30) × Ile	0.967	< 0.001
Leucine	NB = -86.09 (±5.50) + 1.73 (±0.10) × Leu	0.980	< 0.001
Phenylalanine	NB = -112.15 (±5.30) + 3.55 (±0.20) × Phe	0.955	< 0.001
Glycine	NB = -91.03 (±5.00) + 2.63 (±0.20) × Gly	0.984	< 0.001
Histidine	NB = -108.50 (±5.50) + 26.53 (±1.80) × His	0.953	< 0.001

The mean protein content in the carcasses was estimated to be 180g/kg of protein. This value was used to calculate the maintenance requirement based on body protein content ($mg/BP_m^{0.73} \times \mu$). Since the roosters were at maturity, the μ value (BP/BP_m) was considered equal to 1. Therefore, the maintenance requirements expressed in the four scales (mg/bird, mg/BW_{kg}, $mg/BW_{kg}^{0.75}$, and $mg/BP_m^{0.73} \times \mu$ per day) and the ideal essential amino acid ratio for maintenance are presented in Table 4.

The different scales did not influence the ideal amino acid ratio for maintenance. The analysed amino acid composition of the body free of feathers was Lys 117, Met 37, Trp 12, Thr 70, Arg 111, Val 80, Ile 67, Leu 123, Phe 65, Gly 134 and His 41 mg. For feathers the analysed amino acid composition was: Lys 66, Met 23, Trp 25, Thr 161, Arg 224, Val 207, Ile 155, Leu 265, Phe 157, Gly 237 and His 24 mg. Thereafter, these values were plotted against the amino

acid requirements of Table 4 for comparison as presented in Figure 1.

Table 4. Amino acid requirements for maintenance estimated in four scales and the ideal ratio relative to Lysine (100)

Amino acid	Daily maintenance requirement per scale*				Ideal ratio relative to Lys
	mg/bird	mg/BW_{kg}	mg/$BW_{kg}^{0.75}$	mg/$BP_m^{0.73} \times \mu$ †	
Lysine	40.62	6.77	10.60	38.40	100
Methionine	112.24	18.71	29.28	106.11	276
Tryptophan	19.41	3.23	5.06	18.35	48
Threonine	89.54	14.92	23.36	84.65	220
Arginine	189.88	31.65	49.53	179.51	467
Valine	111.76	18.63	29.15	105.66	275
Isoleucine	80.42	13.40	20.98	76.03	198
Leucine	298.54	49.76	77.87	282.23	735
Phenylalanine	189.56	31.59	49.45	179.21	467
Glycine	207.64	34.61	54.16	196.29	511
Histidine	24.54	4.09	6.40	23.20	60

* Mean value for body weight (BW_{kg}) used in the calculations was 6 kg.

† Analyzed protein content in the body was 180g/kg and was used to calculate mature body protein content (BP_m). Since the rooster were mature, μ (BP/BP_m) was considered equal to 1.

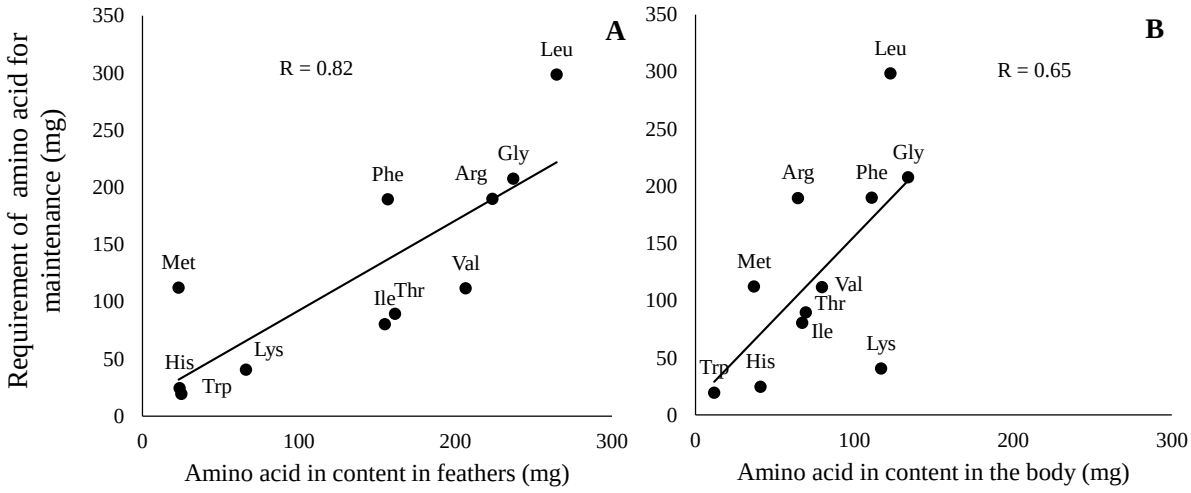

Figure 1. Graphical representation of the correlation between the predicted amino acid requirement for maintenance (●) and the observed (—) pattern of amino acid content in the feather **A** (Pearson´s correlation, R=0.82 and p<0.05) and body free of feathers **B** of adult roosters (Pearson´s correlation, R=0.65 and p<0.05).

The amino acid profile of the feathers was closer to the estimated amino acid requirements for maintenance (R=0.82) than the amino acid profile of the body free of feathers (R=0.65). However, as observed in Figure 1, it seems that methionine and leucine does not follow this trend.

2.4. DISCUSSION

The aim of this study was to determine the ideal profile of essential amino acids to meet the maintenance requirement of poultry using adult roosters. For this purpose it was used the deletion method to update and obtain new coefficients for maintenance requirement in poultry as suggested by Fuller et al. (1989), but with some adaptations to improve the methodology: 1) the experimental feed was provided by tube to ensure that the diet was completely consumed, and 2) the NF diet was provided ad libitium to ensure a positive energy balance minimizing amino acid catabolism from body tissues. These procedures were also applied in recent studies to estimate maintenance requirements (Burnham and Gous, 1992; Nonis and Gous 2008; Bonato et al., 2011).

Although the technique used have its sampling difficulties, we choose to use nitrogen excretion to determine nitrogen balance due to the advantages that the birds are not destroyed and this technique can theoretically be used over small periods of time in comparison to the determinations in the body lean mass. According to Abboudi et al. (2006), the period of measurement must be long enough to measure significant differences in protein growth but short enough to ensure that maintenance requirements will not vary during their evaluation. The same authors concluded that with longer measurement periods (>1 month) there is a decline of nitrogen excretion, affecting the estimations.

In this study, it was assumed that three points are enough to establish a linear regression, since the nitrogen balance data must provide: 1) a negative nitrogen balance; 2) a nitrogen balance close to zero; and 3) a positive nitrogen balance. Therefore, even with fewer points it was possible simultaneously determining all essential amino acids in a single trial. Thus, this methodology could be useful for the reassessment of the coefficients used for maintenance in different situations.

The maintenance requirement of lysine was estimated to be 6.77 mg/BW_{kg} per day or 40.62 mg/bird per day. Considering the requirement of lysine per bird, it was similar to 43 mg/bird per day estimated by Bonato et al. (2014). However, this value is lower than those reported in the literature, e.g. 37 mg/BW_{kg} per day estimated by Nonis and Gous (2008). In the other hand, it was stated that the most crucial difference between the amino acid pattern required for growth and that required for maintenance concerns lysine (Jansen, 1974). The same authors also explains that of all essential amino acids, lysine turnover is quite low and this implies that lysine required for maintenance is also quite low (Jansen, 1974).

The arginine maintenance requirement was estimated to be 31.65 mg/BW_{kg} in the present study which is lower than the estimated by Leveille et al. (1960) of 54 mg and higher than Ishibashi (1972) estimated requirement of 25 mg/BW_{kg}. According to Ball et al. (2007) arginine requirement varies widely and poultry have high requirements. Moreover, this requirement represents the metabolic arginine requirement for protein synthesis plus that required to replace the arginine that is degraded by renal arginase (Ball et al., 2007) and its requirement can be also related to the lysine requirement due to its well know antagonistic relationship (NRC, 1994).

In the present study, methionine was supplied in the feed but not cystine. However, the methionine maintenance requirement of 18.71 mg/BW_{kg} was identical to the requirement of 19

mg/BW_{kg} estimated by Bonato et al. (2011). On the other hand, this methionine requirement was lower than 39 mg/BW_{kg} estimated by Leveille et al. (1960) and 50 mg/BW_{kg} by Ishibashi (1972), both estimated in the absence of dietary cystine. According to NRC (1998), the higher value can be explained by the fact that methionine was converted to cystine; thus, when no cystine is available, methionine can meet the requirement for total sulphur amino acids (TSAA).

The phenylalanine maintenance requirement of 31.59 mg/BW_{kg} was close to 38 mg/BW_{kg} per day estimated by Leveille et al. (1960) in the absence of tyrosine. Similarly to methionine, phenylalanine can meet the total requirement for aromatic amino acids because it can be converted to tyrosine (NRC, 1998). However, tyrosine can satisfy at least 50 percent of the total need for these aromatic amino acids (Robbins and Baker, 1977) as well as cystine can satisfy approximately 50 percent of the need for TSAA (Roth and Kirchgessner, 1989) that could be estimated by a rough calculation.

Considering the branched chain amino acids (BCAA), the estimates for valine of 18.63 mg/BW_{kg} and isoleucine of 13.40 mg/BW_{kg} per day were close to the estimates from Ishibashi (1972) of 17 and 15 mg of valine and isoleucine per kg of body weight, respectively. The estimate of 49.76 mg of leucine per kg of body weight was closer to Leveille et al. (1960) estimate of 54 mg/BW_{kg}, which was higher than the range proposed by Ishibashi (1972) of 10 to 20 mg/BW_{kg}. The higher requirement of leucine in comparison to other BCAAs reflects its importance in regulating the catabolism of all BCAAs by controlling it own degradation and that of isoleucine and valine (NRC, 2006), which collaborate to maintain body weight.

For tryptophan, the requirement for maintenance estimated of 3.24 mg/BW_{kg} was lower than the range propose by Ishibashi (1972) of 5 to 10 mg/BW_{kg} and was lower than the recommendation of Leveille et al. (1960) of 7 mg/BW_{kg}. For threonine, the maintenance requirement was estimated to be 14.92 mg/BW_{kg} which was close to the estimate of Bonato et

al. (2011) of 17 mg/BW_{kg}, being very low in relation to 55 mg found by Leveille et al. (1960) and higher than that found by Ishibashi (1972) of 10 mg/BW_{kg}. Both amino acids are important for protein synthesis but also are important for maintenance of immune functions and, in the case of threonine, for replacement of gut mucosal losses (Baker, 2005).

For adult rooster, Leveille and Fisher (1959) concluded that histidine is not necessary to maintaining nitrogen balance, but Ishibashi (1972) suggests that its requirements could be in between 0 and 8 mg/BW_{kg}. Later, Amend et al. (1979) observed that histidine in fact is necessary to maintain body weight and adequate blood haemoglobin level as well as to prevent its decrease in brain and muscle tissue. In the present study, histidine was estimated to be 4.09 mg/BW_{kg} and it is in accordance to the range proposed by Ishibashi (1972) and was lower than 0.11% estimated by Amend et al. (1979), considering 24.54 mg of histidine per bird per day (Table 4) and a daily intake of 40g, which results in 0.06%.

Although glycine can be synthesized the rate is not adequate to support optimal growth (Featherston, 1976) but it is possible that this rate is enough for maintaining body weight since serine can be converted to glycine and this reaction is reversible (NRC, 1994). However, in the present study it was estimated that glycine maintenance requirement is 34.61 mg/BW_{kg}. Compared to other amino acids studied this requirement is expressively high. This is important since it was reported in chicks that glycine deficiency can lead to poor growth, inefficient feed utilization and lack of feathering (Graber and Baker, 1973) that could also affect adult birds.

The scales applied in this study (Table 4) are commonly used when expressing maintenance requirements, and can be used regardless of bird size. The scale mg/bird does not take into account bird weight; the mg/BW_{kg} per day scale considers body weight, but not body composition; mg/$BW_{kg}^{0.75}$ per day also enables a comparison to be made between requirements of birds of different sizes (mass : surface ratio) but not of different body composition. Since

amino acids are not required to maintain the content of water, fat, or minerals in the body (Gous, 2007), the scale mg/$BP_m^{0.73}$×u per day, which accounts for metabolic protein weight, is the preferred scale for expressing maintenance requirements for amino acids. The low values of maintenance requirement using the body protein weight (Table 4) indicate that at maturity small amounts of amino acids are required to maintain body at nitrogen equilibrium, being the nutrients that exceed their requirements deposited as fat (Forbes, 1995).

Another important aspect suggested by Leveille and Fisher (1959) is that the requirements an adult rooster are largely determined by feather synthesis. By plotting the amino acid content in feathers and body free of feathers (Figure 1) it was possible to verify a relative higher correlation between amino acid estimated for maintenance and amino acid composition in feather (R = 0.82) rather than in carcass free of feathers (R = 0.65). However, it seems that methionine and leucine don not fit this hypothesis. The same aspect can be observed in the study of Li et al. (2003) by comparing the amino acid pattern for carcass, feathers and maintenance.

The essential amino acid ratio presented in Table (4) was similar to that presented by Li et al. (2003). However, some differences in the ratios can be attributed to the different physiological states of the birds, i.e. in this study was used adult birds in maturity while in the study of Li et al. (2003) was used growing birds. Therefore, the order of importance of the amino acids used to synthetize a specific tissue (e.g. muscle or feathers) could possibly be the consequence of this variation.

In conclusion, the most important observation in this study is that the ideal ratio for maintenance is quite different from that observed for growth (Dorigam et al., 2015). Therefore, using only one ideal ratio for essential amino acids by the ideal protein concept to establish the requirement of other amino acids could not be reliable; instead, the calculation could be more

accurate if considering also an ideal ratio for maintenance. Moreover, this study presents an updated information of the amino acid required for maintenance of poultry in different scales for modelling use, being more indicated to express maintenance requirement as a function of metabolic body protein weight.

2.5. ACKNOWLEDGMENTS

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CAPÍTULO 3 - A comparison of two approaches for determining the optimum dietary amino acid ratios of fast-growing broilers

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A comparison of two approaches for determining the optimum dietary amino acid ratios of fast-growing broilers

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ABSTRACT

Two approaches using amino acid (AA) deletion method, were used to re-evaluate the ideal AA ratios (IAAR) between the essential AA for Cobb500 broilers during three periods (I: 6 to 21, II: 22 to 37, and III: 38 to 53 d). Per trial, 120 male broilers were housed in metabolic cages. The experimental design was composed by 12 treatments, 10 replications and one bird per cage. One balanced diet (BD) was formulated to meet exactly the nutritional requirements and eleven limiting diets were formulated by diluting BD with corn starch to meet 70% of these requirements. Except for the test AA, other nutrients were supplemented to achieve the same concentration as in the BD diet. For the "Louvain approach" a group of birds were killed at the beginning and at the end of each period to measure nitrogen deposition in limiting (ND_{EAA}) and balanced diets (ND_{BD}). These data was used to calculated the requirements by the equation: requirement=$(EAA)_{BD}$x(2-DEL-(ND_{EAA}/ND_{BD})), where DEL is the relation between the AA in limiting diet with the AA in the BD diet (EAA_{BD}). The relation between the AA requirements with Lys requirements provided de IAAR. By the "Goettingen

approach", the excreta was collected during 10 days after five days of adaptation in each period to quantify nitrogen retention (NR). Also, nitrogen intake (NI) was quantified in each period. Thus, the utilization efficiency (b) was calculated by: $b = (\ln NR_{max}T - \ln(NR_{max}T - NR))/NI$, where $NR_{max}T$ is the maximum "theoretically" nitrogen retention estimated in a previous study. By dividing the b value by the concentration of the AA in the dietary protein (c) gives the AA utilization efficiency (bc^{-1}). The IAAR was obtained dividing the bc^{-1} of Lys by the bc^{-1} of other AA.The IAAR determined by "Louvain approach" were: Lys 100, Met+Cys 65, Thr 66, Trp 17, Arg 108, Val 79, Ile 61, Leu 122, Phe+Tyr 128, Gly+Ser 155, and His 41. The IAAR determined by "Goettingen approach" were: Lys 100, Met+Cys 72, Thr 65, Trp 17, Arg 106, Val 76, Ile 67, Leu 107, Phe+Tyr 115, Gly+Ser 137, and His 35. There are some differences among the results obtained by the two methods, but "Goettingen approach" presented better results with more consistence and lower variability in the estimates.

Keywords: amino acid, ideal ratio, comparative slaughter, nitrogen balance

3.1. INTRODUCTION

In poultry feed formulation the quality of a dietary protein is associated with its amino acid (AA) composition and its bioavailability. In another words, the quality of a dietary protein can be considered to be the degree to which the composition of the absorbed AA mixture satisfies the amino acid balance required by animal (Wang and Fuller, 1989). Consequently the estimation of the EAAs required by an animal can be used as an assessment of quality of any dietary protein based on the AA pattern of a reference protein considered to be 'ideal'. Thus the knowledge about the optimal dietary AA pattern in broiler diets is continuously being improved with the use of several procedures with different physiological bases and accuracy.

Conventionally the EAA requirements have been assessed by dose-response studies using the technique of graded supplementation (Baker and Han, 1994; Mack et al., 1999; Baker et al., 2002). However, this method is expensive and time-consuming (Rollin et al., 2003) because multiple assays are needed. Therefore, another practical method has been employed to measure the composition of the EAA in the ideal protein required for swine (Fuller et al. 1989; Wang and Fuller, 1989) and this was later adapted for broilers (Gruber et al., 2000; Roth et al., 2001). The amino acid deletion method relies on a single experiment to determine optimum ratios of all EAA. This method is based on the concept that the reduction of a non-limiting AA has no effect on N deposition. Thus, the changes in N deposition measured on removal of a proportion of each EAA in turn are used to calculate a dietary AA pattern in which all EAA were equally limiting. The advantage of this method is that all amino acid ratios are determined simultaneously using the same stock of animals and the same balanced

diet (Green and Hardy, 2002). Consequently this allows better uniformity and consistency, facilitating the precision to determine the optimum AA ratios.

For practical use, a group of researchers from the Catholic University of Louvain proposed an equation to determine the AA "requirement" (Rollin et al., 2003). According to these researchers, the equation assumes that response to EAA is described by the "broken-line" regression approach. Although it was suggested that the utilization of a limiting EAA is well described by a non-linear model (Fatufe et al., 2004; Samadi and Liebert 2008), the inflection point of a broken-line model can predict minimal requirement values that are desirable for calculating EAA ratios (Baker, 2003). This method also assumes that the efficiencies of the EAAs are similar independent of their dietary concentration. Actually, it is known that the efficiency of utilization of the dietary AA is an important factor that affects amino acid requirements (Samadi and Liebert, 2006, 2007). Considering an equal protein deposition, the AA requirement is only dependent on its efficiency of utilization (Samadi and Liebert, 2008). In this way, it is possible to compare the efficiency of utilization of individual AA directly to evaluate the optimal AA ratio (Samadi and Liebert, 2008). This procedure to derive a scale of optimal AA ratios within one experiment is still under evaluation.

Nitrogen deposition can be assessed when using the deletion method either by the nitrogen balance technique, where deposited nitrogen is obtained as the difference between nitrogen intake and nitrogen excretion, or by comparative slaughter technique, where deposited nitrogen corresponds to the difference in body nitrogen of broilers between the start and the end of the experimental period. However, the undetected and additive losses of feed and excreta would overestimate nitrogen deposition by nitrogen balance technique and losses by

comparative slaughter technique would lead to errors (Just et al., 1982). Studies comparing both techniques indicate that the difference between these estimations of nitrogen deposition are variable (Just et al., 1982) but the description of its influence on determining the EAA pattern is examined only in a small number of studies (Zhengling, 2001).

In this context, the objective of the present study was to compare two approaches using amino acid (AA) deletion method, one with comparative slaughter and another with nitrogen balance technique, to re-evaluate the actual assumptions of an ideal ratio between the essential amino acids (EAA) : lysine for growing broilers.

3.2. MATERIAL AND METHODS

One nitrogen balance trial was performed per age period (I: 6 to 21, II: 22 to 37, and III: 38 to 53 d) using male broilers of Cobb 500 genotype. The experimental design consisted of twelve experimental diets and ten replicates per treatment. This study included two approaches for determining the optimum dietary amino acid ratio. For the nitrogen balance experiment a group of birds was utilized to study the effects of deletion of individual AA on protein quality (Samadi and Liebert, 2008). Simultaneously to this nitrogen balance trial, a group of birds was slaughtered at the start and end of the experimental period to provide data needed to calculate a dietary amino acid pattern in which all the amino acids were equally limiting by the requirement equation proposed by Rollin et al. (2003).

3.2.1. Animals and housing

Table 1. Composition of the experimental diets (g/kg in dry matter) to determinate the optimum ratio between the amino acids for male broilers (Cobb 500)

	Composition of the Balanced diets (g/kg in dry matter)		
	Period I (6 to 21 d)	Period II (22 to 37 d)	Period II (38 to 53 d)
Corn	740	779	804
Soy protein concentrate (60)	136	97.7	62.2
Soybean oil	12.7	15.4	21.9
Dicalcium Phosphate	17.2	14.9	12.8
L-Alanine (99%)	40.7	41.6	46.8
Limestone	8.95	8.42	7.51
L-Lysine HCl (78%)	7.16	7.05	7.01
DL-Methionine (99%)	4.40	3.99	3.54
Choline chloride(60%)	2.82	2.76	2.73
Glycine (96%)	6.19	4.47	4.51
Salt	4.80	4.58	4.49
Potassium chloride	1.41	2.69	4.03
L-Threonine (96%)	2.91	2.74	2.62
Vitaminic\ Mineral mix[1]	1.00	1.00	1.00
L-Arginine (99%)	3.84	4.07	4.34
L-Isoleucine (99%)	2.67	2.76	2.78
L-Valine (98%)	3.11	3.07	2.96
L-Phenylalanine (99%)	2.55	2.68	2.88
L-Histidine (99%)	0.76	0.77	0.80
L-Tryptophan (98%)	0.62	0.77	0.81
Calculated nutritional composition of the Balanced diets (g/kg in dry matter)			
Calcium	8.20	7.30	6.40
ME (MJ/kg)	13.0	13.2	13.4
Crude Fiber	16.6	16.2	15.6
Available Phosphorus	3.90	3.40	3.00
Potassium	5.90	5.80	5.80
Crude Protein	210	190	175
Analyzed content of amino acids (g/kg in dry matter) and EAA: Lysine ratio in the balanced diets			
Arginine	13.2 (108)	11.6 (108)	10.2 (109)
Phenylalanine+Tyrosine	14.0 (115)	12.3 (115)	10.8 (115)
Glycine+Serine	17.9 (147)	14.4 (135)	12.6 (134)
Histidine	4.5 (37)	4.0 (37)	3.5 (37)
Isoleucine	8.2 (67)	7.3 (68)	6.4 (68)
Leucine	13.0 (107)	11.6 (108)	10.2 (109)
Lysine	12.2 (100)	10.7 (100)	9.4 (100)
Methionine+Cystine	8.8 (72)	7.8 (73)	6.9 (73)
Threonine	7.9 (65)	7.0 (65)	6.1 (65)
Tryptophan	2.1 (17)	1.9 (18)	1.7 (18)
Valine	9.4 (77)	8.4 (79)	7.3 (78)

[1]Containing/kg: vit. A 15000000 UI, vit. D3 1500000 UI, vit. E 15000 UI, vit. B1 2 g, vit. B2 4 g, vit. B6 3 g, vit. B12 0.015 g, nicotinic acid 25 g, pantotenic acid 10 g, vit. K3 3 g, folic acid 1 g zinc bacitracine 10 g, selenium 250 mg, manganese 80 g; iron 80 g; zinc 50 g; copper 10 g; cobalt 2 g; iodine 1 g.

