

**UNIVERSIDADE ESTADUAL PAULISTA “JULIO DE MESQUITA
FILHO”**

**FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CÂMPUS DE JABOTICABAL**

**RELAÇÃO IDEAL DOS AMINOÁCIDOS ESSENCIAIS PARA
POEDEIRAS EM CRESCIMENTO E PRODUÇÃO**

Letícia Soares

Zootecnista

2018

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Letícia Soares

Orientador: Profa. Dra. Nilva Kazue Sakomura

Coorientador: Dr. Juliano Cesar de Paula Dorigam

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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Orientador: Nilva Kazue Sakomura

Banca examinadora: Edney Pereira da Silva, Angela Sunder, Nei
Andre Arruda Barbosa, Euclides Braga Malheiros

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TÍTULO DA TESE: RELAÇÃO IDEAL DOS AMINOCÍDIOS ESSENCIAIS PARA POEDEIRAS EM CRESCIMENTO E PRODUÇÃO

AUTORA: LETÍCIA SOARES

ORIENTADORA: NILVA KAZUE SAKOMURA

COORIENTADOR: JULIANO CESAR DE PAULA DORIGAM

Aprovada como parte das exigências para obtenção do Título de Doutora em ZOOTECNIA, pela Comissão Examinadora:

Prof. Dra. NILVA KAZUE SAKOMURA
Departamento de Zootecnia / FCAV / UNESP - Jaboticabal

Prof. Dr. EDNEY PEREIRA DA SILVA
Departamento de Zootecnia / FCAV / UNESP - Jaboticabal

Prof. Dra. ANIELA SUNDER (Videokonferência)
Departamento de Ciências Animal / University Göttingen / Alemanha

Prof. Dr. NELSON ANDRÉ ARRUDA BARBOSA (Videokonferência)
Departamento de Nutrição Animal / Escola de Veterinária e Zootecnia / Universidade de São Paulo / São Paulo/SP

Prof. Dr. EDUARDO SPAGA MALHEIROS
Departamento de Ciências Exatas / FCAV / UNESP - Jaboticabal

Jaboticabal, 06 de agosto de 2018

DADOS CURRICULARES DO AUTOR

LETÍCIA SOARES – filha de Roosevelt José Soares e Marisa Soares, nasceu no dia 24 de outubro de 1989, na cidade de Jundiaí, São Paulo. Em março de 2008 ingressou no curso de Zootecnia na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista – Campus de Jaboticabal, São Paulo, graduando-se em março de 2013. No período de agosto de 2011 a julho de 2012 foi bolsista de iniciação científica pelo CNPq, sob orientação da Profa. Dra. Sandra Aidar de Queiroz. Em março de 2013 ingressou no curso de mestrado em Zootecnia pela mesma instituição, onde obteve bolsa pelo CNPq, sob orientação da Profa. Dra. Nilva Kazue Sakomura e defendendo sua dissertação em fevereiro de 2015. Em março de 2016 iniciou o curso de Doutorado em Zootecnia na Faculdade Ciências Agrárias e Veterinárias da Universidade Estadual Paulista – Campus Jaboticabal, São Paulo, onde obteve bolsa pela FAPESP, sob orientação da Profa. Dra. Nilva Kazue Sakomura, defendendo esta tese em 2018 para obtenção do título.

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*“[...] Porque aquilo que a vida traz, traz para o mundo e não só para ti
Só dê importância à força positiva que te faz seguir
Nunca deixe se levar pelo desespero
Siga até o fim, siga até o fim! [...]”
(Um dia lindo – O Rappa)*

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

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.


Prof.ª Dr.ª Paola Castro Moraes
Coordenadora – CEUA

RELAÇÃO IDEAL DOS AMINOÁCIDOS ESSENCIAIS PARA POEDEIRAS EM CRESCIMENTO E PRODUÇÃO

RESUMO – O tradicional método dose-resposta usado para determinar a relação ideal dos aminoácidos essenciais (IAAR) se baseia na resposta da ave ao aumento gradativo da concentração do aminoácido teste nas dietas, tem sido considerado muito oneroso, uma vez que é necessário um ensaio para cada aminoácido avaliado. Por outro lado, pelo método da deleção é necessário apenas um ensaio para determinar IAAR, sendo assim mais rápido e prático. Dessa forma, o objetivo desta tese foi determinar a relação ideal dos aminoácidos essenciais para frangas e galinhas poedeiras utilizando o método da deleção. O primeiro estudo teve como objetivo determinar a exigência de nitrogênio para manutenção (NMR) e o máximo potencial de retenção de nitrogênio (NR_{maxT}) para frangas em crescimento. Três ensaios de balanço de nitrogênio foram conduzidos utilizando 56 frangas Hy-line W-36 