The experiments were carried out at the facilities of the Laboratory of poultry science of Faculty of Agricultural and Veterinary Sciences. Within the age periods, the birds were randomly allocated to treatments and individually housed in metabolism cages with wire floors, equipped with individual feeders and self-drinking systems. The temperature was controlled using a negative-pressure system, starting with 32°C (1 day old chicks) and decreased continuously to 24°C by 53 days.

3.2.2. Experimental diets

A balanced diet (BD) was formulated according to actual recommendations of the Brazilian Tables for poultry and swine (Rostagno et al., 2011) for the ideal protein in growing broilers for each period. The content of nitrogen and amino acids of the BD was supplied by corn, soy protein concentrate and a mixture of crystalline L-AA. Experimental diets with different limiting AAs were created by deletion of the BD with corn starch to achieve 0.70 of the AA level in BD and supplemented with crystalline AAs, except the AA under study. In all experimental diets, the remaining nutrient and energy contents were the same respectively. The composition, AA and nutritional composition of the BD in each period are shown in Table 1.

3.2.3. Procedures

The nitrogen balance trials were divided into adaptation period (five days) and two consecutive periods of excreta collection (5 days each). During this period the experimental diets were supplied until the end of excreta collection. At the beginning of the adaptation

period, diets were supplied *ad libitum* to predict the feed intake (according to metabolic body weight) for the collection period. The feed was supplied until the beginning of third day of the adaptation period. Based on the measured consumption of the last three days of adaptation the feed supply was slightly adapted for the next two days. At the beginning of collecting period the feed intake was measured again and the feed supply for each individual was kept constant to the end of the collecting period. This procedure was suggested as an acceptable adaptation to increasing feed intake in fast growing chicken (Samadi and Liebert, 2008). The excreta were collected directly from trays (free of feathers) and immediately stored in freezer at -20°C until further analysis. Body nitrogen deposition was also determined by the comparative slaughter method. At the beginning of the trial two birds of each replicate with similar body weight were euthanized using CO_2 after a fasting period of 36 h in order to determine nitrogen content in whole-body (carcass plus feathers) and at the end of each assay all remaining birds were also slaughtered. The slaughtered birds were also stored in freezer at -20°C for further processing.

3.2.4. Chemical analysis

The excreta stored in freezer were thawed, homogenized and weighed. The samples were then freeze-dried (Edwards 501, Thermo®, NC, USA) at -90°C for 72 h. The dried samples were ground in a micromill (A11 Basic, IKA®, Germany) and then stored in a freezer (-20°C) until analysis. The initial and final groups of slaughtered birds stored in freezer were autoclaved for 6 h once the temperature reached 127°C at a pressure of 1 atm. The birds were then homogenized in an industrial blender and the samples weighed before being placed in a forced draft oven at 55°C for 72 h. The dried samples were milled in ball mill and kept in

freezer (-20°C) until analysis. The diets were analyzed for dry matter, crude protein and AA. The excreta and whole-body composition were analyzed for dry matter and crude protein. Samples were analyzed using the following conventional procedures (AOAC, 2002): DM by drying at 105°C for 16 h, crude protein (N x 6.25) by the Kjeldahl method (method n. 2001.11) after acid digestion. Daily N gain was calculated on the basis of whole-body composition analysis. The AA compositions of diets were measured by hydrolysis with 6 N hydrochloric acid for 24 h. Amino acids released in the acid hydrolysis were separated by high-performance liquid chromatography (HPLC) reverse phase and detected by UV at 254 nm. Additionally, apparent metabolizable energy content of BD was calculated according to Rostagno *et al*. (2011).

3.2.5. Data analysis

All statistics were performed using a SAS statistical package (version 9.1). Data were submitted to variance analysis and the AA ratios were compared by the F test at 0.05 of probability. To confirm that the AA studied was limiting in the test diets in comparison to the BD treatment, the reference variables of nitrogen deposition (as a percentage of nitrogen intake) obtained in the Louvain approach and the protein quality (b) obtained by the Goettingen approach were tested using the Dunnett's test at 5% of significance.

In the nitrogen balance study the dietary protein quality (*b*) in each treatment was estimated according to following equation (Samadi and Liebert, 2008):

$$b = \frac{\ln NR_{max}T - \ln(NR_{max}T - NR)}{NI} \qquad \text{Eq. 3.1}$$

Where: $NR_{max}T$ is the theoretical maximum for N retention (mg $N/BW_{kg}^{0.67}$/d), NI is the N intake (mg $N/BW_{kg}^{0.67}$/d) and NR is the N retention (mg $N/BW_{kg}^{0.67}$/d) calculated as the sum of the N deposition (ND) and N maintenance requirement (NMR). The NMR values were determined previously (Dorigam, 2012) and were 219 mg $N/BW_{kg}^{0.67}$/d (6 to 21d); 264 mg $N/BW_{kg}^{0.67}$/d (22 to 37d) and 276 mg $N/BW_{kg}^{0.67}$/d (38 to 53d). The $NR_{max}T$ value is considered 'theoretical' because this value is not the same as for practical performance data, but estimates the genetic potential (Samadi and Liebert, 2006). The $NR_{max}T$ value for Cobb 500 genotype was estimated in a previous study (Dorigam, 2012) according to the procedure of Samadi and Liebert (2007). The $NR_{max}T$ values inserted in the equation were 3966 mg $N/BW_{kg}^{0.67}$/d (6 to 21d); 3401 mg $N/BW_{kg}^{0.67}$/d (22 to 37d) and 2480 mg $N/BW_{kg}^{0.67}$/d (38 to 53d). The slope of the linear function between dietary LAA concentration "*c*" (g AA/100 g CP) and the feed protein quality "*b*" was directly utilized as model parameter (bc^{-1}) indicating the efficiency of LAA utilization (Samadi and Liebert, 2006) and it is only valid when the AA is in limiting position. Consequently, it is possible to compare the model parameters (bc^{-1}) of individual AA directly. Using this procedure for evaluating the optimal AA ratio, comparisons are only allowed within equal age periods because $NR_{max}T$ varies with body weight and affects the established value of (bc^{-1}). The relationship between lysine efficiency (reference) and efficiency of AA under study is utilized to derive ideal AA ratios (IAAR):

$$\text{IAAR} = \frac{bc^{-1}_{\text{Lys}}}{bc^{-1}_{LAA}} \qquad \text{Eq. 3.2}$$

In the comparative slaughter study, the analyzed nitrogen content of whole-body composition was used to determine the nitrogen deposition using the following equation proposed by Rollin *et al*. (2003):

$$\text{N-Deposition} = \frac{(W_f \times N_f) - (W_i \times N_i)}{\frac{1}{2}\left(\left(\frac{W_f}{1000}\right)^{0.67} + \left(\frac{W_i}{1000}\right)^{0.67}\right) \times \Delta t} \qquad \text{Eq. 3.3}$$

Where: W_f and W_i are the mean final and initial live body weights (g), Δt is the duration of the feeding period (d), N_f and N_i are the mean N contents of the whole-body of broiler at the end and at the beginning of the experimental period (g/g), respectively. The coefficient for metabolic body weight used in the equation is 0.75 but, for data comparison, the coefficient 0.67 was used. From the relation of N deposition obtained in the amino acid deletion experiment it was possible to determine an optimum dietary amino acid pattern. In practice, Rollin *et al*. (2003) proposed to calculate the EAA requirement values (g/kg DM) for a given EAA as follows:

$$\text{requirement} = (EAA)_{BD} \times \left(2\text{-DEL-} \left(\frac{ND_{EAA}}{ND_{BD}}\right)\right) \qquad \text{Eq. 3.4}$$

Where: $(EAA)_{BD}$ is the concentration of the considered EAA in the BD (g/kg DM), DEL is the deletion rate calculated by dividing the EAA concentration in the deficient diet by the EAA concentration in BD, ND_{EAA} is the N deposition (mg $N/BW_{kg}^{0.67}$/d) corresponding to the EAA diet and ND_{BD} is the N deposition observed on the BD (mg $N/BW_{kg}^{0.67}$/d). This method is based on the assumption that N retention is a linear function of dietary essential amino acid content when a particular amino acid is limiting. An optimal balance between the EAA was derived by dividing the estimated requirement for each EAA by the estimated requirements for lysine (base lysine = 100).

3.3. RESULTS

All experimental diets were well accepted by the broilers. No mortality was observed during the trial but feather abnormalities were observed in broilers on the treatments in which valine and leucine were deficient. As the individual feed supply was controlled and kept constant during the experimental period, nitrogen intake was similar between dietary treatments (Table 2 and 3). The results of the single dietary EAA deletion on nitrogen deposition and protein efficiency ratio (PER) obtained in the comparative slaughter study are presented in Table 2 and the results of the N balance studies in each age period (relative effects on protein quality) are summarized in Table 3.

High nitrogen deposition and protein efficiency ratios ($p<0.05$) were observed in the BD treatment during trial. Deletion of individual EAA significantly reduced nitrogen deposition, but the extent of reduction depended on the EAA removed. For each EAA, a 30% reduction was sufficient to set it in limiting position. The protein efficiency ratio was also significantly reduced by EAA deletion. The deletion of valine promoted the greatest reduction in N deposition ($p<0.05$) in period I (6 to 21d), followed by leucine in period II and III (22 to 37 and 38 to 53d). Based on the data obtained in the comparative slaughter technique and assuming a linear response between N deposition and EAA intake when a given AA is limiting, the quantity of each EAA that can be removed from the BD without affecting N deposition was determined. From these data, and assuming that each EAA is equally limiting, the ideal dietary EAA profile relative to lysine (=100) is presented in Figure 1. Expressed as g/kg of dry matter (DM), the optimal balance was estimated and are presented in Table 2.

The effects of deletion of individual EAA on protein quality in the experimental diets are also of fundamental importance for evaluation of the applied procedure. Nitrogen deposition and protein quality were higher in BD ($p<0.05$) than in reduced EAA treatments during the trial. However, when comparing the nitrogen deposition obtained with the two techniques, the nitrogen deposition determined by comparative slaughter technique was lower than nitrogen deposition obtained in nitrogen balance. The observed protein quality in this study declined following deletion of crystalline EAA under study. The deletion of histidine caused a lower protein quality in period I (6 to 21d), followed by lysine and threonine in periods II and III (22 to 37d and 38 to 53 d), respectively. In each treatment the efficiency of utilization of dietary AA (bc^{-1}) was calculated. The ideal ratio between EAA was derived by dividing efficiency of utilization of lysine by the efficiency of utilization of the other EAA. The ratio between the EAA in each period using this procedure is presented in Figure 1.

In the present study significant differences ($p<0.05$) were observed in amino acid profiles between the two methods in periods I and II, but in period III they did not differ significantly ($p = 0.649$). In the whole growth period the ideal ratios were significantly different ($p<0.05$) when using the Louvain approach, but with the Goettingen approach the amino acid ratios in periods II and III were similar ($p = 0.287$). In this way we can consider the ratios between period II and III as comparable to the Goettingen Approach.

Table 2. Effect of deleting single amino acids from the diet on nitrogen deposition (ND) and protein efficiency ratio (PER) of fast growing broilers (Cobb500)[1] using comparative slaughter technique by Louvain approach

Diets	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Period I (6 to 21 d)												
W_i	123±3.5	123±3.6	123±3.7	123±3.7	123±3.3	123±3.3	123±3.6	123±3.8	123±3.9	123±3.9	123±4.6	123±4.9
W_f	913±57	686±47	798±87	731±75	646±42	739±41	723±64	778±73	671±56	669±54	835±71	695±33
ND	2427±23	2022±28	2135±30	2059±19	1958±16	1988±10	1956±42	2054±43	1969±28	1979±27	2096±33	2076±66
NI	4200±85	4214±87	4389±69	4306±102	4041±52	4201±170	4038±8.8	4121±155	4102±41	4206±24	4052±52	4146±46
PER	3.38±0.19	2.78±0.12	2.95±0.18	2.84±0.24	2.77±0.16	2.94±0.11	3.00±0.19	3.09±0.09	2.80±0.14	2.73±0.17	3.30±0.20	2.85±0.12
ND(% NI)	58.2±1.1	48.5*±0.9	47.3*±0.7	50.2*±1.0	48.2*±0.5	44.0*±2.1	48.6*±0.2	46.5*±1.2	48.4*±0.4	47.0*±0.2	52.3*±0.8	51.1*±0.6
AAR	-	10.64±0.04	7.20±0.05	1.76±0.01	7.41±0.04	11.58±0.03	9.15±0.02	5.08±0.06	12.61±0.04	14.76±0.16	16.64±0.10	4.27±0.12
Period II (22 to 37 d)												
W_i	975.6±14	975.6±12	975.6±13	975.2±12	975.2±10	975.6±11	975.8±11	975.8±10	975.6±10	975.6±10	975.8±10	975.0±12
W_f	2139±106	1939±91	2045±65	1863±189	1872±108	1903±88	1889±91	1958±86	1773±76	2032±104	2074±108	2080±82
ND	1982±15	1674±28	1752±30	1603±28	1715±20	1647±33	1591±38	1714±19	1279±28	1680±9	1644±24	1722±9
NI	3105±9	3537±83	3615±34	3152±80	3444±138	3340±243	3294±189	3464±165	2923±21	3503±124	3395±161	3427±107
PER	3.01±0.22	2.28±0.15	2.43±0.13	2.36±0.35	2.22±0.25	2.38±0.31	2.35±0.15	2.38±0.21	2.37±0.20	2.48±0.18	2.63±0.08	2.63±0.15
ND(% NI)	63.8±0.2	47.3*±0.9	48.5*±0.3	50.9*±1.0	49.8*±1.5	49.3*±3.0	48.3*±2.4	49.5*±2.0	43.8*±0.3	48.0*±1.4	48.4*±2.0	50.2*±1.3
AAR	-	9.41±0.04	6.50±0.12	1.72±0.03	6.40±0.05	10.92±0.09	8.04±0.07	6.50±0.08	12.24±0.20	11.96±0.04	14.83±0.23	4.05±0.01
Period III (38 to 53 d)												
W_i	2591±41	2591±40	2591±38	2591±38	2591±38	2591±38	2591±38	2591±38	2591±38	2591±38	2591±38	2591±38
W_f	3747±103	3419±145	3448±90	2883±375	3026±385	3326±164	3390±101	3157±70	3045±50	3435±194	3286±29	3453±164
ND	1321±19	812±19	1018±16	943±15	947±14	805±30	962±14	889±15	694±11	814±7	849±12	1012±19
NI[3]	2210±22	2011±39	2205±100	1988±55	2210±64	1897±8	2072±13	2082±29	1982±33	2034±5	2083±27	2167±26
PER[4]	2.58±0.20	2.09±0.29	1.99±0.22	1.65±0.31	1.78±0.06	1.99±0.42	1.97±0.22	1.43±0.15	1.22±0.11	2.10±0.45	1.73±0.04	2.01±0.32
ND(% NI)	59.7±0.5	40.4*±0.8	46.2*±1.6	47.4*±1.4	42.8*±1.0	42.4*±0.3	46.4*±0.2	42.7*±0.4	35.0*±0.5	40.0*±0.1	40.7*±0.6	46.7*±0.3
AAR	-	11.61±0.13	6.70±0.08	1.80±0.02	7.08±0.11	11.38±0.11	7.54±0.06	7.70±0.05	13.66±0.02	13.87±0.06	17.37±0.17	4.47±0.04

W_i = Initial weight (g); W_f = final weight (g); ND=nitrogen deposition (mgN/$BW_{kg}^{0.67}$ per day); NI=nitrogen intake (mgN/$BW_{kg}^{0.67}$ per day); PER =protein efficiency ratio (g of weight gain/ g of crude protein intake); AAR = amino acid requirement (g/kg).

*Significantly different from balanced diet (BD) treatment ($p<0.05$) by Dunnett's test.

[1] Mean ± standard error of mean (SEM).

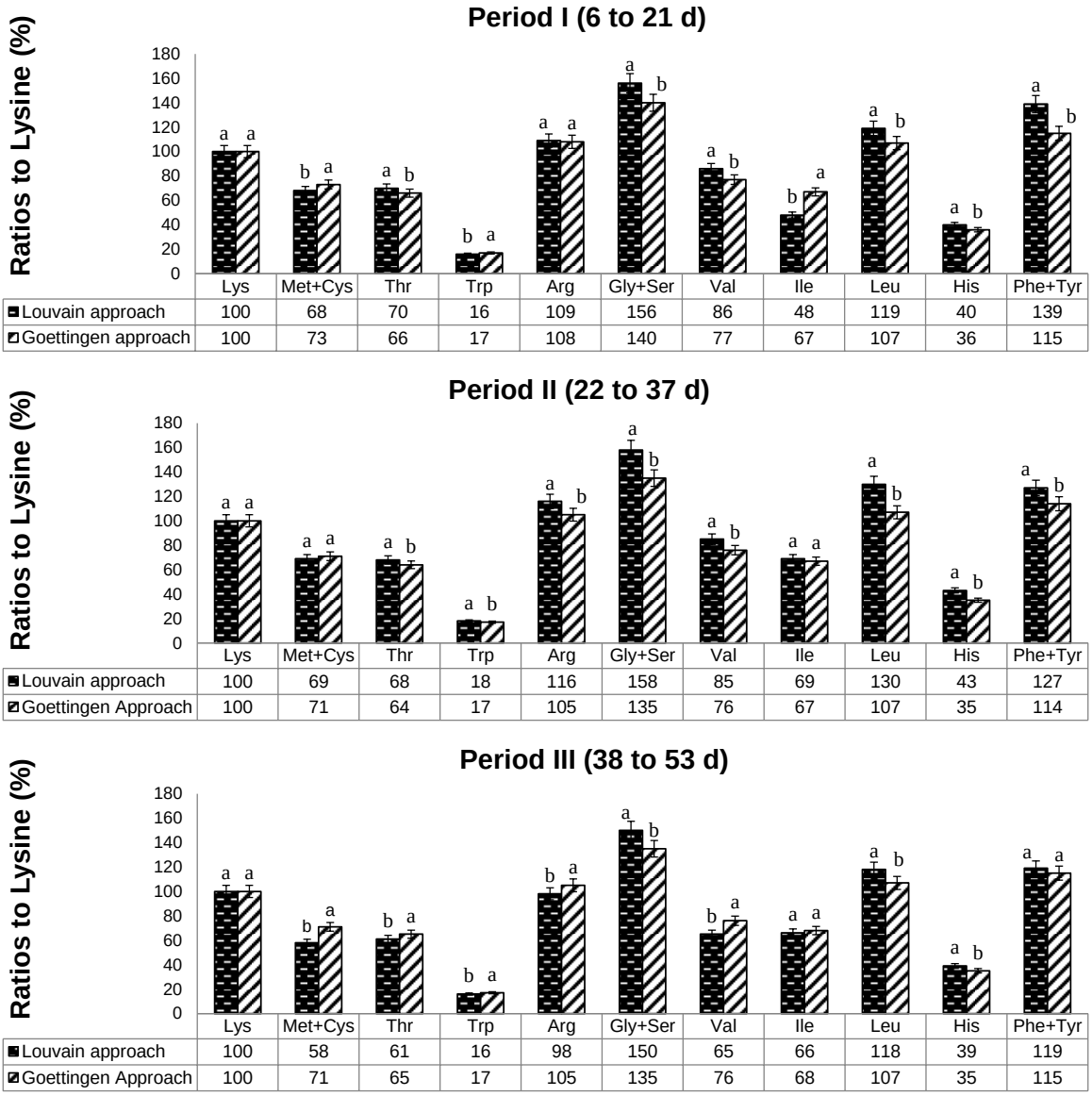
Table 3. Effect of deleting a single amino acid from the diet on protein quality (b) and nitrogen deposition (ND) of fast growing broilers (Cobb500)[1] in N balance trials by "Goettingen approach"

Diets	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Period I (6 to 21 d)												
BW	534±85	434±61	507±70	443±75	394±70	472±68	446±73	483±74	427±66	444±64	515±74	449±60
FI[2]	72±6	57±4	71±5	61±6	51±6	59±6	55±6	62±6	53±4	56±4	65±5	58±3
NI	4430±121	4238±117	4545±143	4364±95	4004±41	4078±32	3850±138	4203±152	4002±101	3904±136	3993±153	4192±252
ND	2960±28	2466±34	2604±36	2510±23	2387±19	2424±12	2386±52	2505±52	2402±35	2414±33	2556±41	2532±80
NR	3179±28	2685±34	2823±36	2729±23	2606±19	2643±12	2605±52	2724±52	2621±35	2633±33	2775±41	2751±80
b	366±1.8	267*±1.8	275*±3.0	268*±2.5	268*±1.7	269*±0.7	279*±1.9	277*±0.8	271*±2.2	280*±4.5	303*±4.5	208*±1.7
c	-	3.26	2.46	0.572	2.14	3.59	2.61	2.25	3.52	3.94	5.22	0.91
bc^{-1}	-	82±0.6	112±1.2	468±4.4	125±0.8	75±0.2	107±0.7	123±0.3	77±0.6	71±1.1	58±0.9	229±1.3
Period II (22 to 37 d)												
BW[2]	1603±136	1512±106	1514±158	1469±115	1437±130	1506±121	1490±112	1526±114	1392±121	1593±110	1580±127	1566±131
FI[2]	131±6	117±4	123±7	102±5	114±5	110±4	104±3	120±4	75±4	121±6	118±5	117±8
NI	3448±54	3557±118	3625±115	2943±72	3441±88	3291±130	3211±120	3469±93	2381±69	3436±37	3371±79	3232±58
ND	2417±18	2042±34	2137±36	1955±35	2092±25	2009±40	1940±47	2091±23	1559±34	2049±11	2005±29	2100±11
NR	2681±18	2306±34	2401±36	2219±35	2356±25	2273±40	2204±47	2355±23	1823±34	2313±11	2269±29	2364±11
b	451±0.5	320*±2.1	339*±2.0	360*±0.9	344*±2.7	337*±2.5	326*±1.2	341*±3.4	323*±2.4	332*±2.3	327*±2.7	368*±4.8
c	-	3.08	2.32	0.581	2.10	3.40	2.38	2.19	3.33	3.61	4.25	1.222
bc^{-1}	-	104±0.7	146±0.8	620±1.5	164±1.3	99±0.7	137±0.5	156±1.6	97±0.7	92±0.6	77±0.6	301±3.9
Period III (38 to 53 d)												
BW[2]	3205±163	3042±117	3028±112	2993±146	3027±92	2989±119	2998±123	2807±120	2811±97	3014±147	2910±126	3032±121
FI[2]	153±4	109±4	140±1	121±2	131±2	105±4	129±2	114±4	86±1	108±2	112±3	132±3
NI	2346±64	1824±58	2203±40	1936±39	2213±44	1752±69	2065±36	1983±46	1429±25	1725±29	1893±46	2088±60
ND	1610±23	990±23	1241±19	1150±18	1155±18	982±37	1173±18	1084±18	847±14	992±9	1035±15	1234±24
NR	1886±23	1266±23	1517±19	1426±18	1431±18	1258±37	1449±18	1360±18	1123±14	1268±9	1311±15	1510±24
b	611±0.9	393*±2.9	430*±1.7	442*±1.1	389*±2.4	405*±1.3	425*±1.8	402*±1.9	422*±3.6	416*±5.5	398*±5.8	451*±3.0
c	-	3.14	2.44	0.613	2.04	3.40	2.59	2.17	3.61	3.85	4.28	1.246
bc^{-1}	-	125±0.9	176±0.7	721±1.8	191±1.2	119±0.4	164±0.7	185±0.9	117±1.0	108±1.4	93±1.3	362±2.4

BW=mean body weight (g); FI=feed intake (g/d); NI=nitrogen intake (mgN/$BW_{kg}^{0.67}$ per day); NR=nitrogen retention (mgN/$BW_{kg}^{0.67}$ per day); b=Protein quality ($\times 10^{-6}$); c=amino acid concentration in the dietary protein (g AA/ 100g CP); bc^{-1} = efficiency of utilization ($\times 10^{-6}$).

*Significantly different from balanced diet (BD) treatment ($p<0.05$) by Dunnett's test.

[1]Mean ± standard error of mean (SEM).



	Lys	Met+Cys	Thr	Trp	Arg	Gly+Ser	Val	Ile	Leu	His	Phe+Tyr
Louvain approach	100	68	70	16	109	156	86	48	119	40	139
Goettingen approach	100	73	66	17	108	140	77	67	107	36	115

	Lys	Met+Cys	Thr	Trp	Arg	Gly+Ser	Val	Ile	Leu	His	Phe+Tyr
Louvain approach	100	69	68	18	116	158	85	69	130	43	127
Goettingen Approach	100	71	64	17	105	135	76	67	107	35	114

	Lys	Met+Cys	Thr	Trp	Arg	Gly+Ser	Val	Ile	Leu	His	Phe+Tyr
Louvain approach	100	58	61	16	98	150	65	66	118	39	119
Goettingen Approach	100	71	65	17	105	135	76	68	107	35	115

Figure 1. The ideal dietary EAA profiles relative to lysine in each age period for fast growing broilers (Cobb500) determined using the Louvain approach with comparative slaughter technique and by Goettingen approach with nitrogen balance study. Different letters in the graph indicate significant differences by F test at 0.05 probability.

3.4. DISCUSSION

Amino acids in excess to the animal's requirement are oxidized and used as an energy source, when dietary energy is limiting, and their nitrogen is excreted (Wang and Fuller, 1989). In the present study non-protein energy was supplied according to the broiler's requirement for age and maintained constant in diets to prevent energy being a limiting factor for protein accretion. Also, in all treatments, apart from those with amino acid deletions, all other dietary conditions (e.g. dietary energy and fiber) were the same. Another point is that the diets in the deletion method use industrial AAs to precisely modify the EAA composition of the experimental diets. However, the lower utilization efficiency of crystalline AAs compared to protein bound AAs suggested by many authors (Dabrowski and Guderly, 2002) may influence the estimation of EAA pattern. In this study, all the essential amino acids were supplemented in each experimental diet to ensure a balanced mixture and instead of feeding the animals once daily, the animals were fed several times a day ad libitum.

The observed changes in nitrogen deposition when dietary amino acids were reduced from the BD were used to calculate the optimum balance between the EAA in the N balance trial. In both methods, the deletion of valine promoted the greatest reduction in N deposition in period I (6 to 21d) followed by leucine in period II and III (22 to 37 and 38 to 53d). This indicates that valine and leucine were the first limiting AA in BD given in period I, II and III, respectively. This would lead to the feather abnormalities observed in valine and leucine treatments, similar to those observed by Gruber et al. (2000). As dietary valine and leucine levels decreased, a similar progressive feather abnormality became apparent and gave the feathers a ragged appearance (Robel, 1977) and is responsible for the decrease in body weight and feed conversion (Farran and Thomas, 1992).

As protein is essential for both growth and maintenance, nitrogen deposition is affected by the level of protein intake and by the quality of the dietary protein (Wang and Fuller, 1989). As the individual feed supply was controlled and kept constant during the experimental period, nitrogen intake was similar between dietary treatments. The decrease in protein efficiency ratio observed during the experimental period may be explained largely by the fact that as body weight gain increased with age, protein requirements for maintenance also increased (Brody, 1945) while feed or protein intake did not increase proportionately (Scott et al., 1969); hence a decrease in the quantity of protein available for growth (weight gain). However, protein efficiency ratio is not a good indicator of protein quality because this method does not consider the quantity of protein used for maintenance and values of protein efficiency ratio vary with levels of protein intake. Additionally, body weight gain does not necessarily correspond to body protein gain. However, protein quality (b) calculated using the Goettingen approach is a parameter that plots the slope of the exponential function. Thus, the dietary protein quality (b) is independent of NI but is linearly dependent on the concentration of the limiting AA in the feed protein (c). The observed protein quality (b) declined following deletion of crystalline AA under study. Due to EAA deletion protein quality was significantly affected. According to Samadi and Liebert (2008), for equal daily protein deposition, the daily AA requirement is only dependent on the efficiency of utilization of the individual dietary AA under study, and this was established by the model parameter (bc^{-1}). Consequently, it is possible to compare the model parameters (bc^{-1}) of individual AA directly. Using this procedure for evaluating the optimal AA ratio, comparisons are only allowed within equal age periods because varying $NR_{max}T$ depending on age affected the established value of bc^{-1}. Thus, the optimal lysine to EAA ratio was derived by dividing the efficiency of utilization of lysine by the efficiency of utilization of EAA.

The deletion method is generally accepted as an efficient and rapid tool to estimate the ideal EAA profile (Baker, 2003). This method was initially outlined by Wang and Fuller (1989) in pigs and is based on the concept that each EAA is equally limiting to protein accretion. In broiler chickens, estimation of the ideal dietary EAA profile by the deletion method has already been applied (Gruber et al., 2000 Roth et al., 2001) but only from 7 to 28 d post-hatching. Roth et al. (2001) estimated the EAA profile for broiler chicks by the deletion method and values obtained (Table 4) are very similar to those estimated in the present study by Goettingen approach. The data are also consistent with the recommendations of Brazilian tables (Rostagno et al. 2011) for periods I and II and the Illinois ideal protein pattern (Baker and Han 1994) in period I.

Additionally, in the whole grower period the ideal ratios differed with the Louvain approach, but using the Goettingen approach the amino acid ratios in periods II and III were similar ($p = 0.287$). Studies indicate that the ideal dietary amino acid profile for birds scarcely undergoes change in the whole grower period (Baker and Han, 1994) but the differences between period I to II and III (22 to 53 d) indicate the opposite. This difference is due to total requirement pattern depending on the relative contribution of maintenance and growth requirements (Zhen et al., 1999) in which maintenance contribution is smaller in young period and increases with the age.

Table 4. Ideal protein patterns for broilers based on literature

	Baker and Han[1] (1994)	Roth et al. (2001)	Rostagno et al. (2011)		Mack et al. (1999)	Present study[2]					
						GA	LA	GA	LA	GA	LA
Period	8-21d	8-28d	1-21d	22-56d	21-42d	6-21d		22-37d		38-53d	
Lys	100	100	100	100	100	100	100	100	100	100	100
Met+Cys	72-75	70	72	73	75	73	68	71	69	71	58
Trp	16-17	14	17	18	19	17	16	17	18	17	16
Thr	67-70	66	65	65	63	66	70	64	68	65	61
Arg	105-108	108	108	108	112	108	109	105	116	105	98
Val	77-80	81	77	78	81	77	86	76	85	76	65
Ile	67	63	67	68	71	67	48	67	69	68	66
Leu	109	108	107	108	-	107	119	107	130	107	118
Phe+Tyr	105	121	115	115	-	115	139	114	127	115	119
Gly+Ser	-	-	147	134	-	140	156	135	158	135	150
His	32-35	38	37	37	-	36	40	35	43	35	39

[1]Ranges in the amino acid ratios are due to differences in the response criteria and in the model adjusted data. [2] GA is Goettingen approach and LA is Louvain approach

In the present study, the nitrogen deposition measured by nitrogen balance technique was higher compared to nitrogen deposition estimated by comparative slaughter technique. This explains in part the small difference in EAA profiles determined by the two techniques, since the variation between the two estimates can represent approximately 0.16 (Just et al., 1982). Compared with nitrogen balance technique, the differential of nitrogen in carcass analysis by the comparative slaughter technique has an additional advantage of not overestimating the N gain due to potential unrecorded N losses (Heger and Frydrych, 1985) but the nitrogen balance technique is mostly preferable because of the association with animal welfare.

According to Rollin et al. (2003), the "requirement" equation relies on two main assumptions. In the first assumption, the equation assumes that the responses to EAA are well described by the 'broken-line' regression approach. In broilers chickens, most of the reported requirements

have been estimated according to the broken-line model (Baker et al., 2002). However, there are differences seen in published results due to criterion optimized and in the model fitted data. Some authors observed a continuously diminishing when the curve approach to a maximum response (Gahl et al. 1991; Gahl et al. 1994) and, in the other hand, some researchers believe that the animal's responses are linear models (Dunkin et al. 1986; Campbell et al., 1984; Campbell et al., 1985). Following the concept of the diminishing returns, the curvilinear models are most appropriated to describe the animal's response with better physiological background and due to this aspect these models have been used (Robbins et al. 1979; Rodehutscord et al. 1997). The "requirement" equation also relies on the assumption that EAA are utilized with similar efficiencies. In the Goettingen approach, the slope (bc^{-1}) calculated assumes that the dietary protein quality (b) is linearly dependent on the concentration of the limiting AA in the feed protein (c) (Samadi and Liebert, 2008). However, in this approach the calculated efficiencies (bc^{-1}) for each EAA are different and would be responsible for the differences in the estimation of the EAA ratio by the two methods. Clearly, research is still needed in these fields in order to clarify the potential limitations of these methodologies.

Results of this study support the premise that the deletion method can be used to estimate the optimum EAA pattern. Although this methodology relies on some assumptions that need to be further clarified, fast growth of animals and rigorous dietary formulation are expected to improve consistency of results. Comparing the two methods, the estimation of optimum amino acid ratio by Goettingen approach gave values closer to those in the literature and also show lower variation.

3.5. ACKNOWLEDGMENTS

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CAPÍTULO 4 - Modelling lysine requirements in broiler breeder hens based on potential for nitrogen retention and efficiency of dietary lysine utilization

Este capítulo é apresentado de acordo com as normas da **Animal Feed Science and Technology**

Modelling lysine requirements in broiler breeder hens based on potential for nitrogen retention and efficiency of dietary lysine utilization

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ABSTRACT: The current feeding programs used for broiler breeder hens need information on how to make use of the genetic potential more efficiently by modelling the amino acid (AA) requirements. Thus, this study aimed to determine the model parameters for maximum nitrogen retention ($NR_{max}T$), nitrogen maintenance requirement (NMR) and the efficiency of lysine utilization (bc^{-1}) to determine the lysine (Lys) requirements of broiler breeder hens. Nitrogen balance trials were performed in two periods (I: 31-35 wks and II: 46-50 wks). Seven treatments were used with eight replicates and one hen per cage; the treatments consisted of seven diets with protein levels ranging from 58.8 to 311.9 g/kg of feed, with Lys being limiting in the dietary protein (c = 3.91 g of Lys in 100 g of CP). For each period, the data of nitrogen intake (NI), nitrogen excretion (NEX), nitrogen in egg mass (NEM), nitrogen deposition (ND) and nitrogen retention (NR) were obtained in a N balance trial of 25 days. The NMR was calculated by the exponential relationship between NEX and NI. The $NR_{max}T$ and b (slope related to protein quality) were estimated by the exponential fit between NR and NI. The bc^{-1} was obtained dividing b by c. Based on the likelihood ratio test for the model parameters, the obtained values were 255 $mg/BW_{kg}^{0.67}$ for NMR, 0.000117 for b and 1684 $mg/BW_{kg}^{0.67}$ (period I) and 1484 $mg/BW_{kg}^{0.67}$ (period II) for $NR_{max}T$. The Lys intakes were estimated by the function $Lys = (\ln(NR_{max}T)-\ln(NR_{max}T-NR)):(16\times bc^{-1}))$, which resulted in the Lys intakes of 915 and 876 mg/d for breeder hens to achieve 80% of the $NR_{max}T$ in the periods I and II, respectively. The optimal dietary lysine concentration between the age periods were equal and they increased from 0.54 to 0.67% of Lys within the simulated feed intakes. The current study concludes that the optimal Lys requirement is in range with literature data, but the recommendations can be adapted according to feed intake, aimed protein deposition and dietary AA efficiency.

Keywords: dilution technique, egg mass, exponential, lysine, maintenance

4.1. INTRODUCTION

Over the past years, breeder companies made significant progress in producing more efficient broiler breeder hens with higher egg production. On the other hand, to maintain optimum production of hatching eggs, broiler breeder are fed restrictively to prevent overweight related reproductive problems (Joseph et al., 2000). Therefore, the amino acid (AA) recommendations for broiler breeder hens should be made considering these conditions.

The description of the potential performance of the animal as determined by the genotype is the first step in the determination of amino acid requirements (Emmans and Fisher, 1986). The modelling procedure as proposed by "Goettingen approach" (Samadi and Liebert, 2006a,b; Samadi and Liebert, 2007a,b; Samadi and Liebert, 2008; Khan et al. 2015; Liebert 2015) makes use of the estimated upper limit of an exponential function for body nitrogen retention (NR) depending on nitrogen intake (NI) to characterize the genetic potential. For breeder hens, this parameter is important because current feeding programs need information how to make use of the genetic potential more efficiently and amino acid requirements therefore. However, the only experimental data from adequate dose response studies are available from Halle et al. (1984b), but insufficiently related to modern broiler breeder hens' genotype.

In poultry nutrition, lysine is an important amino acid for the synthesis of egg and body protein, and the absence of this amino acid results in lower egg production (Domingues et al., 2012). In addition, lysine is the second limiting amino acid in practical diets and is mostly used as reference AA to relate other essential amino acids by the ideal protein concept (Emmert and Baker, 1997). Therefore, the lysine requirement should be known at high level of validity. However, the NRC (1994) recommendation for the daily total lysine requirement

is 765 mg, which is lower than 893 mg for the average hen or 1080 mg for a flock of breeder hens as reported by Fisher (1998). In addition, some studies on AA requirements are confounded by differences in dietary protein intake (Harms and Ivey, 1992). It can be summarized that, the experimental base to derive AA requirements of breeding hens is rather weak. In consequence, the aim with this study was assessing the digestible lysine requirements of broiler breeder hens based on model application (Goettingen approach) taking into account the potential for body nitrogen retention and dietary lysine efficiency.

4.2. MATERIAL AND METHODS

4.2.1. Ethics approval

This study was approved by the Ethics Committee on Animal Use of the Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal (no 9999/14).

4.2.2. Birds, housing and experimental design

Two nitrogen (N) balance trials were conducted at the Laboratory of Poultry Sciences of the University of Agrarian and Veterinary Sciences – FCAV / UNESP, Jaboticabal, São Paulo, Brazil (geographic coordinates: latitude 21º 15' 16" S longitude 48º 19' 19" W, altitude 607 m). Both trials were performed from 31 to 35 weeks (Period I) and from 46 to 50 weeks of age (Period II). Each utilized 56 broiler breeder hens of Ross 308AP genotype. The hens were individually allotted in a completely randomized design with seven dietary treatments and eight replicates each. The experimental units contained metabolic cages with wire floors,

individual feeders and water drinkers. The facilities were equipped with a negative-pressure ventilation system with controlled humidity and temperature, which was kept at 21^{o}C. The lightning program used was 13 hours of light according to Ross parent stock manual (Aviagen, 2013).

4.2.3. Experimental diets

Principles of the diet dilution technique were applied, as described by Fisher and Morris (1970). Initially, a lysine limiting summit diet (N6) was formulated based on corn and soybean meal to contain 12.18 gLys/kg feed. The Lys level in this diet was provided approximately 1.9 times of the recommendations for Ross parent stock nutrition (Avigen, 2013). The content of other amino acids was adjusted to provide 2.1 times the assumed requirement level, ensuring that the diets were limiting in lysine supply. According to diet dilution technique, a dilution diet (N0) was formulated to meet the nutritional requirements, except for protein and AAs. The diet composition is presented in Table 1.

The proportion of the N6 to N0 diet used in the preparation of the experimental diets was 14:86 for N1, 31:69 for N2, 49:51 for N3, 66:34 for N4, 83:17 for N5, and 100:0 for N6. Therefore, the lysine levels obtained were N1=2.30 g/kg, N2=4.35 g/kg, N3=6.90 g/kg, N4=8.57 g/kg, N5=11.25 g/kg, N6=12.18 g/kg. The seventh diet (N7) was used to confirm that lysine was the first limiting amino acid in the experimental diets. The level in the N7 diet was obtained by adding a small quantity of the industrial amino acid to the diet with the same composition as N1 (N7 = N1 + 2.62 g L-Lysine HCL 78%). In this case, if the nitrogen deposition of the hens are improved due to the small addition of the lysine in the N1 diet, then, this amino acid was limiting in the experimental diets. The N7 diet was used only to

confirm the limiting amino acid and the data is not used in the regression analysis. The nutritional composition of the diets are presented in Table 2.

Table 1. Composition of the experimental diets

Ingredients (g/kg)	Diets	
	Summit (N6)	Dilution (N0)
Corn	367.28	-
Soybean meal (45%)	351.82	-
Corn gluten meal (60%)	180.00	-
Limestone	68.48	63.00
Dicalcium phosphate	12.96	18.90
Soybean oil	9.57	65.00
Salt	4.63	3.30
DL-Methionine (99%)	2.86	-
Mineral and vitaminic supplement[1]	2.00	2.00
L-Tryptophan (98%)	0.27	-
L-Lysine HCl (78%)	0.10	-
L-Threonine (99%)	0.03	-
Potassium chloride	-	11.40
Corn starch	-	570.00
Inert (sand)	-	60.00
Rice husk	-	178.00
Sugar	-	28.40

[1]Content/kg - vit. A = 9000000 IU, vit. D3 = 2600000 IU, vit. E = 14000mg, vit. B1 = 2200mg, vit. B2 = 6000mg, vit. B6 = 3000mg, vit. B12 = 10000mg, Niacin = 30000mg, pantothenic acid = 15000mg, vit. K = 1600mg, folic acid = 600mg, selenium = 200mg, manganese = 70000mg, iron = 50000mg, zinc = 50000mg, copper = 8000mg, iodine = 1200mg.

4.2.4. Management and data collection

Body weight of hens was monitored weekly and egg production was monitored daily until the breeder hens reached the peak og egg production. According to body weight and egg production hens were allotted to the experimental units. The first N balance trial started at 31 weeks of age to obtain data for the peak egg production. The second N balance trial started

after 15 weeks (46 weeks of age) when a decline of egg production was observed. The daily amount of feed provided was 152g from 31 to 35 weeks and slightly decreased to 147g from 46 to 50 weeks, according to recommendations for hens kept in cages (Van Daele, 2014).

Table 2. Nutritional composition and digestible amino acid content of the diets

	Nutritional composition (g/kg) of the diets						
	N1	N2	N3	N4	N5	N6	N7
Crude protein	58.75	111.25	176.25	219.37	287.50	311.87	58.75
Ether extract	62.40	56.84	51.29	45.73	40.18	34.62	62.40
Crude fiber[1]	64.31	56.93	49.55	42.18	34.80	27.42	64.31
Calcium[1]	30.00	30.00	30.00	30.00	30.00	30.00	30.00
Sodium[1]	1.57	1.66	1.74	1.83	1.91	2.00	1.57
Potassium[1]	6.25	6.55	6.84	7.14	7.44	7.74	6.25
Available Phosphorus[1]	3.50	3.50	3.50	3.50	3.50	3.50	3.50
AMEn (Mcal/kg)[2]	2.80	2.80	2.80	2.80	2.80	2.80	2.80
	Amino acid composition[3]						
Lysine	2.30	4.35	6.90	8.57	11.25	12.18	4.41
Arginine	2.84	5.34	8.37	10.57	13.88	15.43	2.94
Histidine	1.37	2.54	3.98	5.15	6.58	7.29	1.49
Isoleucine	2.20	4.23	6.58	8.15	10.74	11.58	2.55
Leucine	6.17	11.73	18.65	23.25	30.16	32.74	7.24
Methionine+Cystine	2.36	4.42	7.07	8.65	11.45	12.38	2.69
Methionine	1.49	2.84	4.54	5.57	7.42	7.97	1.60
Threonine	2.24	4.24	6.69	8.27	10.88	11.80	2.51
Phenylalanine	3.08	5.73	9.09	11.32	14.29	16.07	3.60
Phenylalanine+Tyrosine	4.99	9.43	14.91	18.60	24.37	26.50	5.72
Tryptophan	0.52	0.99	1.56	1.96	2.55	2.80	0.60
Valine	2.58	4.88	7.73	9.61	12.65	13.63	3.14

[1]Calculated composition using data from Rostagno et al. (2011).

[2]Apparent metabolizable energy corrected by nitrogen balance, calculated.

[3]Analyzed composition of the amino acids in the experimental diets.

In each age period, N balance trials were divided into five days of adaptation period and 20 days of quantitative collection both of excreta and of eggs. A total of 20 days collection period was applied due to the irregularity of egg production of these broiler hens and to minimize the influence of this factor on N balance data. The excreta were collected in

the trays, stored in plastic pots and weighed at the end of each collection period. The hens were weighed at the beginning and at the end of the experiment to measure body weight. The leftovers were weighed daily to measure real feed intake. The egg production and egg weight were measured daily to calculate egg mass production.

4.2.5. Chemical analysis

The excreta and eggs collected from each experimental unit during each period were homogenised. The total samples were frozen and freeze-dried for 72 hours at -80°C under 800 mbar of pressure (Edwards® 501 Modulyo freeze drier, West Sussex, United Kingdom). The samples were weighed to quantify the dry matter content and were milled using a Micro Mill (IKA® A11 Basic Analytical Mill, Staufen, Germany). The N content of the diets, excreta and eggs were analysed in a N distiller (Kjeltec™ 8400 Foss, Foss, Hillerod, Denmark) using the Kjeldhal method (Method No. 2001.11) according to AOAC (2005). The factor 6.25 was used to convert nitrogen content to crude protein (CP). The total amino acid content of the ingredients and the experimental diets was analysed by high-performance liquid chromatography (HPLC). The digestible AA data were calculated based on the tables of Rostagno et al. (2011).

4.2.6. Statistical analysis

The nitrogen balance data were analyzed by a one-way ANOVA using a GLM procedure and were fitted to exponential models using PROC NLIN procedure in SAS (Statistical Analysis System, version 9.1); the Levenberg-Marquardt algorithm was used to

converge in a solution for these models. A regression analysis between nitrogen intake (NI, mg/$BW_{kg}^{0.67}$ kg per day) and nitrogen excretion (NEX, mg/$BW_{kg}^{0.67}$ per day) was used in the fitting of the exponential function:

$$NEX = NMR \times e^{b \times NI} \qquad \text{Eq. 4.1}$$

Where NMR is the nitrogen requirement for maintenance (mg/$BW_{kg}^{0.67}$ per day), b is the slope of the exponential curve and *e* is the Euler number. The NMR was estimated by considering the intercept of the curve on the y-axis (NEX) when NI = 0. The intersection point in the y-axis derived from the exponential regression between NEX and NI reflects the inevitable metabolic nitrogen losses (Wecke and Liebert, 2009). The nitrogen balance or nitrogen deposition (ND, mg/$BW_{kg}^{0.67}$ per day) was calculated as the difference between NI and NEX. Nitrogen retention (NR, mg/$BW_{kg}^{0.67}$ per day) was considered as the sum of nitrogen in egg mass (NEM, mg/$BW_{kg}^{0.67}$ per day), ND and NMR. A regression analysis between NI and NR was performed to fit another exponential model as presented in several studies with growing animals (Samadi and Liebert, 2006a,b):

$$NR = NR_{max}T \times (1 - e^{-b \times NI}) \quad \text{Eq. 4.2}$$

Where $NR_{max}T$ is the "theoretical" maximum nitrogen retention (mg/$BW_{kg}^{0.67}$ per day), b is the slope of the nitrogen retention curve and *e* is the Euler number. The $NR_{max}T$ is the asymptotic value in the exponential model, which was estimated by a statistical procedure following several iterations by the Levenberg-Marquardt algorithm until the sum of the squares of the residual was minimized. Therefore, the attribute "theoretical" is given to this

parameter because the value estimated is not attainable in practical condition, even if the hens are bred under perfect conditions. However, this is an important parameter used for the modelling procedure of the amino acid requirements.

The fitted models (1) and (2) for broiler breeder hens in each period were compared using a statistical analysis to verify the similarity of model parameters. The following hypotheses were tested:

(1) H_0: $NMR_{(period\ I)}$ or $NR_{max}T_{(period\ I)}$ = $NMR_{(period\ II)}$ or $NR_{max}T_{(period\ II)}$ = $NMR_{(period\ I+II)}$ or $NR_{max}T_{(period\ I+II)}$ vs. H_1: not all NMRs or NR_{max}Ts are equal;

(2) H_0: $b_{(period\ I)}$ = $b_{(period\ II)}$ = $b_{(period\ I+II)}$ vs. H_1: Not all b are equal;

(3) H_0: $NMR_{(period\ I)}$ or $NR_{max}T_{(period\ I)}$ = $NMR_{(period\ II)}$ or $NR_{max}T_{(period\ II)}$ = $NMR_{(period\ I+II)}$ or $NR_{max}T_{(period\ I+II)}$ and $b_{(period\ I)}$ = $b_{(period\ II)}$ = $b_{(period\ I+II)}$ vs. H_1: at least one parameter is not equal (NMR, NR_{max}T or b);

Based on these hypotheses, the following models were adjusted: Ω = unrestricted model, where the two parameters (NMR or NR_{max}T and b) were adjusted for each period; ω1 = restricted model, where the NMR or NR_{max}T parameter is common for both periods; ω2 = restricted model, where the b parameter is common for both periods; ω3 = restricted model, with all parameters common for both periods. The likelihood ratio test was used to test these hypotheses according to statistical procedures proposed by Regazzi (2003) and Carvalho et al. (2010). After deciding on the model parameter value to be used for the modelling procedure, the lysine requirement was calculated after a logarithimic transformation of the equation (2) according to several publications (Samadi and Liebert, 2006a,b; Samadi and Liebert, 2007a,b; Samadi and Liebert, 2008):

$$LAAI = (\ln(NR_{max}T) - \ln(NR_{max}T - NR)) / (16 \times bc^{-1}) \qquad \text{Eq. 4.3}$$

Where LAAI is the necessary daily intake of lysine (mg/$BW_{kg}^{0.67}$), c is the concentration of the lysine in feed protein (g/100g CP or g/16g N), and bc^{-1} is the efficiency of the utilization of dietary lysine (slope between b and c). The number 16 results from limiting amino acid concentration in the dietary protein (g/16g N). The optimum level of dietary lysine was calculated as the Lys requirement (g/d) divided by the feed intake (g) multiplied by 100. The feed intake used in the calculations was based on the metabolizable energy (ME) in the feed and the daily ME requirement of 472 and 456 kcal for hens with 31 and 46 weeks of age, respectively, according to Ross 308AP parent stock manual (Aviagen, 2013). In addition, it was considered the required ME intake for breeder hens raised in floor and considering 10% and 20% lower ME requirement for hens raised in cages to simulate the feed intake in these three scenarios as mentioned by Van Daele (2014).

4.3. RESULTS

The results obtained from the nitrogen balance trials in periods I and II are presented in Table 3. The nitrogen retention of the hens fed the control diet (N7) was in-between the nitrogen retention of the hens fed the N1 and N2 diets, therefore, confirming that the lysine was in fact the limiting amino acid in the experimental diets and the response of hens were not affected by the protein content of the diets. No significant differences were observed in body weight between the treatments for period I and II ($P>0.05$). The nitrogen content in egg mass produced (per metabolic body weight) did not differ between treatments in periods I and II ($P>0.05$). The effect of the diets were observed in the nitrogen retention in periods I and II ($P<0.05$), which raised sharply from N1 to N4 and then stabilized the response up to N6 diet.

Although the NR response stabilized from N5 to N6, the NEX in function of the NI increased exponentially in both periods as can be seen in the Figure 1.

Table 3. Data of initial weight (W_i, kg), final weight (W_f, kg) feed intake (FI, g/d), daily nitrogen intake (NI, mg/$BW_{kg}^{0.67}$), daily nitrogen excretion (NEX, mg/$BW_{kg}^{0.67}$), daily nitrogen deposition (ND, mg/$BW_{kg}^{0.67}$), daily nitrogen deposition in egg mass (NEM, mg/$BW_{kg}^{0.67}$) and nitrogen retention (NR, mg/$BW_{kg}^{0.67}$) obtained in the nitrogen balance trials with broiler breeder hens receiving graded levels of protein limiting in lysine (3.91 g Lys/100 g protein)[1]

	Period I (31-35 wks)								
Diets[1]	N1	N2	N3	N4	N5	N6	N7[2]	RSD[3]	p-value
W_i	3.95	3.98	4.00	4.00	4.06	4.02	3.90	0.07	NS
W_f	3.73	4.00	4.32	4.44	4.42	4.42	3.72	0.29	NS
FI	148	151	150	151	151	150	148	2.76	NS
NI	572	1063	1633	2003	2647	2854	658	64.81	**
NEX	305	418	853	1095	1742	1888	273	85.35	**
ND	267	646	780	908	905	966	384	43.17	**
NEM	357	338	409	356	425	374	351	46.81	NS
NR	878	1233	1443	1519	1585	1595	989	11.71	**
	Period II (46-50 wks)								
W_i	4.05	4.06	4.06	4.02	4.10	4.00	3.96	0.05	NS
W_f	3.84	4.00	4.24	4.30	4.67	4.42	3.86	0.48	NS
FI	144	144	145	146	144	145	144	3.19	NS
NI	539	1007	1573	1971	2467	2755	619	67.59	**
NEX	344	495	822	1145	1497	1748	351	106.43	**
ND	195	512	751	826	970	1007	268	73.21	**
NEM	189	214	237	277	230	231	192	76.15	NS
NR	638	980	1242	1356	1454	1492	714	17.91	**

[1]N1 = 2.30 g lysine/kg (9.40 g protein/kg), N2 = 4.35 g lysine/kg (17.80 g protein/kg), N3 = 6.90 g lysine/kg (28.20 g protein/kg), N4 = 8.57 g lysine/kg (35.10 g protein/kg), N5 = 11.25 g lysine/kg (46.00 g protein/kg), N6 = 12.18 g lysine/kg (49.90 g protein/kg).

[2]N7 is the counter-proof treatment (N1 + 2.62 g of L-lysine HCl (78 %) per kg of feed).

[3]Residual standard deviation, expressed in the same units as the related variable.

NS, not significant, **$p < 0.05$

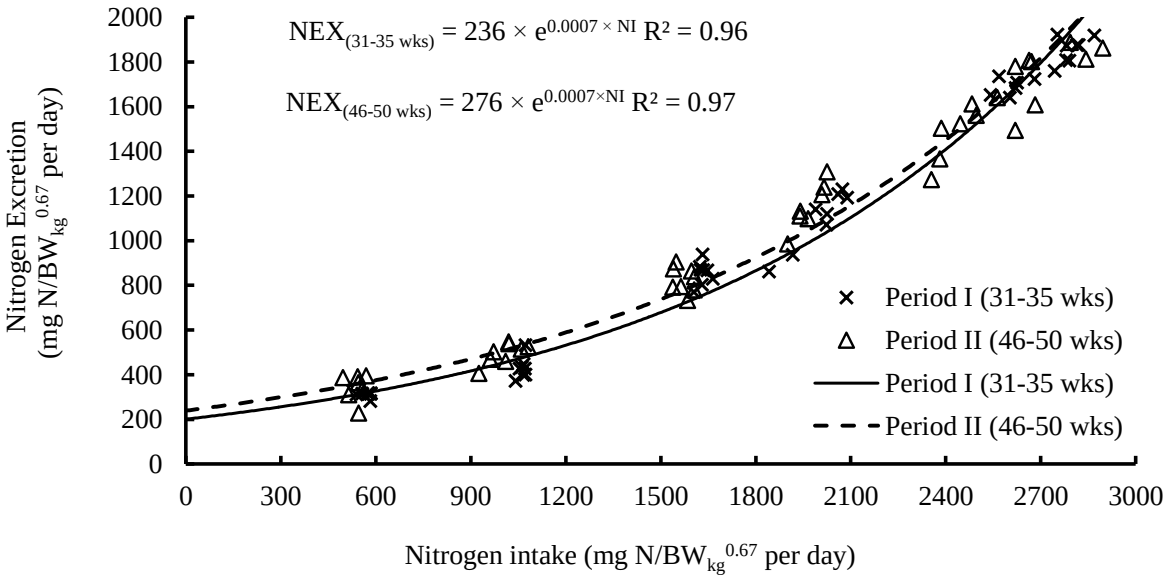

Figure 1. Estimation of the nitrogen requirements for maintenance by fitting an exponential function between the nitrogen intake (NI) and nitrogen excretion (NEX) during a gradual increase in supplied protein limited in lysine for broiler breeder hens in periods I (31-35wks) and II (46-50wks). *e* = Euler number. Observed (×) and predicted (—) values for period I. Observed (**Δ**) and predicted (-----) values for broiler breeder in period II.

Table 4. Estimative of the NMR and *b* parameters of the unrestricted (Ω) and restricted exponential models (ω_1 to ω_3) from the nitrogen balance data obtained in the period I and period II (*n*=94)

	Exponential model parameters						Statistics			
	Period I (31-35wks)		Period II (46-50wks)		Period I + II					
Adjusted models[1]	$NMR_{(31\text{-}35wks)}$	$b_{(31\text{-}35wks)}$	$NMR_{(46\text{-}50wks)}$	$b_{(46\text{-}50wks)}$	$NMR_{(period\ I + II)}$	$b_{(period\ I + II)}$	RSS	χ^2	d.f.	*P*
Ω	236.1	0.000735	275.7	0.000693			7367			
ω_1		0.000712		0.000719	254.1		7566	3	1	0.465
ω_2	251.1		257.1			0.000716	7482	1	1	0.583
ω_3					255.1	0.000714	7599	3	2	0.287

NMR = nitrogen maintenance requirement; *b*=slope of the exponential function; RSS = residual sum of squares; $\chi 2$ = Chi-square; d.f. = degrees of freedom;

[1]Adjusted models: Ω = unrestricted exponential model, where the two parameters (NMR and *b*) were adjusted to periods I and II; ω_1 = restricted exponential model, where the NMR parameter was common to periods I and II ($NMR_{(period\ I + II)} = NMR_{(period\ I)} = NMR_{(period\ II)}$); ω_2 = restricted exponential model, where the *b* parameter was common to periods I and II ($b_{(period\ I + II)} : b_{(period\ I)} = b_{(period\ II)}$); ω_3 = restricted exponential model, where the NMR and *b* parameters were common to periods I and II ($NMR_{(period\ I + II)} = NMR_{(period\ I)} = NMR_{(period\ II)}$ and $b_{(period\ I + II)} = b_{(period\ I)} = b_{(period\ II)}$).

[2]Probability of significance 5%;

According to the results of the statistical analysis it is possible to use the same exponential equation to describe the nitrogen maintenance requirement in both periods (P>0.05) since that there is no difference in the parameters estimated. Therefore, the adopted NMR value for further calculation is 255 mg/$BW_{kg}^{0.67}$ per day for both periods.

The results of the non-linear regression fitting between NI and NR demonstrated that $NR_{max}T$ decreased from 1,640 mg/$BW_{kg}^{0.67}$ per day (period I) to 1,554 mg/$BW_{kg}^{0.67}$ per day (period II) but with almost similar b value, as shown in Figure 2.

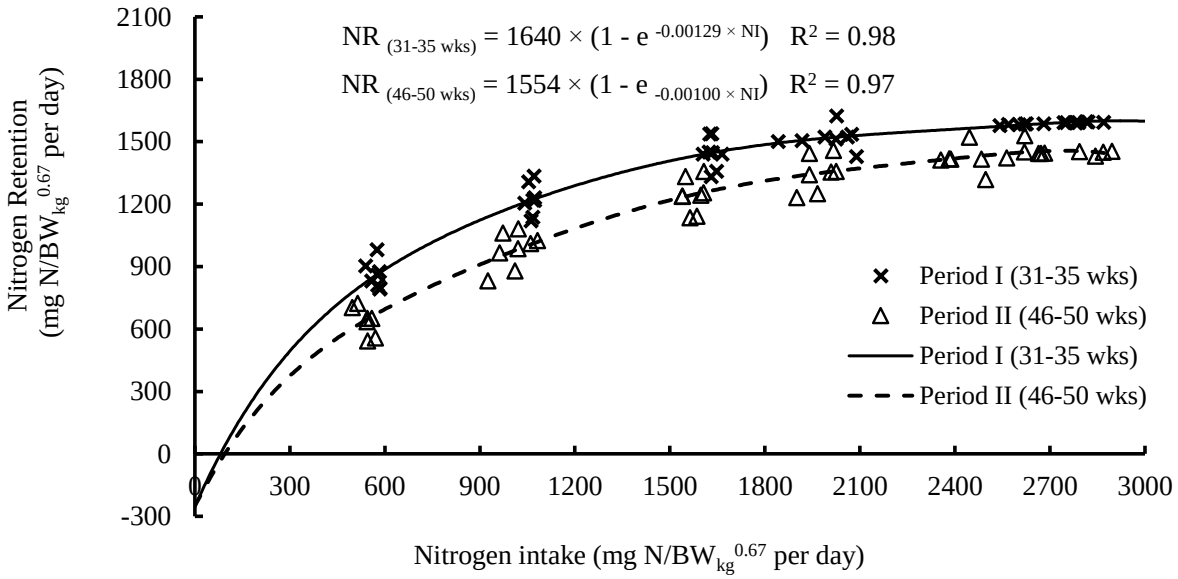

Figure 2. Estimation of the theoretical potential for nitrogen retention in broilers breeder hens of the Ross 308AP genotype based on the exponential fitting between the daily nitrogen intake (NI) and the daily nitrogen retention (NR) in periods I (31-35 wks) and II (46-50wks). *e* = Euler number. Observed (×) and predicted (—) values for period I. Observed (**Δ**) and predicted (----) values for broiler breeder in period II.

The model parameters $NR_{max}T$ and b, obtained in each period were submitted to the likelihood test to verify the equality of the models and the results are presented in Table 5.

Table 5. Estimative of the $NR_{max}T$ and *b* parameters of the unrestricted (Ω) and restricted exponential models (ω_1 to ω_3) from the nitrogen balance data obtained in the period I and period II (*n*=94)

Adjusted models[1]	Exponential model parameters						Statistics			
	Period I (31-35wks)		Period II (46-50wks)		Period I + II					
	$NR_{max}T$ (31-35wks)	*b* (31-35wks)	$NR_{max}T$ (46-50wks)	*b* (46-50wks)	$NR_{max}T$ (period I + II)	*b* (period I + II)	RSS	χ^2	d.f.	*P*
Ω	1639.9	0.001290	1554.2	0.001000			133			
ω_1		0.001340		0.000908	1617.6		392	101	1	0.078
ω_2	1684.3		1484.0			0.001160	862	176	1	0.059
ω_3					1595.7	0.001140	7809	383	2	0.003

$NR_{max}T$=maximum theoretical nitrogen retention; *b*=slope of the exponential function; RSS=residual sum of squares; χ^2=Chi-square; d.f.=degrees of freedom;

[1]Adjusted models: Ω = unrestricted exponential model, where the two parameters ($NR_{max}T$ and *b*) were adjusted to periods I and II; ω_1 = restricted exponential model, where the $NR_{max}T$ parameter was common to periods I and II ($NR_{max}T_{(period\ I + II)}=NR_{max}T_{(period\ I)}=NR_{max}T_{(period\ II)}$); ω_2 = restricted exponential model, where the *b* parameter was common to periods I and II ($b_{(period\ I + II)} : b_{(period\ I)} = b_{(period\ II)}$); ω_3 = restricted exponential model, where the $NR_{max}T$ and *b* parameters were common to periods I and II ($NR_{max}T_{(period\ I + II)} = NR_{max}T_{(period\ I)} = NR_{max}T_{(period\ II)}$ and $b_{(period\ I + II)} = b_{(period\ I)} = b_{(period\ II)}$).

[2]Probability of significance 5%

According to the results of the statistical analysis it is not possible to use the same exponential equation to describe the nitrogen retention in both periods ($P<0.05$). Although the statistical analysis provided no significant differences in the models when one $NR_{max}T$ value is considered for both periods ($P>0.05$), this hypothesis was rejected because egg mass production decrease with age and, thus, the NR and $NR_{max}T$. On the other hand the hypothesis that the b value is the same for both periods was accepted ($P>0.05$), because the same diet was used for both periods. Therefore, the adopted b value is 0.00117 for both periods and the $NR_{max}T$ values for period I and II are 1684 and 1484 $mg/BW_{kg}^{0.67}$ per day, respectively. Thus, the bc^{-1} value for period I and II was calculated as 0.000299.

Model calculation of Lys requirements depending nitrogen retention and observed average dietary Lys efficiency (Tables 6) used different standards for comparison ($mg/BW_{kg}^{0.67}$ per day; mg/day; percentage of the diet). To calculate Lys requirements according to hens´ production performance as expected under more practical feeding conditions 80% of the threshold value ($NR_{max}T$) were applied as levels of production performance. Based on the calculated parameters and the response of the broiler breeder hens in this study, the daily Lys intakes required to achieve 80% of $NR_{max}T$ were 915 mg and 876 mg in the period I and II, respectively. To calculate the optimal Lys concentration in the diets, predictions for daily feed intake in line with the observed restrictive feed intake were used. According to the simulation in Table 6, the optimal dietary lysine concentration do not differed between the age periods but they must be increased with the type of rearing system when the allowed feed intake becomes lower due to the metabolizable energy requirement of hens reared in cages in comparison to those reared in floor.

Table 6. Model calculation of the lysine requirement (Lys) for broiler breeder hens (Ross 308AP) in age period I (31-35 wks) and II (46-50 wks) depending on the determined efficiency of lysine utilization and different predictions for feed intake

Model parameters	Period I (31-35 wks)		Period II (46-50 wks)	
$NR_{max}T$ (mg $N/BW_{kg}^{0.67}$ per day)	1684		1484	
NR (mg $N/BW_{kg}^{0.67}$ per day)	1371		1188	
Lys efficiency (bc^{-1})	0.000299		0.000299	
Lys intake (mg $N/BW_{kg}^{0.67}$ per day)	352		337	
Lys intake (mg/day)	915		876	
	Optimal dietary lysine concentration			
	FI (g/day)[1]	Lys (%feed)	FI (g/day)[2]	Lys (%feed)
	169	0.54	163	0.54
	152	0.60	147	0.60
	135	0.67	130	0.67

FI=feed intake; $NR_{max}T$= maximum theoretical nitrogen retention; NR= nitrogen retention; MBW=metabolic body weight.

[1] The feed intake was calculated based on the recommendation for a daily metabolizable energy (ME) of 472 kcal/hen per day (31 weeks-old hens) and 2800 kcal ME/kg of feed, simulating three conditions: floor (100% of ME), cage (90% of ME) and cage (80% of ME).

[2] The Feed intake was calculated based on the recommendation for a daily metabolizable energy (ME) of 456 kcal/hen per day (46 weeks-old hens) and and 2800 kcal ME/kg of feed, simulating three conditions: floor (100% of ME), cage (90% of ME) and cage (80% of ME).

The optimal dietary lysine concentration between the age periods were equal but they increase as the simulated feed intakes became lower.

4.4. DISCUSSION

The objective of this study was to determine the model parameters NMR and $NR_{max}T$ for broiler breeder hens to use in the modelling procedure of the lysine requirements. Preliminary results for NMR shown an increase in maintenance requirements from 236 (31-35wks) to 276 mg/$BW_{kg}^{0.67}$ per day (46-50wks). Probably because the nitrogen losses from endogenous protein degradation is lower at higher rates of egg production (Ekmay, et al. 2013b), which could partially have contributed to the lower maintenance requirement in period I (31-35wks). However, as these values had no apparently difference (40 mg/$BW_{kg}^{0.67}$ of difference), it was used the likelihood test ratio for non-linear models to confirm the equality of these two values (Regazzi, 2003). The results indicated that one exponential model can be used to described the maintenance requirements for both periods, since that no difference was observed in these model parameters. Therefore, the value of 255 mg/$BW_{kg}^{0.67}$ was adopted as "working value" for the daily nitrogen maintenance requirement of broiler breeders with the age periods under study. The observed NMR in this study 255 mg/$BW_{kg}^{0.67}$ is close to the NMR value of 259 mg/$BW_{kg}^{0.67}$estimated by Halle et al. (1984a) in the top laying period (29-30wks). A most recent prediction for maintenance was derived by Rabello et al. (2002) indicates that daily inevitable nitrogen losses would be 248 mg/$BW_{kg}^{0.67}$ for broiler breeder hens, which is also close to our results.

The protein deposition is the result of the balance between synthesis and degradation ratio of protein, which can be affect by genotype, age, gender and nutrition (Samadi and Liebert, 2006a). In the case of broiler breeder hens, the egg production can also alter protein synthesis and degradation ratio (Ekmay et al., 2013b). Although the statistical results indicated that one $NR_{max}T$ can be used for both periods (Table 5), this hypothesis was discarded due to the differences in egg production rate in the two periods, which directly affect the NR response. In addition the same experimental diets were provided for these hens

in both periods, and since the b value indicates the protein quality (Samadi and Liebert, 2006a) they should not be different. Therefore, the hypothesis that one b value can be used for both periods was accepted due to these assumptions. In this case, the threshold value ($NR_{max}T$) was 1684 mg/$BW_{kg}^{0.67}$ in the peak production (31-35wks) and decreased to 1484 mg/$BW_{kg}^{0.67}$ from 46 to 50 wks. The only information found in the literature about the upper limit for nitrogen retention of breeder hens was in the study of Halle et al. (1984b), which estimated 1,440 mg/$BW_{kg}^{0.67}$ for broiler hens at peak production (29-30wks). This value is 244 mg/$BW_{kg}^{0.67}$ lower than the estimated value in the current study for peak production and we can assume that this difference could be due to the genetic improvement of the broiler breeder hens over the past 31 years.

The nitrogen retention observed in this study are close from the estimated $NR_{max}T$ values, which was approximately 80% of the $NR_{max}T$. One would wonder about reaching this maximum nitrogen would be impossible due to the restrictive feed intake, but in fact, this value is obtained from a mathematical extrapolation provided by the exponential model and it is not attainable in practical conditions (Samadi and Liebert, 2008). The “theoretical” upper limit is utilized only for ranking the performance data within the procedure of modelling of amino acid requirements (Wecke and Liebert, 2009). In the case of broiler breeder hens, the percent of $NR_{max}T$ should be used to maximize the body nitrogen retention without compromise egg mass production.

According to the utilized model, both variation of daily nitrogen retention parameters and predicted feed intake may yield remarkable changes of the concluded optimal Lys concentration in the diet (Samadi and Liebert, 2007a). This fact may create difficulties when results of modeling are compared with recommendations, which are not able to take these important factors of influence into account. For comparison of current data with latest results

in broiler breeders, the database is scarce. However, the estimated optimal lysine intake in this study was in the data range given in literature. Fisher (1998) calculated lysine intake as 893 mg per day from 27 to 33 weeks for the average individual, which was close to the lysine intake of 915 mg/day for broiler breeder hens from 31 to 35 weeks calculated in the current study. For maximum egg mass production, Ekmay et al. (2013a) determined in two consecutive trials the daily requirements of digestible lysine as 909 mg and 919 mg in peak production, respectively, which was also closer to the Lys requirement in the current study. Fakhraei et al. (2010) suggested total daily Lys requirement for 60-wks-old broiler breeders at 1012 mg, but assuming 87% of digestibility as Ekmay et al. (2013a) suggested, the daily requirement would be 881 mg and is closer to the requirements estimated in this study.

An important consideration in this study is that energy maintenance requirement for broiler breeder hens kept in cages can be 10%-20% lower than that for hens kept in floor due to the lower activity and lower heat production (Van Daele, 2014). Because of the restricted feed program of the breeder hens, the energy intake will always be a limiting nutrient and will determine the desired feed intake; therefore, once the amino acid requirement is established, the dietary level will depend on the feed intake (Fisher, 1998). Therefore, it was simulated three conditions which broiler breeders can be reared (Table 6). Since the feeding standards for broiler breeders in cages are practically non-existent (Daghir and Jones, 2008) the focus in the comparisons will be the recommendations for birds reared in floor pens. According to Rostagno et al. (2011), the dietary lysine concentration for a breeder hen with 3kg and daily feed intake of 159 g would be 0.57% digestible Lys, which is close to the dietary concentration calculated with our data. Bowmaker and Gous (1991) suggests that a broiler breeder hen with 3kg, egg mass production of 45 g and consuming 150 g of feed requires 0.53% of digestible Lys, which is the closer to our calculations (0.54%). The dietary Lys

concentrations are also in agreement with the Ross parent stock nutrition specifications (Avigen, 2013) of 0.56% of digestible Lysine. These recommendations for the breeder hens reared in cages are slight above those recommendations to compensate the lower feed amount. On the other hand, Van Daele (2014) suggests that there is no need to adjust the amino acid and protein intake, because this dietary management this help to control body weight gain without affecting egg production under cage conditions.

In general, it was observed that most of the earlier data in the literature are similar to our data calculated for the performance in nitrogen retention obtained in the current study. Furthermore, the modelling procedure proposed here contribute to formulate nutritional strategies to feed these hens for each rearing system, since the $NR_{max}T$ and the lysine utilization efficiency is known and can be also used as an interesting tool to describe the nitrogen utilization based on physiological interpretations of interrelationships between metabolism and

In conclusion, although the optimal Lys intake and dietary concentrations presented here are similar to those of the literature, it is important to consider that the optimal in-feed recommendations depend on many factors, such as the variation in the daily nitrogen retention and feed intake. In the case of broiler breeder hens, the egg production rate also have directly influence in the nitrogen retention which would affect the requirements of lysine. In general, this modelling approach presented have a number of advantages for improving the methodical base for the calculation of the amino acid requirements for both growth and egg production.

4.5. ACKNOWLEDGEMENTS

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4.6. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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CAPÍTULO 5 - Assessment of the optimal dietary amino acid ratio based on individual amino acid efficiencies for broiler breeder hens

Este capítulo é apresentado de acordo com as normas da **Animal Feed Science and Technology**

Assessment of the optimal dietary amino acid ratio based on individual amino acid efficiencies for broiler breeder hens

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ABSTRACT: An optimal feed formulation for breeder hens depends on the adequacy of the dietary amino acid (AA) balance that strictly meet its requirements, considering both genetic potential and individual dietary AA utilization. Thus, the aim of this study was to apply the individual AA efficiency data (bc^{-1}) of Lys and each essential AA to derive an ideal AA ratio (IAAR) for breeder hens. N-balance trials were performed from 31 to 35 wks and from 46 to 50 wks. Twelve treatments with eight replicates and one hen per cage were used. A balanced diet (BD) was formulated to meet the IAAR and the requirement of other nutrients for breeder hens. The limiting diets were formulated diluting BD with corn starch and refilled with crystalline AAs and other feed ingredients, except for the AA under study. In each period, the data of N-intake (NI), N-excretion (NEX), N in egg mass (NEM), N-deposition (ND) and N-retention (NR) were obtained in a balance trial of 25 days. The b values (protein quality) were estimated by $b = (\ln(NR_{max}T)-\ln(NR_{max}T-NR)):(NI))$, where $NR_{max}T$ is the potential for maximum nitrogen retention of breeder hens. The parameter b in the limiting diets declined significantly in comparison to the b value in BD ($P < 0.05$), confirming the limiting position of the studied AA. In both periods, the dilution of the histidine led to a greater impairment of the dietary protein quality (b). The bc^{-1} values were obtained dividing b by the dietary AA concentration (c, g AA/16g N). The IAAR was obtained by dividing the bc^{-1} from lysine by the bc^{-1} value from each other AA in each period. As the IAAR in each period do not difer numerically, an average IAAR value for both periods was derived: Lys (100), Met+Cys (83), Trp (24), Thr (81), Arg (114), Val (90), Ile (92), Leu (106), Phe+Tyr (109), Gly+Ser (95), and His (35). The IAAR was in the line with the recommendation from the literature, validating this alternative procedure for predicting dietary IAAR for broiler breeder hens and propovided an updated IAAR for broiler breeder hens.

Keywords: Amino acid efficiency, ideal ratio, deletion method, genetic potential

5.1. INTRODUCTION

Over the past decades body composition of broiler breeder hens has dramatically changed due to the influence of the market demands. As consequence, increasing performance potential likely leads to changes in nutrient and thus amino acid (AA) requirements. Actually, the biggest challenge of feeding the broiler breeder hens is how to supply AAs and other nutrients without the large appetite of these hens affect its weight gain (Silva, et al. 2012). Normally this is accomplished through the use of feed restriction, but feeding must be adjusted to meet the daily nutritional requirements.

In the laying period, it is important to supply adequate AA quantities and ratios to obtain maximum egg mass production at peak production to produce a chick with better quality (Silva, et al. 2012). The supply of the essential AAs demands proper understanding on their metabolic effects on egg production and other reproductive traits, since the unbalanced dietary essential AAs can be deleterious to hen's metabolism, e.g. when there are AA antagonisms (Silva et al. 2012). The ideal protein concept can play an important role in breeder hens' nutrition because it provides precise ratio of AAs. This concept suggests that, theoretically, the ratios between the essential AAs and lysine are not influenced while the quantitative AA requirement is affected by many factors. Thus, a maximized correspondence between dietary AA supply and physiological requirements can improve metabolic efficacy within the physiological possibilities and provide sustainability in animal production (Wecke and Liebert, 2013).

Currently, the AA ratios are derived from dose response studies making use of the graded amino acid supplementation (Baker and Han, 1994; Mack et al., 1999; Baker et al., 2002). However, this procedure is expensive and time-consuming because multiple assays are

needed (Rollin, et al. 2003; Dorigam et al., 2015). On the other hand, Wang and Fuller (1989) have developed another approach based on the responses in nitrogen retention with the individual deletions of the AAs from a complete diet. The observed slope of the response criteria between complete and deleted diets is utilized for conclusion of optimal AA ratios. The advantage of this approach is that all AA ratios are determined simultaneously using the same stock of animals and the same balanced diet (Green and Hardy, 2002; Dorigam et al., 2015). Consequently this allows better uniformity and consistency facilitating the precision needed to determine the optimum AA ratios (Dorigam, et al. 2015).

The deletion method concept was used in the present study, but the effect of the individual AA deletion was directly measured by amino acid efficiency from modelling of the observed nitrogen balance as proposed by Wecke and Liebert (2013). Thus, the aim of this study was to apply the efficiency data observed from the nitrogen balance study for Lysine, Methionine+Cystine, Threonine, Tryptophan, Arginine, Valine, Isoleucine, Leucine, Phenylalanine+Tyrosine, Glycine+Serine and Histidine to derive an optimal amino acid ratios for broiler breeder hens.

5.2. MATERIAL AND METHODS

5.2.1. Ethics approval

This study was approved by the Ethics Committee on Animal Use of the Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal (no 9999/14).

5.2.2. Birds, housing and experimental design

Two nitrogen balance trials were conducted at the Laboratory of Poultry Sciences of the University of Agrarian and Veterinary Sciences – FCAV / UNESP, Jaboticabal, São Paulo, Brazil (geographic coordinates: latitude 21º 15' 16" S longitude 48º 19' 19" W, altitude 607 m). The trials were performed from 31 to 35 weeks (Period I) and from 46 to 50 weeks of age (Period II). For each balance trial, 96 broiler breeder hens of Ross 308AP genotype were used. The hens were individually distributed in a completely randomized design with 12 treatments and eight replicates each. The experimental units contained metabolic cages with wire flooring equipped with individual feeders and water drinkers. The facilities were equipped with a negative-pressure ventilation system with controlled humidity and temperature, which was kept at 21ºC. The lightning program used was 13 hours of light according to Ross parent stock manual (Aviagen, 2013).

5.2.3. Experimental diets

In this study, a balanced diet (BD) was formulated strictly to meet the nutritional requirements and the ideal amino acid ratio provided by the Brazilian Tables for poultry and swine (Rostagno et al., 2011) for broiler breeder hens. The content of nitrogen and amino acids in the BD was supplied by corn, soybean meal, corn gluten meal and a mixture of industrial L-AA (Table 1).

The other eleven experimental diets with different limiting AAs were created by dilution of the BD with corn starch to achieve 45% of the AA level in BD and then supplemented with industrial AAs, except the AA under study. Nitrogen content was refilled by the supplementation of L-Alanine. The remaining nutrients and energy contents were refilled with feed ingredient (vitaminic and mineral supplements, cellulose, dicalcium

phosphate, limestone, soybean oil, sodium and potassium chloride) to the same concentration as the BD (Table 2). The nutritional composition of the diets are presented in Table 3.

Table 1. Composition of the balanced diet (g/kg) to determinate the optimum ratio between the amino acids for broiler breeder hens (Ross 308AP)

Ingredients (g/kg)	Balanced diet (T1)
Corn	643.0
Soybeal meal (45%)	32.7
Cellulose	78.8
Limestone	66.3
Sucrose	60.0
Dicalcium Phosphate	16.4
Potassium Chrolide	6.7
Corn Gluten Meal (60%)	24.8
Salt	3.2
Mineral and vitamin premix[1]	1.0
Amino acid mixture (g/kg)	
L-Alanine (96%)	45.4
L-Lysine HCl (78%)	4.7
DL-Methionine (99%)	2.8
L-Threonine (96%)	2.0
L-Arginine (99%)	4.1
L-Isoleucine (99%)	2.6
L-Valine (98%)	2.2
L-Phenylalanine (99%)	1.3
L-Histidine (99%)	0.1
L-Tryptophan (98%)	0.8

[1]Content/kg - vit. A = 9000000 IU, vit. D3 = 2600000 IU, vit. E = 14000mg, vit. B1 = 2200mg, vit. B2 = 6000mg, vit. B6 = 3000mg, vit. B12 = 10000mg, Niacin = 30000mg, pantothenic acid = 15000mg, vit. K = 1600mg, folic acid = 600mg, selenium = 200mg, manganese = 70000mg, iron = 50000mg, zinc = 50000mg, copper = 8000mg, iodine = 1200mg.

5.2.4. Management and data collection

The body weight was monitored weekly and egg production was monitored daily until the breeder hens reach the peak production. Thus, the hens were separated according to the

body weight and egg production to be distributed in the experimental units. The first nitrogen balance trial started at 31 weeks of age to obtain data for the peak production of these hens. The second nitrogen balance trial started after 15 weeks (46 weeks of age) when a decrease in the egg production was observed. The daily amount of feed provided was 152 g from 31 to 35 weeks and slightly decreased to 147 g from 46 to 50 weeks, according to the recommendation for hens kept in cages (Van Daele, 2014). In each period, the nitrogen balance trials were divided into five days of adaptation to the experimental diets followed by 20 days of excreta and egg collection. In this study it was considered 20 days of collection period due to the irregularity in the egg production of these hens, which are characterized by a certain number of pauses during the laying period. The excreta were collected in the trays, stored in plastic pots and weighed at the end of each collection period to measure total excreta production. The hens were weighed at the end of the experiment to measure body weight. The leftovers were weighed daily to measure feed intake. The egg production and egg weight were measured daily to calculate egg mass production.

Table 2. Composition of the experimental diets with individual AA deletion

Ingredients (g/kg)	Lys	Met+Cys	Thr	Trp	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Balanced diet	451.7	451.8	451.1	442.9	451.9	450.0	445.3	450.0	447.5	446.8	452.4
Corn starch	307.4	308.6	306.9	311.6	302.7	309.3	312.6	312.4	314.0	306.8	304.9
Cellulose	61.4	61.4	61.5	62.4	61.4	61.6	62.1	61.6	61.9	61.9	61.3
Inert	30.2	29.6	31.1	31.0	30.6	29.5	29.3	27.4	27.8	32.7	31.1
Limestone	35.4	35.4	35.5	36.0	35.4	35.5	35.8	35.5	35.7	35.7	35.4
Soybean oil	13.4	13.4	13.4	13.6	13.4	13.5	13.6	13.5	13.5	13.5	13.4
Dicalcium phosphate	10.4	10.4	10.4	10.5	10.4	10.4	10.5	10.4	10.5	10.5	10.4
Mineral and vitamin premix[1]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Potassium chloride	6.3	6.3	6.3	6.4	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Salt	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
L-Alanine (96%)	48.4	46.7	47.0	46.3	52.9	47.5	47.5	48.6	47.5	49.5	46.6
L-Leucine (98%)	5.3	5.3	5.3	5.3	5.3	5.3	5.3	0.0	5.3	5.3	5.3
L-Phenylalanine (99%)	4.4	4.4	4.4	4.5	4.4	4.4	4.5	4.4	0.0	4.5	4.4
L-Arginine (99%)	4.4	4.4	4.4	4.4	0.0	4.4	4.4	4.4	4.4	4.4	4.4
Glycine (96%)	3.5	3.5	3.5	3.6	3.5	3.6	3.6	3.6	3.6	0.0	3.5
L-Valine (98%)	3.1	3.1	3.1	3.2	3.1	0.0	3.2	3.1	3.2	3.2	3.1
L-Isoleucine (99%)	2.9	2.9	2.9	3.0	2.9	2.9	0.0	2.9	3.0	3.0	2.9
DL-Methionine (99%)	3.1	0.0	3.1	3.2	3.1	3.1	3.2	3.1	3.2	3.2	3.1
L-Threonine (96%)	2.7	2.7	0.0	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
L-Histidine (99%)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.0
L-Tryptophan (98%)	0.8	0.8	0.8	0.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8
L-Lysine HCl (78%)	0.0	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2

[1]Content/kg - vit. A = 12000000 IU, vit. D3 = 22000000 IU, vit. E = 30000mg, vit. B1 = 2200mg, vit. B2 = 6000mg, vit. B6 = 3300mg, vit. B12 = 16000mg, Niacin = 53000mg, pantothenic acid = 13000mg, vit. K = 2500mg, folic acid = 1000mg, selenium = 250mg, antioxidant = 100000mg, manganese = 75000mg, iron = 50000mg, zinc = 70000mg, copper = 6500mg, cobalt = 200 mg, iodine = 1500mg.

Table 3. Nutritional composition of the experimental diets with individual AA deletion

Nutrients (g/kg)	BD	Lys	Met+Cys	Thr	Trp	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Linoleic acid[1]	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9
Digestible Arginine[2]	7.9	7.9	7.9	7.9	7.9	3.6	7.9	7.9	7.9	7.9	7.9	7.9
Calcium[1]	3.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Chloride[1]	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Metabolizable Energy (Mcal/kg) [1]	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80
Digestible Phenylalanine[2]	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	2.3	5.2	5.2
Digestible Phe+Tyr[2]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	3.6	8.0	8.0
Crude fiber[1]	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3
Available phosphorus[1]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Digestible glycine[2]	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	2.8	6.2
Digestible histidine[2]	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	1.0
Digestible Isoleucine[2]	5.3	5.3	5.3	5.3	5.3	5.3	5.3	2.4	5.3	5.3	5.3	5.3
Digestible Leucine[2]	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4	4.2	9.4	9.4	9.4
Digestible Lysine[2]	6.0	2.7	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Digestible Met+Cys[2]	5.6	5.6	2.5	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6
Digestible Methionine[2]	4.3	4.3	1.9	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3
Potassium[1]	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Crude protein[2]	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0
Sodium[1]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Digestible Threonine[2]	4.7	4.7	4.7	2.1	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7
Digestible Tryptophan[2]	1.4	1.4	1.4	1.4	0.6	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Digestible Valine[2]	5.6	5.6	5.6	5.6	5.6	5.6	2.5	5.6	5.6	5.6	5.6	5.6

[1]Calculated.

[2]Analyzed content of diets and calculated based on true digestibility of the amino acids in the diets (Analysis performed by Ajinomoto)

5.2.5. Chemical analysis

The excreta and eggs collected from each experimental unit during each period were homogenised. The samples were frozen and freeze-dried for 72 hours at -80°C under 800 mbar of pressure (Edwards® 501 Modulyo freeze drier, West Sussex, United Kingdom). The samples were weighed to quantify the dry matter content and were milled using a Micro Mill (IKA® A11 Basic Analytical Mill, Staufen, Germany). The nitrogen content of the diets, excreta and eggs were analysed in a nitrogen distiller (Kjeltec™ 8400 Foss, Foss, Hillerod, Denmark) using the Kjeldahl method (Method No. 2001.11) according to AOAC (2005). The factor 6.25 was used to convert nitrogen content to crude protein (CP). The total amino acid contents of the ingredients and the experimental diets were analysed by Ajinomoto Ltd. using high-performance liquid chromatography (HPLC), and the correction of these values for digestible amino acids was performed using the tabulated digestibility coefficients (Rostagno et al., 2011).

5.2.6. Statistical analysis

The nitrogen balance data were analyzed by a one-way ANOVA using a GLM procedure in SAS (Statistical Analysis System, version 9.1). The nitrogen deposition (ND, $mg/BW_{kg}^{0.67}$ per day) was calculated by the difference between nitrogen intake (NI, $mg/BW_{kg}^{0.67}$ per day) and nitrogen excretion (NEX, $mg/BW_{kg}^{0.67}$ per day). The nitrogen retention was considered as the sum of nitrogen in egg mass (NEM, $mg/BW_{kg}^{0.67}$ per day), nitrogen deposition ($mg/BW_{kg}^{0.67}$ per day) and nitrogen maintenance requirement (NMR, $mg/BW_{kg}^{0.67}$ per day). The NMR value considered for the whole-experimental period was 255

$mg/BW_{kg}^{0.67}$ per day determined in the previous study (Data not published). The dietary protein quality (b) in each treatment was estimated according to the equation of Samadi and Liebert (2008):

$$b = (\ln(NR_{max}T)-\ln(NR_{max}T-NR)):NI \qquad \text{Eq. 5.1}$$

Where $NR_{max}T$ is the theoretical maximum nitrogen retention ($mg/BW_{kg}^{0.67}$ per day). The $NR_{max}T$ value is considered to be 'theoretical' because the value estimated is not attainable in practical condition, even if the hens are bred under perfect conditions. However, this is an important parameter used for the modelling procedure of the amino acid requirements (Samadi and Liebert 2006a,b). The $NR_{max}T$ values for Ross 308AP genotype used in the calculations were 1,684 $mg/BW_{kg}^{0.67}$ per day from 31 to 35 weeks and 1,484 $mg/BW_{kg}^{0.67}$ per day from 46 to 50 weeks, both estimated in a previous study (Data not published) according to the procedure of Samadi and Liebert (2007a,b). Significant differences between the b values obtained in the limiting diets and those obtained in the control diet (BD) were tested using Dunnett's test and the p-values < 0.05 were deemed statistically significant. According to Pastor et al. (2015), the individual AA under study must be definitely confirmed by evaluation of parameter b.

The association between dietary limiting amino acid (LAA) concentration c (g AA/16g N) and the feed protein quality b was directly utilized to determine the efficiency of the LAA utilization (bc^{-1}), which is valid only when the AA is in a limiting position. Consequently, it is possible to compare the model parameters bc^{-1} of an individual AA directly. Using this procedure for evaluating the optimal AA ratio, comparisons are only allowed within equal age periods because $NR_{max}T$ varies with body weight and affects the

established bc^{-1} value (Samadi and Liebert, 2008). The relationship between lysine efficiency (reference) and the efficiency of the LAA under study is utilized to derive ideal AA ratios (IAAR), according to the calculation (Wecke and Liebert, 2013):

$$IAAR = bc_{Lys}^{-1} : bc_{LAA}^{-1} \qquad \text{Eq. 5.2}$$

5.3. RESULTS

No pathological signs or mortality were observed within the experimental periods. The broiler breeder hens in limiting treatments did not consume the allotted amount of feed provided in both periods. Results of the nitrogen balance trials are summarized in Table 4. From these data, both dietary protein quality (b) and AA efficiency (bc^{-1}) data were derived (Table 4).

Table 4. Effect of deleting the amino acid from the diet on protein quality and nitrogen retention of broiler breeder hens in N balance trials

	Period I (31 to 35 wks)											
	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
BW	4.01±0.14	3.94±0.13	3.92±0.12	3.84±0.21	3.73±0.17	4.04±0.12	3.76±0.15	3.82±0.17	3.92±0.11	3.81±0.10	4.01±0.09	4.00±0.17
FI	150±1	141±8	147±4	133±13	123±15	144±7	106±19	115±18	131±11	102±11	148±4	139±7
NI	1448±34	1368±75	1459±60	1335±115	1235±125	1385±83	1067±190	1117±166	1268±89	1042±113	1427±48	1344±68
NEX	849±28	816±127	928±68	858±79	779±81	873±68	678±105	697±86	694±76	689±105	985±78	927±53
ND	599±14	552±67	531±64	477±58	456±143	512±28	389±143	420±162	574±107	353±38	442±48	417±26
NEM	382±14	279±88	332±57	359±42	359±100	349±30	286±81	276±98	303±113	290±62	401±58	351±21
NR	1236±11	1086±32	1118±25	1091±48	1070±62	1116±38	930±109	951±92	1132±46	898±62	1098±20	1023±32
b	916±25	758*±8	748*±3	785*±15	820*±3	787*±4	762*±5	750*±3	884*±54	734*±5	740*±3	697*±4
c	-	2.04	1.68	0.50	1.76	2.44	1.81	1.83	2.53	2.13	1.89	0.66
bc^{-1}	-	372±4	445±2	1570±6	466±2	323±2	421±3	410±2	349±19	345±2	392±2	1056±6
	Period II (46 to 50 wks)											
BW	4.12±0.17	4.09±0.14	4.02±0.14	3.62±0.15	3.67±0.34	4.32±0.44	3.64±0.21	3.81±0.21	3.99±0.22	3.40±0.15	3.96±0.07	3.93±0.06
FI	144±4	136±13	143±4	118±10	105±10	142±5	87±10	91±26	108±16	70±16	135±9	140±7
NI	1360±62	1282±128	1392±53	1236±115	1069±110	1312±74	891±85	880±220	1032±128	774±184	1319±80	1363±59
NEX	656±70	801±104	850±93	729±60	626±78	825±130	623±131	617±104	613±42	607±99	920±88	934±69
ND	704±64	481±61	542±73	507±84	443±146	487±85	268±90	263±139	419±98	167±153	399±86	429±84
NEM	198±66	228±52	214±76	188±47	194±100	264±100	225±109	211±49	255±38	244±88	316±76	265±72
NR	1157±9	964±57	1011±22	950±54	892±62	1006±32	748±55	729±132	929±71	666±133	970±36	949±25
b	1115±54	823*±3	819*±5	830*±8	864*±9	865*±6	789*±8	782*±14	959*±5	780*±16	805*±5	749*±3
c	-	2.04	1.68	0.50	1.76	2.44	1.81	1.83	2.53	2.13	1.89	0.66
bc^{-1}	-	403±1	487±3	1660±15	491±5	355±2	436±4	427±8	379±3	366±8	426±3	1135±5

BD=Balanced diet; BW=mean body weight (kg); FI=Feed intake (g/d); NI=nitrogen intake (mg/$BW_{kg}^{0.67}$ per day); NEX=nitrogen excretion (mg/$BW_{kg}^{0.67}$ per day); ND=nitrogen deposition (mg/$BW_{kg}^{0.67}$ per day); NEM=nitrogen in egg mass (mg/$BW_{kg}^{0.67}$ per day); NR=nitrogen retention (mg/$BW_{kg}^{0.67}$ per day); *b*=slope of the exponential function or protein quality ($\times 10^{-6}$); c=Amino acid concentration in dietary protein (g AA/100g CP); bc^{-1}= Efficiency of amino acid utilization ($\times 10^{-6}$).

*Significantly different from balanced diet (BD) treatment ($p<0.05$) by Dunnett's test.

[1]Mean ± standard error of mean (SEM).

In both experimental periods, lower nitrogen balance data were obtained with AA deleted diets as compared to the BD, resulting from both reduced supply of individual AA and total nitrogen intakes as presented in Table 4. The deletion of the essential AAs significantly reduced the performance of the hens, but the extension of this reduction depended on the essential AA deleted. The deletion of the phenylalanine+tyrosine greatly depressed the nitrogen retention, followed by valine and isoleucine in both periods. The high depression in nitrogen retention followed the low feed intake in the phenylalanine+tyrosine, valine and isoleucine deleted diets in both periods. However, it was not observed a great impact on body weight in these treatments when compared to other limiting diets. As expected, the BD diet yielded the significantly highest protein quality (b). The parameter b in the limiting diets declined significantly in comparison to the b value in BD ($P<0.05$), thus, confirming the limiting position of the AAs under study. In both periods, the dilution of the histidine led to a greater impairment of the dietary protein quality (b). Because in this study the diet composition was the same em both periods, the efficiency data can be compared. In both periods, the efficiency data within the age periods were very similar. The efficiencies (bc^{-1}) between period I and II presented a small range of variation of 4 to 15%, approximately (Table 4). Using the observed individual AA efficiency from Table 4, the results of ideal AA ratios were Lys 100%, Met+Cys 84%, Trp 24%, Thr 80%, Arg 115%, Val 88%, Ile 91%, Leu 107%, Phe+Tyr 108%, Gly+Ser 95%, and His 35% from 31 to 35 weeks and Lys 100%, Met+Cys 83%, Trp 24%, Thr 82%, Arg 114%, Val 92%, Ile 94%, Leu 106%, Phe+Tyr 110%, Gly+Ser 95%, and His 36% from 46 to 50 weeks.

5.4. DISCUSSION

The purpose of this study was to apply an alternative modelling procedure to predict the dietary IAAR for broiler breeder hens, taking into account the quantitative performance data and the AA efficiencies observed with the individual dilutions of the dietary AAs. Due to the severity in the AA reduction, the hens did not consume the amount of the feed allotted. In diets where the limiting nutrient content is severely deficient, a decline in feed intake is expected to occur as the result of the severely constrained growth rates that occur in such feeds, which in turns result in a lower capacity for feed intake when measured over a fixed time period (Emmans 1981; Burnham et al., 1992; Gous et al., 2007). Even with this severe restriction of the AAs in the diets, it seems that the breeder hens were able to maintain the egg production as was also observed in the Bowmaker and Gous (1991) study.

The use of a semi-purified diet with balanced AA profile and the use highly digestive feed ingredients resulted in an enhanced dietary protein efficiency. The decline in the b values of the limiting diets in comparison to the b value obtained from the balanced diet, as observed in the Table 4, validate the limiting position of the AA under study as suggested by Wecke and Liebert (2013). On the other hand, if the diluted diets yielded equal protein quality as compared to b value from balanced diet, the calculation for the IAAR would not provide reliable results. In this study, it was possible to calculate the IAAR for all AAs under evaluation, which are in line with the ratios presented in the literature for breeder hens.

The estimated IAAR are close to the ideal AA profile preconized by the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011), except for Leu, Phe+Tyr and Gly+Ser. The ideal ratios of 135% for Leu:Lys, 132% for Phe+Tyr:Lys and 102% for Gly+Ser:Lys seems to be very high in comparison to our results and the literature (Fisher, 1998; Aviagen, 2013; Ekmay et al., 2013). However, there are few information about the ideal ratios for these AAs for broiler breeder hens. A modelling procedure proposed by Fisher (1998) shown that

the ratios for these AAs should be 112% and 111% at 29-wks and 117% and 116% at 64-wks for Leu:Lys and Phe+Tyr:Lys, respectively. Although these values are close to the IAAR estimated in this study, for every other AA, the recommendation of Fisher (1998) is lower than our estimates and those found in the literature (Ekmay et al., 2013). The IAAR calculated from the nutritional specifications in the Ross parent stock manual (Aviagen, 2013) is a little bit lower than our estimates, probably because of the higher recommendation for Lys. As Lys has been used as reference in the ideal protein concept, the IAAR would likely to change within the response criteria used such as body weight gain, egg mass production or feed intake (Han and Baker, 1991). In the current study, the applied nitrogen utilization model provides a measure of the individual AA efficiency, which is directly used for the IAAR calculations (Samadi and Liebert, 2008).

Although the dietary Met+Cys:Lys ratio was in agreement with the results found in the literature for broiler breeder hens (Rostagno et al., 2011; Aviagen, 2013), it is important to highlight that in this study Cys was not provide in the diets resulting in lower Cys to Met ratio (30:70). It was recommended by Ekmay et al. (2013) to add dietary Cys equal to fifty percent of the Met+Cys requirement to ensure that Met is being primarily used for methylation reactions and protein synthesis and not for transsulfuration reactions noted for Cys synthesis. Since in this age period the Cys would be mainly used for feather keratin synthesis, it is possible that in part Met was used in these transsulfuration reactions for Cys synthesis, contributing to a higher Met+Cys to lysine ratios. As well as Met and Cys, phenylalanine and tyrosine follows the same concept (Ekmay, et al. 2013).

The higher Thr:Lys ratio found in the literature was calculated using data from Silva et al. (2015), which resulted in 87%, followed by our estimates of 80% in the period of 31-35wks and 82% in period for 46-50wks. It seems that broiler breeder hens have higher

requirements for threonine to maintain their body weight and the egg mass production (Kidd and Kerr, 1996). As well as threonine, arginine have an important role in the uric acid synthesis. Additionally, the antagonism between Arg and Lys increases the renal arginases inducing the arginine breakdown, thus increasing the metabolic requirement of arginine (Silva, et al. 2012). In this study, the ideal Arg:Lys ratio of 115% and 114% was similar to the Arg:Lys ratios of 115% recommended by the Brazilian Tables (Rostagno et al., 2011) and 112% proposed by Ekmay et al. (2013).

The antagonism between the branched chain amino acids (BCAA) such as Leu, Val and Ile has been documented in the literature, and the surplus of one of these AAs (mostly Leu) can decline feed intake (Edmonds and Baker, 1987) and reproductive performance (Rocha et al., 2013). Therefore, the depression in feed intake was observed in this study with diets limiting in Val and Ile and probably occurred due to the severity in the dilution of these AAs in relation to Leu creating an unbalance between them. In the present study, the IAARs of Val, Ile, Leu and Lys was calculated to be 88:91:107:100 (31-35wks) and 92:94:107:100 (46-50wks), which are similar to the IAARs for Val:Ile:Lys of 87:91:100 estimated by Ekmay et al. (2013), except for Leu that they not determined. The information on the ideal Leu:Lys ratio is difficult to find and they vary from 111% (Fisher, 1998) to 135% (Rostagno et al., 2011) for broiler breeder hens. Considering the importance of the antagonistic effect between the BCAA, further studies should also consider the ideal ratio for Leu.

Tryptophan has been considerate the third limiting amino acid in diets based on corn and soybean meal for laying hens (Peganova and Eder, 2003), which is also important for better performance and egg mass production (Lima et al. 2012). The ideal Trp:Lys of 24.5% determined by Lima et al. (2012) is closer to our results of 24% for broiler breeder hens. The information in the literature show that the recommendation of Trp:Lys ratio have a less

significant variation of 21-24% (Fisher, 1998; Rostagno et al., 2011; Lima et al., 2012). The His:Lys seems to present less variation in the recommendations (33-36%), but these recommendation are based only in calculations (Fisher, 1998; Rostagno et al., 2011) and was not determined in feeding trials. The Gly+Ser:Lys ratios also is practically inexistent for broiler breeder hens and only Rostagno et al. (2011) have recommended a ratio of 102% for Gly+Ser:Lys for breeder hens, which is higher than our estimate (95%). Although Gly has been categorized as a nonessential amino acid in poultry, there might be conditions when it becomes limiting, especially when at low levels of protein in diets (Corzo et al., 2004). Glycine and Serine are important for acid uric synthesis for excretion of excessive nitrogen and our results showed that broiler breeder hens requires a smaller amount of Gly+Ser (95%) than growing-broilers (147%) in earlier stages (Rostagno et al., 2011).

Due to variations in available data, further experiments to study the factors influencing the dietary EAA efficiency and the ideal ratio of EAA to Lys were found to be necessary, especially for glycine+serine and phenyalalnine+tyrosine.

In conclusion, the results presented in this study give further support to the advantages in using a nonlinear modelling procedure for improving the IAAR data in broiler breeder hens as well as presented in previous study with growing broilers (Wecke and Liebert, 2013). The IAAR for several amino acids were able to be determined in this current study, many of them were similar to the recommendations presented in previous reports. However, since there are few information on the IAAR for broiler breeder hens, it would be wise to validate the current IAAR in further investigations in performance trials.

5.5. ACKNOWLEDGEMENTS

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5.6. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

5.7. REFERENCES

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CAPÍTULO 6 - Optimal in-feed amino acid ratio for broiler breeder hens based on deletion studies

Este capítulo é apresentado de acordo com as normas da **Animal Physiology and Animal Nutrition**

TITLE PAGE

Short title: Ideal amino acid ratio for breeders

Title: Optimal in-feed amino acid ratio for broiler breeder hens based on deletion studies

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SUMMARY

An optimal ideal amino acid ratio (IAAR) for breeder hens is needed for maximum nitrogen (N) retention (NR) taking into account the N deposition in body (NDB), feathers (NDF) and N in the egg mass (NEM) to minimize N excretion and improve dietary protein efficiency. Thus, the aim of this study was to apply the deletion method to derive an IAAR for broiler breeder hens. The N balance trials were performed from 31 to 35 wks and from 46 to 50 wks. Twelve treatments with eight replicates and one hen per cage were used. A balanced diet (BD) was formulated to meet the IAAR and the requirement of other nutrients. The other diets were formulated diluting BD with corn starch and refilled with amino acids (AA) and other feed ingredients, except for the AA under study. Each feeding trial lasted 25 days. The feather losses, egg production and egg weight were recorded daily and the samples were stored to further determine NEM and N in feather losses (NDFL), respectively. At the start and the end of each period, a group of breeder hens were slaughtered to further determine NDB and NDF. The NR was calculated as the sum of NDB, NDF, NDFL, NEM, and the N maintenance requirement (NMR=255 mg/$BW_{kg}^{0.67}$). During the peak production the contribution of the NEM in the total NR is approximately 31% greater than that for NMR and from 46 to 50wks the contribution of the NMR becomes approximately 34% higher than for NEM. The percentual reduction in NR and the percent of the AA to delete from the BD were used to calculate the AA requirement. The relation between the AA and Lys requirements provided an average IAAR of Lys 100, Met+Cys 86, Trp 23, Thr 80, Arg 113, Val 90, Ile 91, Leu 133, Phe+Tyr 108, Gly+Ser 94, and His 35. The IAAR was in line with the recommendation from the literature, validating deletion method with the advantages from a rapid and low-cost procedure. In addition, the correction in the NR values decreased the variability in the results.

Keywords: Egg mass, deletion method, nitrogen retention, optimal amino acid ratio

6.1. INTRODUCTION

The modern broiler breeder hens have a high potential to produce hatching eggs while maintaining the growth potential as the modern broilers (Richards, et al. 2010). To take advantages of the full potential of these broilers breeder hens it is necessary to ensure that the correct amount of protein and other nutrients are being provided (Lopez and Leeson, 1995). The proper control of the daily protein intake is needed due to the impacts on body composition and egg size in the laying phase (Joseph et al., 2000). Also, the utilization efficiency of the dietary protein is determined by the amino acid (AA) composition and the AA balance in relations to the broiler breeder hens' requirements (Lopez and Leeson, 1995). For that reason, it is necessary to determine the AA requirements accounting for the physiological changes and provide the correct amount of AA to fulfill the maintenance requirements, support growth and meet the requirements for egg mass production (Fisher and Gous, 2009).

The current procedures to determine the AA requirements and their optimal balance utilize dose response studies making use of graded AA supplementation (Baker et al., 2002; Baker, 2003), which the diets are formulated to meet the recommendations from NRC (1994), except for the AA under study. Estimates of the AA requirement are derived by the first intersection of the quadratic response curve with the plateau from broken-line analysis, which is used in the conclusion of the optimal dietary AA ratios. However, this procedure is expensive and time-consuming because multiple assays are needed (Rollin, et al. 2003; Dorigam et al., 2015). On the other hand, it is possible to derive the optimal AA ratios from the responses obtained with the individual AA deletion of an AA balanced diet in only one assay (Wang and Fuller, 1989). This balanced diet is supplemented with crystalline AA to strictly meet the AA requirements of the animal and then, the AA under study is individually deleted from the

balanced diet. Both responses with the balanced and the deleted diets are measured and the observed slope of the response criteria between these diets was utilized for conclusion of optimal dietary ratios between individual AAs. The concepts of this approach are applied in the current study. However, as concluded in the previous study with broilers (Dorigam et al., 2015), it seems that this approach produce results with higher variation in the estimates and in part this occurred because the maintenance requirement, feather losses and the nitrogen deposition in the different tissues were not considered separately in the estimates of the amino acid requirements. The new hypothesis presented in this study suggest that the partition of the nitrogen retention may contribute to decrease this variation and produce more reliable results. Thus, the aim of this study was to apply the deletion method to derive an IAAR for broiler breeder hens based on the partition of the nitrogen retention.

6.2. MATERIAL AND METHODS

6.2.1. Ethics approval

This study was approved by the Ethics Committee on Animal Use of the Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal (nº 9999/14).

6.2.2. Birds, housing and experimental design

Two nitrogen balance trials were conducted at the Laboratory of Poultry Sciences of the University of Agrarian and Veterinary Sciences – FCAV / UNESP, Jaboticabal, São Paulo, Brazil (geographic coordinates: latitude 21º 15' 16" S longitude 48º 19' 19" W, altitude 607 m).

The nitrogen balance trials were performed from 31 to 35 weeks (Period I) and from 46 to 50 weeks of age (Period II). For each balance trial, 96 broiler breeder hens of Ross 308AP genotype were used. The hens were individually distributed in a completely randomized design with 12 treatments and eight replicates each. The experimental units contained metabolic cages with wire flooring equipped with individual feeders and drinkers. The facilities were equipped with a negative-pressure ventilation system with controlled humidity and temperature, which was kept at 21^{o}C. The lightning program used was 13 hours of light according to Ross parent stock manual (Aviagen, 2013).

6.2.3. Experimental diets

In this study, a balanced control diet (BD) was formulated strictly to meet the nutritional requirements and the ideal amino acid ratio provided by the Brazilian Tables for poultry and swine (Rostagno et al., 2011) for broiler breeder hens. The content of nitrogen and amino acids in the BD was supplied by corn, soybean meal, corn gluten meal and a mixture of industrial L-AA (Table 1).

Table 1. Composition of the balanced diet (g/kg) to determinate the optimum ratio between the amino acids for broiler breeder hens

Ingredients (g/kg)	Balanced diet (T1)
Corn	643.0
Soybeal meal (45%)	32.7
Cellulose	78.8
Limestone	66.3
Sucrose	60.0
Dicalcium Phosphate	16.4
Potassium Chrolide	6.7
Corn Gluten Meal (60%)	24.8
Salt	3.2
Mineral and vitamin mix[1]	2.0
L-Alanine (96%)	45.4
L-Lysine HCl (78%)	4.7
DL-Methionine (99%)	2.8
L-Threonine (96%)	2.0
L-Arginine (99%)	4.1
L-Isoleucine (99%)	2.6
L-Valine (98%)	2.2
L-Phenylalanine (99%)	1.3
L-Histidine (99%)	0.1
L-Tryptophan (98%)	0.8

[1]Content/kg - vit. A = 9000000 IU, vit. D3 = 2600000 IU, vit. E = 14000mg, vit. B1 = 2200mg, vit. B2 = 6000mg, vit. B6 = 3000mg, vit. B12 = 10000mg, Niacin = 30000mg, pantothenic acid = 15000mg, vit. K = 1600mg, folic acid = 600mg, selenium = 200mg, manganese = 70000mg, iron = 50000mg, zinc = 50000mg, copper = 8000mg, iodine = 1200mg.

The other eleven experimental diets with different limiting AAs were created by dilution of BD with corn starch to achieve 45% of the AA level in BD and then supplemented with industrial AAs, except the AA under study (Table 2). The N content was refilled by the supplementation of L-Alanine. The remaining nutrients and energy contents were refilled with feed ingredient (premixes, cellulose, dicalcium phosphate, limestone, soybean oil, sodium and potassium chlorides) to the same concentration as BD. The nutritional composition of the diets are presented in Table 3.

6.2.4. Management and data collection

The body weight was monitored weekly and egg production was monitored daily until the breeder hens reach the peak production. Thus, the hens were separated according to the body weight and egg production to be distributed in the experimental units. The first nitrogen balance trial started at 31 weeks of age to obtain data for the peak production of these hens. The second nitrogen balance trial started after 15 weeks (46 weeks of age) when a decrease in the egg production was observed. The daily amount of feed provided was 152g from 31 to 35 weeks and slightly decreased to 147g from 46 to 50 weeks, according to the recommendation for hens kept in cages (Van Daele, 2014). The nitrogen balance trials were divided into five days of adaptation to the experimental diets followed by 20 days for egg collection. In addition, feathers were collected from trays to measure the amount lost in the experimental period. In this study it was considered 20 days of collection period due to the irregularity in the egg production of these hens, which are characterized by a certain number of pauses during the laying period. The hens were weighed at the start and at the end of the experiment to measure body weight. The leftovers were weighed daily to measure feed intake. The egg production and egg weight were measured daily to calculate egg mass production and the feathers were collected from trays to quantify the feathers losses. At the start and at the end of the experiment, a group of broiler breeder hens were slaughtered with CO_2 to further quantify the N content in feathers and body free of feathers (separately) by the comparative slaughter technique.

Table 2. Composition of the experimental diets with individual AA dilution

Ingredients (g/kg)	Lys	Met+Cys	Thr	Trp	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Balanced diet	451.7	451.8	451.1	442.9	451.9	450.0	445.3	450.0	447.5	446.8	452.4
Corn starch	307.4	308.6	306.9	311.6	302.7	309.3	312.6	312.4	314.0	306.8	304.9
Cellulose	61.4	61.4	61.5	62.4	61.4	61.6	62.1	61.6	61.9	61.9	61.3
Inert	30.2	29.6	31.1	31.0	30.6	29.5	29.3	27.4	27.8	32.7	31.1
Limestone	35.4	35.4	35.5	36.0	35.4	35.5	35.8	35.5	35.7	35.7	35.4
Soybean oil	13.4	13.4	13.4	13.6	13.4	13.5	13.6	13.5	13.5	13.5	13.4
Dicalcium phosphate	10.4	10.4	10.4	10.5	10.4	10.4	10.5	10.4	10.5	10.5	10.4
Mineral and vitamin mix[1]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Potassium chloride	6.3	6.3	6.3	6.4	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Salt	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
L-Alanine (96%)	48.4	46.7	47.0	46.3	52.9	47.5	47.5	48.6	47.5	49.5	46.6
L-Leucine (98%)	5.3	5.3	5.3	5.3	5.3	5.3	5.3	0.0	5.3	5.3	5.3
L-Phenylalanine (99%)	4.4	4.4	4.4	4.5	4.4	4.4	4.5	4.4	0.0	4.5	4.4
L-Arginine (99%)	4.4	4.4	4.4	4.4	0.0	4.4	4.4	4.4	4.4	4.4	4.4
Glycine (96%)	3.5	3.5	3.5	3.6	3.5	3.6	3.6	3.6	3.6	0.0	3.5
L-Valine (98%)	3.1	3.1	3.1	3.2	3.1	0.0	3.2	3.1	3.2	3.2	3.1
L-Isoleucine (99%)	2.9	2.9	2.9	3.0	2.9	2.9	0.0	2.9	3.0	3.0	2.9
DL-Methionine (99%)	3.1	0.0	3.1	3.2	3.1	3.1	3.2	3.1	3.2	3.2	3.1
L-Threonine (96%)	2.7	2.7	0.0	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
L-Histidine (99%)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.0
L-Tryptophan (98%)	0.8	0.8	0.8	0.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8
L-Lysine HCl (78%)	0.0	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2

[1]Content/kg - vit. A = 12000000 IU, vit. D3 = 22000000 IU, vit. E = 30000mg, vit. B1 = 2200mg, vit. B2 = 6000mg, vit. B6 = 3300mg, vit. B12 = 16000mg, Niacin = 53000mg, pantothenic acid = 13000mg, vit. K = 2500mg, folic acid = 1000mg, selenium = 250mg, antioxidant = 100000mg, manganese = 75000mg, iron = 50000mg, zinc = 70000mg, copper = 6500mg, cobalt = 200 mg, iodine = 1500mg.

Table 3. Nutritional composition of the experimental diets with individual AA dilution

Nutrients (g/kg)	BD	Lys	Met+Cys	Thr	Trp	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Linoleic acid[1]	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9	12.9
Digestible Arginine[2]	7.9	7.9	7.9	7.9	7.9	3.6	7.9	7.9	7.9	7.9	7.9	7.9
Calcium[1]	3.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Chloride[1]	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Metabolizable Energy (Mcal/kg) [1]	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80
Digestible Phenylalanine[2]	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	2.3	5.2	5.2
Digestible Phe+Tyr[2]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	3.6	8.0	8.0
Crude fiber[1]	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3	44.3
Available phosphorus[1]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Digestible glycine[2]	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	2.8	6.2
Digestible histidine[2]	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	1.0
Digestible Isoleucine[2]	5.3	5.3	5.3	5.3	5.3	5.3	5.3	2.4	5.3	5.3	5.3	5.3
Digestible Leucine[2]	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4	4.2	9.4	9.4	9.4
Digestible Lysine[2]	6.0	2.7	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Digestible Met+Cys[2]	5.6	5.6	2.5	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6
Digestible Methionine[2]	4.3	4.3	1.9	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3
Potassium[1]	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Crude protein[1]	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0
Sodium[1]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Digestible Threonine[2]	4.7	4.7	4.7	2.1	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7
Digestible Tryptophan[2]	1.4	1.4	1.4	1.4	0.6	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Digestible Valine[2]	5.6	5.6	5.6	5.6	5.6	5.6	2.5	5.6	5.6	5.6	5.6	5.6

[1]Calculated.

[2]Analyzed content of diets and calculated based on true digestibility of the amino acids in the diets (Analysis performed by Ajinomoto)

6.2.5. Chemical analysis

The carcasses (without feathers) were minced in a meat mill and homogenised manually. The excreta and eggs collected from each experimental unit during each period were homogenised in an industrial blender. The carcasses, eggs and excreta samples were frozen and freeze-dried for 72 hours at -80°C under 800 mbar of pressure (Edwards® 501 Modulyo freeze drier, West Sussex, United Kingdom). The feather were processed in the mill and then dried in a forced draught oven at 55°C for 72 h. These samples were weighed to quantify the dry matter content and were milled using a Micro Mill (IKA® A11 Basic Analytical Mill, Staufen, Germany). The nitrogen content of the diets, carcasses, feathers, excreta and eggs were analysed in a nitrogen distiller (Kjeltec™ 8400 Foss, Foss, Hillerod, Denmark) using the Kjeldahl method (Method No. 2001.11) according to AOAC (2005). The total amino acid contents of the ingredients and the experimental diets were analysed by Ajinomoto Ltd. using high-performance liquid chromatography (HPLC), and the correction of these values for digestible amino acids was performed using the tabulated digestibility coefficients (Rostagno et al., 2011).

6.2.6. Statistical analysis

The N balance data were analyzed by a one-way ANOVA using a GLM procedure in SAS (Statistical Analysis System, version 9.1). Significant differences between the N retention obtained in the limiting diets and those obtained in the balanced control diet (BD) were tested using Dunnett's test and the p-values < 0.05 were deemed statistically significant. The results with the individual amino acid deletions were used to determine an optimal in-feed amino acid ratio following the principles of the approach presented by Green and Hardy (2003). The

proportional reduction in N retention (RNR) resulting from individual amino acid deletions relative to the balanced control diet was calculated as:

$$RNR_i = 100 \times (1 - NR_i/NR_{control}) \quad \text{Eq. 6.1}$$

where RNR_i is the reduction in N retention corresponding to each individual amino acid deletion or $IAAD_i$ associated with each limiting diet; NR_i is the N retention associated with each limiting diet calculated as the sum of the N deposition in the body free of feathers (NDB, $mg/BW_{kg}^{0.67}$ per day), N deposition in the feathers (NDF, $mg/BW_{kg}^{0.67}$ per day), N deposition in the feathers lost (NDFL, $mg/BW_{kg}^{0.67}$ per day), N deposition in egg mass (NEM, $mg/BW_{kg}^{0.67}$ per day) and N maintenance requirement (NMR, $mg/BW_{kg}^{0.67}$ per day) estimated in the previous study with the same hens as 255 $mg/BW_{kg}^{0.67}$ per day (*data no published*); $NR_{control}$ is the N retention associated with the balanced control diet.

To account for small differences in the individual amino acid deletions (IAADs) and to standardize RNRi values, the fractional RNR values were calculated as:

$$\text{fractional RNRi} = \text{RNRi/IAADi} \quad \text{Eq. 6.2}$$

The proportion of each amino acid to delete from the balanced control diet in formulation of the estimated optimum essential amino acid ratio was calculated as:

$$\%\text{AA deletion} = \text{mean IAAD} \times [1 - (\text{fractional } RNR_i/\text{fractional } RNR_{max})] \quad \text{Eq. 6.3}$$

where mean IAAD is the mean of individual amino acid deletions for all experimental diets, and fractional RNR_{max} is the maximum fractional reduction in N retention associated with the most limiting amino acid. This equation scales amino acid deletions from a minimum possible value of 0 to a maximum possible value equal to the mean $IAAD_i$.

The proportion of each amino acid in the formulation of the optimum amino acid pattern for broiler breeder hens was calculated as:

$$\text{Optimum } AA_i = \text{control AA} - [\text{control AA} \times (\%\text{AA deletion}/100)] \qquad \text{Eq. 6.4}$$

where optimum AA_i represents proportion of each essential amino acid in the optimum amino acid formulation and control AA represents percent of each amino acid in the balanced control diet used in the amino acid deletion experiment. The optimum AA_i of each essential amino acid was related to the optimum in-feed lysine requirement to estimate the optimum in-feed amino acid ratios.

6.3. RESULTS

The mean values and their respective standard deviations (±SD) obtained from nitrogen balance assays are presented in Tables 4 and 5. The broiler breeder hens in limiting treatments did not consume the allotted amount of feed provided in both periods. The nitrogen deposition in feathers covering the hen were higher in valine limiting diets in both periods. On the other hand, the nitrogen in the feather losses were higher in hens fed the balanced diet and tryptophan limiting diets in both periods. Nitrogen deposition in the body free of feathers were higher in hens fed the balanced diet in both periods but, on the contrary, the deletion of the threonine at

peak production (Table 4) and glycine+serine in period from 46-50wks resulted in lower body nitrogen deposition (Table 5). The nitrogen deposited in egg mass with hens fed the limiting diets were not different from the hens fed the balanced diet, except in the phenylalanine+tyrosine, glycine+serine and histidine treatments which were higher (P>0.05). As expected, the broiler breeder hens fed the balanced control diet yielded the significantly highest nitrogen retention ($P<0.05$). The relative contributions of the nitrogen depositions in body free of feathers, feathers, feather losses, in egg mass and the maintenance requirements in the total nitrogen retained by the hens are represented in Figure 1.

Table 4. Summarized results of the individual amino acid deletions on nitrogen retention of broiler breeder hens in nitrogen balance trials (mean± standard deviation) from 31 to 35 weeks of age

	Period I (31 to 35 wks)											
	BD	Lys	M+C	Trp	Thr	Arg	Val	Ile	Leu	P+T	G+S	His
FI	150±1	141±8	147±4	133*±13	123*±15	144±7	106*±19	115*±18	131*±11	102*±11	148±4	139±7
BW	4.0±0.1	3.9±0.1	3.9±0.1	3.8±0.2	3.7*±0.2	4.0±0.1	3.7*±0.1	3.8±0.2	3.9±0.1	3.8±0.1	4.0±0.1	4.0±0.2
ND_B	1254±43	542*±9	546*±32	525*±51	475*±90	489*±91	513*±88	580*±97	500*±71	883*±11	631*±31	607*±74
ND_F	11.0±0.5	7.0*±0.1	1.0*±0.1	11.0±0.7	5.0*±1.5	4.0*±0.9	16.0*±3.1	7.0*±0.6	6.0*±0.8	4.0*±0.1	8.0±0.4	8.0±1.6
ND_{FL}	9.0±1.3	5.0*±0.4	7.0±0.8	10.0±2.0	6.0*±0.6	7.0±1.2	6.0*±1.5	6.0*±1.0	6.0*±1.4	7.0±0.6	6.0*±0.9	6.0*±0.5
NEM	385±17	305±30	373±46	320±88	353±58	361±38	292±90	249±30	360±18	284±48	386±47	351±21
NR[1]	1913±42	1114*±28	1182*±13	1121*±65	1094*±94	1117*±72	1083*±87	1096*±97	1127*±63	1433*±52	1286*±43	1227*±81
RNR	-	42 (0.76)	38(0.73)	41(0.73)	43(0.73)	41(0.73)	43(0.79)	43(0.78)	41(0.76)	25(0.55)	33(0.64)	36(0.72)
IAAD	-	55	53	57	58	57	55	54	52	46	51	50
DEL	-	1.81	4.12	3.86	3.86	3.63	0.00	0.11	2.10	16.28	9.68	4.53
AAR	-	0.552	0.474	0.132	0.440	0.631	0.501	0.502	0.723	0.604	0.514	0.194
IAAR	-	100	86	24	80	114	91	91	131	110	93	35

FI=feed intake; BD=Balanced diet; BW=body weight (kg); ND_B= daily nitrogen deposition in body free of feathers ($mg/BW_{kg}^{0.67}$); ND_F=daily nitrogen deposition in feathers ($mg/BW_{kg}^{0.67}$); ND_{FL}=daily nitrogen deposition in feather lost ($mg/BW_{kg}^{0.67}$); NEM=nitrogen in egg mass ($mg/BW_{kg}^{0.67}$ per day); NR=daily nitrogen retention ($mg/BW_{kg}^{0.67}$); RNR = percent reduction in NR (%) and the respective fractional reductions inside the brackets; IAAD = individual amino acid deletions (%); DEL = proportion of the amino acid to be deleted in the balanced diet; AAR = amino acid requirement (%); IAAR=optimal in-feed amino acid ratio (%)

[1]Calculated as $NR=ND_B+ND_F+ND_{FL}+NEM+NMR$, where NMR is the nitrogen maintenance requirement (255 $mg/BW_{kg}^{0.67}$ per day).

Table 5. Summarized results of the individual amino acid deletions on nitrogen retention of broiler breeder hens in nitrogen balance trials (mean± standard deviation) from 46 to 50 weeks of age

	Period II (46 to 50 wks)											
	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
FI	144±4	136±13	143±4	118*±10	105*±10	142±5	87*±10	91*±26	108*±16	70*±16	135±9	140±7
BW	4.1±0.2	4.1±0.1	4.0±0.1	3.6*±0.1	3.7*±0.3	4.3±0.4	3.6*±0.2	3.8±0.2	4.0±0.2	3.4*±0.2	4.0±0.1	3.9±0.1
ND_B	1086±67	505*±52	615*±90	598*±27	556*±31	598*±54	517*±28	450*±8	445*±99	600*±67	435*±37	444*±36
ND_F	12±1	8*±1	7*±2	7*±2	8*±2	9*±1	14±1	6*±1	8*±2	13±1	10±1	7*±1
ND_{FL}	18±2	12*±2	16±1	18±3	12*±1	15±2	11*±1	14±1	13*±2	12*±1	16±2	13*±1
NEM	198±66	228±52	158±59	188±47	164±67	223±142	200±31	262±43	272±79	374*±59	396*±101	351*±21
NR[1]	1568±78	1008*±59	1051*±76	1065*±47	995*±89	1033*±36	997*±61	988*±43	993*±20	1254*±88	1114*±62	1070*±30
RNR	-	36 (0.65)	33(0.63)	32(0.57)	37(0.63)	34(0.60)	36(0.66)	37(0.68)	37(0.67)	20(0.43)	29(0.57)	32(0.64)
IAAD	-	55	53	57	58	57	55	54	52	46	51	50
DEL	-	2.35	4.18	9.00	4.20	6.24	1.60	0.00	0.48	19.41	8.76	3.34
AAR	-	0.549	0.473	0.125	0.437	0.614	0.493	0.503	0.735	0.582	0.519	0.196
IAAR	-	100	86	23	80	112	90	92	134	106	95	36

FI=feed intake; BD=Balanced diet; BW=body weight (kg); FI=feed intake (g/d); ND_B= daily nitrogen deposition in body free of feathers ($mg/BW_{kg}^{0.67}$); ND_F=daily nitrogen deposition in feathers ($mg/BW_{kg}^{0.67}$); ND_{FL}=daily nitrogen deposition in feather lost ($mg/BW_{kg}^{0.67}$); NEM=nitrogen in egg mass ($mg/BW_{kg}^{0.67}$ per day); NR=daily nitrogen retention ($mg/BW_{kg}^{0.67}$); RNR = percent reduction in NR (%) and the respective fractional reductions inside the brackets; IAAD = individual amino acid deletions (%); DEL = proportion of the amino acid to be deleted in the balanced diet; AAR = amino acid requirement (%); IAAR is the optimal in-feed amino acid ratio (%)

[1]Calculated as $NR=ND_B+ND_F+ND_{FL}+NEM+NMR$, where NMR is the nitrogen maintenance requirement (255 $mg/BW_{kg}^{0.67}$ per day).

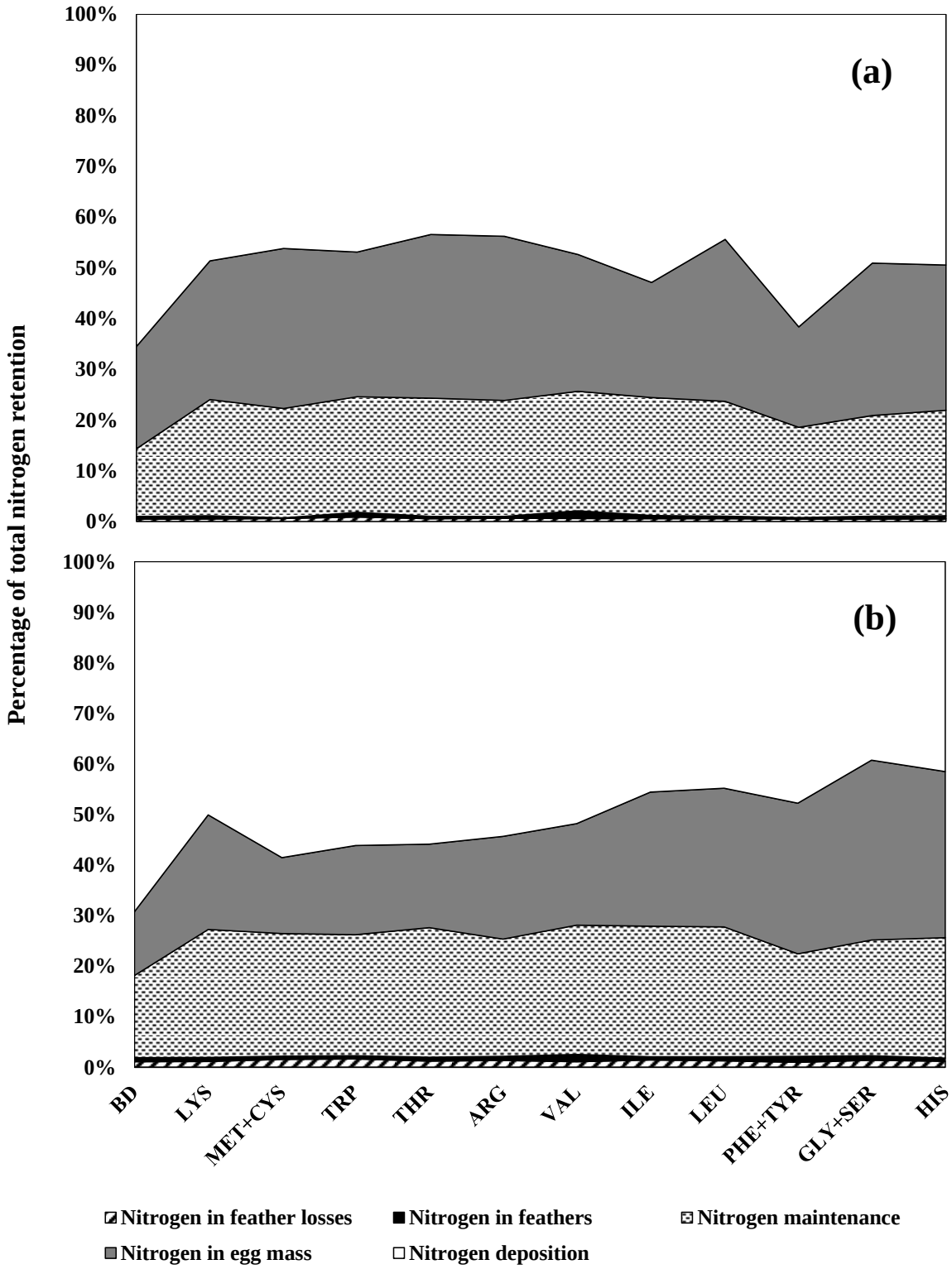

Figure 1. Proportions of the total nitrogen retention distributed for feathers, egg mass production, feathers lost and body free of feather according to the individual amino acid deletions in the diets provided in periods from 31 to 35weeks **(a)** and 46 to 50 weeks **(b)**.

Analyzing separately the components of the nitrogen retention (Figure 1), the proportion of the nitrogen in feathers and feather losses represented less than 1% of the total nitrogen retained in both periods. The greatest proportion of total nitrogen retained is found in the body protein, especially in hens fed the balanced control diet, which represented approximately 66% (31-35wks) and 69% (46-50wks) of the total nitrogen retained. During the peak production the contribution of the nitrogen deposition in egg mass in the total nitrogen retained is approximately 31% greater than that for maintenance. On the other hand, the opposite can be seen. As the hens getting older, the contribution of the nitrogen maintenance requirement becomes approximately 34% higher than for egg mass production except when isoleucine, leucine, phenylalanine+tyrosine, glycine+serine and histidine were limiting in the diets. Thus, taking into account these differences in the proportions of the maintenance requirement and nitrogen depositions on total nitrogen retention was important in further calculations.

The nitrogen retention in the limiting diets declined significantly in comparison to the nitrogen retention of the breeder hens fed the balanced control diet (P <0.05), thus, confirming the limiting position of the AAs under study. The deletion of the phenylalanine+tyrosine in the balanced control diet was lower than previously deducted and, thus, the calculated percent of the phenylalanine+tyrosine to be deleted from balanced control diet was higher in both periods. Even so, the primarily deduction on the reductions for all amino acids were sufficient to make the amino acid evaluated first limiting.

The extension of this reduction in nitrogen retention depended on the essential AA deleted. The deletion of valine greatly depressed the nitrogen retention in peak production (Table 4) while the dilution of the isoleucine greatly depressed the nitrogen retention of the hens from 46 to 50wks of age (Table 5). Subsequently, the optimum dietary amino acid requirements were calculated in relation to these first limiting amino acids in the control pattern

(Tables 4 and 5). As no differences were observed between the optimal in-feed essential amino acid ratios estimated in each period, an average value can be used for both periods. Thus, the optimal balance amongst the essential amino acids relative to lysine (100) from peak production onward were methionine+cystine 86, tryptophan 23, threonine 80, arginine 113, valine 90, isoleucine 91, leucine 133, phenylalanine+tyrsosine 108, glycine+serine 94, and histidine 35.

6.4. DISCUSSION

The purpose of this study was to apply the deletion method to predict the dietary IAAR for broiler breeder hens, taking into account the fractions of the nitrogen deposited in body free of feathers, feathers, feathers losses, nitrogen deposition in egg mass and nitrogen maintenance requirement in the total nitrogen retention observed with the individual deletions of the dietary AAs. The results obtained in the nitrogen balance study were validated after confirming the limiting position of the evaluated amino acids in the diets. During the experimental periods, it was observed that the hens did not consume the amount of the feed allotted (Tables 4 and 5), probably, because of the severity in the AA reductions. As the deficiency in the diets become more severe, the feed intake is depressed because of the increase in the heat production (Bowmaker and Gous, 1991, Bendezu et al., 2015), in part, resulting from the deamination process of other amino acids that are in excess and are not being used for protein synthesis (Cadirci et al., 2009). This is a common characteristic of this type of study due to the formulation technique used for the experimental diets (Bendezu et al., 2015).

In adult hens, an amino acid deficiency results in net catabolism of body proteins in order to supply the amino acids to prevent a distortion in the plasma and tissue amino acid levels (Cadirci and Smith, 2005). Thus, it was proposed by Mackenzie et al. (1992) that a larger body

supplies a greater pool of mobilized proteins when these hens receive an amino acid deficient diet. This pool can act as a source of limiting amino acids when the animal are given a deficient diet (Cadirci and Smith, 2005). In Figure 1 the mobilization of the body protein used to provide a pool of amino acids for protein synthesis is illustrated. The proportion of the body nitrogen content in relation to the total nitrogen in broiler breeders fed the balanced diet was an average value of 68%. At the cost of the body protein, this proportion drops to approximately 50% as these hens fed the limiting diets whereas the proportion of the nitrogen to maintain egg mass production increases. Consequently, the amino acids in the pool that are not being used are catabolized and contribute to increase the proportion of the endogenous nitrogen losses and, thus, nitrogen maintenance requirement.

The egg mass of hens in peak production (Caravalho et al. 2012) and feed intake (Lima et al., 2007) were not affected regardless the Arg: Lys ratio used in previous studies with laying hens. The results from the N balance trial corroborate with these observations (Tables 4 and 5), but different from these studies, the Arg: Lys ratio in the current study affected the nitrogen balance data. This can be partially explained by the increase in the enzyme arginase due to the excess of lysine which in turn increase the degradation of arginine (Leeson and Summers, 2001). As can be seen in Figure 1, as the deficiency of arginine becomes severe the large amount of the nitrogen retained as body protein is mobilized to meet the requirement of arginine and maintain the protein synthesis in egg mass. Thus, the optimum Arg: Lys ratio estimated in the present study as 113% would be used to avoid these issues.

Threonine is involved in important metabolic processes such as uric acid formation and protein synthesis (Rocha et al., 2013). At the peak production, it seems that a great part of the nitrogen retained as body protein was used (Figure 1) to maintain protein synthesis for egg mass production which was not significantly different from the egg mass production of the hens fed

the balanced diet (P>0.05). After 46 weeks, it was observed that this proportion becomes lower (Figure 1) as the protein synthesis requirement also becomes lower, on the contrary, the proportion of the nitrogen retention designated for maintenance becomes higher. Considering these aspects observed in the results, it seems that the Thr: Lys ratio have great importance for egg mass production and this ratios was estimated to be 80% which is higher when compared to the ideal Thr: Lys ratio of 65% required for growing broilers (Rostagno et al., 2011).

Tryptophan is a precursor of serotonin, a neurotransmitter which has been attributed the function of regulating appetite in birds (Calderano et al., 2012). The excess of dietary LNAA (Large neutral amino acids) such as isoleucine compete with tryptophan, causing a deficiency of this amino acid in the brain therefore inhibiting the synthesis and liberation of serotonin resulting in a decreased feed intake (Peganova and Eder, 2003). Due to the deletion of dietary tryptophan, this is a possible explanation for the lower feed intake (Tables 4 and 5). Besides, the effect of the tryptophan deletion follow a similar pattern as threonine in the nitrogen retention partition, with great importance in egg mass production, mainly at the peak production. Therefore, an ideal Trp: Lys ratio of 23% seems to be appropriated to maintain the performance and egg mass production of broiler breeder hens. Also, this estimate perfectly match the ideal ratio in the Brazilian Tables (Rostagno et al., 2011) and the nutritional recommendations for parent stock (Aviagen, 2013).

In previous studies was shown that feed intake has been associated with the excess of one of the branched chain amino acids (BCAA) such as isoleucine, valine and, mainly, leucine (Peganova and Eder, 2002). The leucine consumed in excess results in stimulation of isoleucine and valine oxidation, which reduce body protein deposition (Friedman, 1989) as can be seen in Table 4 and 5. On the other hand, the depletion isoleucine and valine pools could be readily used to maintain the protein synthesis for egg mass production (Figure 1). The elevated rate of

BCAA oxidation would be expected to continue as long as high concentration of the tissue leucine are maintained (Friedman, 1989). Thus, the ideal BCAA to lysine ratio (Val: Ile: Leu: Lys) for broiler breeder hens to maintain body weight and egg mass production was found to be 90:91:133:100, which is close to the recommendations in the Brazilian Tables (Rostagno et al., 2011).

The phenylalanine+tyrosine, glycine+serine and histidine were the least limiting amino acids in the experimental diets, because they do not promoted a great impact in the nitrogen retention as observed in the hens feeding other limiting diets. Surprisingly, the nitrogen deposition in egg mass for these treatments were maintained or improved in the period from 46 to 50 weeks of age. The feed intake was not depreciated in the glycine+serine and histidine treatments, but in the Phenylalanine+tyrosine it was greatly depreciated. The supplementation of the BCAA in the phenylalanine+tyrosine limiting diet could have reduced the tyrosine, thus, increasing the enzyme phenylalanine hydroxylase and decreasing available phenylalanine in plasma (Fernstrom, 2013). Consequently, the mobilization of phenylalanine from body pools is enhanced for its utilization in the egg mass production as observed Figure 1. Since the histidine is an aromatic amino acid (AAA) together with phenyalanine and tyrosine, it is possible to assume that the utilization of this amino acid is affected by the BCAA ratio. However, there is an absence of studies on the effects of the BCAA: AAA ratio on poultry nutrition in the scientific literature. The excess of the BCAA also affects the synthesis of the amino acid glycine (Pedroso, et al. 2015). In this case, as the glycine is the one of the most abundant amino acid in the body protein pool (Wu, 2013) a great proportion of this amino acid would be mobilized from body to promote egg mass production. In addition, there is a possibility that these amino acids were supplied in excess relative to the initial assumption of the ideal requirement in the balanced diet. This excess is confirmed by results in Tables 4 and 5, which

indicates that the amount of Phe+Tyr to be deleted was 16% and 19% of the balanced diet in the peak production and after 46 wks, respectively. The same pattern is observed for Gly+Ser and His. This means that is possible that the Phe+Tyr: Lys ratio (132%) recommended for broiler breeder hens by the Brazilian Tables (Rostagno et al, 2011) is overestimated. The same is true for Gly+Ser: Lys ratio (102%) in both periods. In the present study, it seems that the optimal Phe+Tyr :Lys and Gly+Ser: Lys ratios are 108% and 94%, respectively.

Lysine is an important amino acid for the synthesis of egg and body protein, and the absence of this amino acid results in lower egg production (Hiramoto et al., 1990). The body protein have an important role as a source of amino acids for egg formation (Ekmay et al., 2013). More recently, these authors suggested that lysine is incorporated at the rate of 78% in body protein in comparison to 22% present in the egg protein. In lysine limiting diets the proportion of the nitrogen deposition in egg mass in relation to the nitrogen retained was approximately 27% in peak production and 23% after 46 wks, which (Figure 1). On the other hand, the proportion of the nitrogen in the body protein reduced from an average 68% (balanced diet) to approximately 50% (Figure 1), corroborating with the assumption that the body protein is fundamental to provide lysine to maintaining the egg mass production (Ekmay e tal., 2014).

Apart from lysine, methionine+cystine also have an important role in the synthesis of egg and body protein (Gomes et al., 2011). At the peak production, it is possible to observe a great mobilization of body protein content to supply methionine+cystine in the synthesis of protein for egg mass production which contributes for 32% of the total nitrogen retention. However, this is not observed in the period from 46 to 50 wks and the proportion was similar to that from the balanced diet treatment. Since in this age period the Cys would be mainly used for feather keratin synthesis, it is possible that in part Met was used in the transsulfuration reactions for Cys synthesis (Ekmay et al., 2013) to replace the protein in feather losses which

represented 0.02% of total nitrogen retention. This author also suggest to add dietary Cys equal to fifty percent of the Met+Cys requirement to ensure that Met is being primarily used for methylation reactions and protein synthesis. However, the optimal Met+Cys: Lys ratio supported by this author was higher (98%) in comparison to our results (86%) and 87% from Brazilian Tables (Rostagno et al., 2011) and 89% from the nutritional guidelines (Aviagen, 2013). Nevertheless, it seems that Met+Cys is more important for broiler breeder hens than for growing broilers which requires approximately 72% (Rostagno et al., 2011; Dorigam et al., 2015).

In contrast to the deletion method applied in this study, previous estimates of the IAAR for broiler breeder hens were based on experiments conducted in different ages, birds and basal diets. This issue was also recognized in the past studies, suggesting that the IAAR ratio should be determined in only one experiment using the same basal diet and birds at the same period to ensure a valid measurement (Baker, 2003). Another important aspect is that the requirements estimated here are not valid for a nutritional recommendation, being used only as a reference for the calculation of the IAAR (Rollin et al., 2003). Further investigation should be conducted in order to better describe the interaction of certain amino acids in relation to the nitrogen retention, because it is not clear why the nitrogen in egg mass was improved with the deletion of the AAA and glycine, for example. Nevertheless, the IAAR for several amino acids were able to be determined in this current study, many of them were similar to the recommendations presented in previous reports. However, since there are few information on the IAAR for broiler breeder hens, it would be wise to validate the current IAAR in further investigations in performance trials.

The fractioning of the nitrogen retention improved the estimates of the amino acid requirements to derive an optimal amino acid ratio. The optimal in-feed amino acid ratios from

peak production onwards are similar, therefore, an average ratio can be used for both periods. In addition, the optimal in-feed amino acid ratio estimated was in the line with the recommendation from the literature, validating deletion method to determine the IAAR for broiler breeder hens with the advantages from a rapid and low-cost procedure.

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6.6. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

6.7. REFERENCES

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CAPÍTULO 7 – CONSIDERAÇÕES FINAIS

O conceito da proteína ideal contribui imensamente para melhorar o aproveitamento da proteína dietética e assim formular rações com menor custo e com menor excreção de compostos nitrogenados para o ambiente reduzindo a poluição ambiental. Dessa forma, o conhecimento científico gerado nesta tese contribui com a nutrição de aves, devido à padronização de novas metodologias que permitem explorar melhor a resposta animal na definição de uma relação ideal entre os aminoácidos essenciais para aves.

Até o momento, a relação ideal de aminoácidos essenciais na proteína ideal que é apresentada na literatura e manuais de referência não fazia distinção entre um perfil ideal de aminoácidos exigidos para mantença separado do crescimento ou produção. No segundo capítulo foi determinado um perfil ideal de aminoácidos para mantença, o qual difere muito do perfil ideal de aminoácidos para o crescimento apresentado no terceiro capítulo. Este perfil ideal ganha importância principalmente quando consideramos a abordagem fatorial em que a exigência total do aminoácido é particionada em exigência de crescimento e mantença e/ou produção de ovos. Dessa forma, os resultados obtidos neste estudo permitem agora determinar a exigência dos demais aminoácidos essenciais para mantença pela relação ideal determinada no segundo capítulo e assim aplicar estes valores nos modelos fatoriais e melhorar as estimativas das exigências. Embora este exercício não tenha sido feito nesta tese, é bem provável que a aplicação destas relações nos modelos fatoriais resulte em exigências um pouco maiores do que aquelas observadas na literatura ou manuais de referência em que é utilizado apenas uma relação ideal sem a distinção entre mantença e crescimento.

No terceiro capítulo foram padronizadas duas metodologias para aves em crescimento. Ambas metodologias utilizam os mesmos princípios do método da deleção apresentados no primeiro capítulo, mas usam abordagens bem diferentes para os cálculos e diferentes interpretações biológicas para os parâmetros usados. Enquanto o “método de Goettingen” considera os principais fatores que determinam a resposta animal, como genótipo, idade e dinâmica de crescimento para determinar a eficiência de utilização do aminoácido e assim obter uma relação ideal, o “método de

Louvain" determina as "exigências" por uma simples equação e relaciona as exigências dos aminoácidos com a exigência de lisina. Dentre as duas abordagens usadas, a "metodologia de Goettingen" foi aquela que apresentou resultados mais coerentes com a literatura e com menor variação entre as estimativas ao usar diretamente as eficiências para estabelecer uma relação ideal. Por outro lado, apesar da "metodologia de Louvain" ser mais prática, foi aquela que gerou maior variação entre os resultados e discrepâncias nos valores obtidos quando relacionados com a literatura. Assim, a padronização do método da deleção pela abordagem da Universidade de Goettingen, permitirá uma redução no tempo e custo da pesquisa para estimar as exigências dos aminoácidos essenciais e suas relações com a lisina, quando comparados com o método dose-resposta que tem sido utilizado atualmente para a determinação da relação ideal dos aminoácidos pelo grupo de Illinois. Considerando essas metodologias, um novo perfil ideal de aminoácidos foi proposto e que representa melhor o estado fisiológico da ave (genética e idade).

Uma vez que o "método de Goettingen" foi eleito como o método mais indicado por considerar melhor os aspectos fisiológicos da ave e por proporcionar melhores resultados, decidimos usar esta metodologia para obter uma relação ideal para matrizes pesadas. Embora a metodologia tenha sido padronizada para frangos de corte, ela ainda não havia sido aplicada em matrizes até o momento e os estudos com poedeiras ainda se encontram em fase de estudo pelo grupo de pesquisa da universidade de Goettingen. Assim houve também a necessidade de padronizar esta metodologia para matrizes pesadas, devido principalmente ao estado fisiológico destas aves que neste estudo se encontrava na fase de produção de ovos. Assim, pelas etapas descritas no primeiro capítulo, inicialmente, determinamos o máximo potencial "teórico" de retenção de nitrogênio destas aves ($NR_{max}T$) e suas exigências de mantença no quarto capítulo. Muitos pesquisadores podem se perguntar como é possível atingir um máximo potencial de retenção de nitrogênio em uma situação em que estas aves estão sobre restrição alimentar. Entretanto, cabe lembrar que o valor estimatido de $NR_{max}T$ é um valor teórico usado apenas como referência do máximo potencial usado nas estimativas das exigências de aminoácidos para estas aves. Provavelmente, é possível que a ave se aproxime deste máximo em uma condição sem restrições, uma vez que para as condições deste estudo foi mostrado que mesmo

restrição as aves estavam a 80% do valor de $NR_{max}T$. Porém nesta fase, o ganho de peso corporal destas aves é praticamente em gordura, o que é totalmente desfavorável para a reprodução destas aves. Desta forma, também apresentamos uma forma de modelar as exigências de aminoácidos para matrizes levando em consideração o máximo potencial teórico destas aves e o consumo de ração com a variação na exigência de energia de aves criadas no chão e em gaiola. Assim esta é uma excelente ferramenta que também contribui para estabelecer um programa nutricional adequado de aminoácidos para estas aves.

Dando continuidade ao procedimento para estimar a relação ideal de aminoácidos essenciais para de matrizes pelo “método de Goettingen”, os valores de máximo potencial teórico de nitrogênio e a exigência de nitrogênio para mantença determinados no quarto capítulo foram usados para os cálculos da eficiência de utilização dos aminoácidos. O ensaio foi semelhante àquele apresentado no terceiro capítulo, porém temos um fator a mais a ser considerado, que é o ovo. Diferente dos ensaios com frangos de corte apresentado no terceiro capítulo, aqui, resolvemos fazer um ensaio no pico de postura e outro após onze semanas de criação para averiguar se a relação ideal destas aves muda com a idade e produção de massa de ovos. Assim como no ensaio realizado anteriormente com frangos, foi possível atualizar e obter uma nova relação ideal de aminoácidos essenciais para matrizes usando diretamente as eficiências no cálculo com menor variação entre as estimativas. Embora esta nova relação ideal de aminoácidos essenciais esteja coerente com as relações apresentadas na literatura e manuais de referência para a linhagem, percebemos que as relações dos aminoácidos fenilalanina+tirosina e glicina+serina das tabelas brasileiras se encontravam muito acima dos nossos resultados e dos valores da literatura e podem estar superestimados como apresentado no sexto capítulo. Entretanto, estas diferenças nas relações ideais sobre o desempenho das aves ainda devem se averiguadas antes de qualquer conclusão. Assim, foi possível concluir que a relação ideal dos aminoácidos essenciais não muda no período estudado e com a produção de massa de ovos da matriz. Isto implica que não há necessidade de realizar outro ensaio após o pico de produção, já que a relação ideal é praticamente a mesma durante a produção, como apresentado no quinto capítulo.

No sexto capítulo retomamos a “metodologia de Louvain” com o objetivo de melhorar os resultados obtidos e viabilizar mais esta metodologia para determinar a relação ideal dos aminoácidos essenciais. Desta vez usamos os dados obtidos no ensaio de balanço de nitrogênio que foram conduzidos com matrizes pesadas (quinto capítulo). Neste estudo comprovamos que não só o fracionamento dos componentes do nitrogênio total retido proporcionou melhores estimativas para os cálculos das “exigências” dos aminoácidos essenciais para obter uma relação ideal, como também a correção das retenções pela taxa de deleção do aminoácido diminui a variabilidade nos resultados obtidos pelo balanço de nitrogênio. Assim, este estudo proporcionou também mais uma relação ideal de aminoácidos essênciais para matrizes em produção com valores mais atuais, porém, ainda são necessários estudos para verificar se esta nova relação ideal de aminoácidos para matrizes tem impacto positivo sobre seu desempenho.

Apesar da praticidade desta metodologia, devemos lembrar que ela é vantajosa apenas para determinar a relação ideal dos aminoácidos essenciais e não é recomendada para determinar as exigências nutricionais dos animais já que a técnica usa um número menor de níveis nutricionais. Como enfatizado pelos pesquisadores de Louvain que desenvolveram a equação para estimar a exigência pelo método da deleção, as recomendações resultantes dos cálculos são apenas usadas como referência para determinar a relação ideal dos aminoácidos e não para recomendar níveis práticos. Para determinar as exigências nutricionais dos animais ainda é mais adequado realizar ensaios pelo método dose-resposta sobretudo para lisina, que é o aminoácido referência pelo conceito da proteína ideal.

Como sugestão para estudos futuros, seria interessante utilizar esta metodologia em diferentes situaçãos que possam ocorrer no campo para averiguar se a relação entre os aminoácidos essenciais é afetada quando os animais são desafiados. A correta exigência de aminoácido para frangos de corte mantidos sob desafio sanitário não está devidamente estabelecida, havendo inclusive indícios na literatura de que estas relações podem ser diferentes dependendo da condição (ambiental e sanitária) em que os frangos de corte são submetidos. Dessa forma, a metodologia neste estudo também poderia ser uma excelente ferramente para estabelecer uma relação ideal de aminoácidos em uma condição de desafio sanitário.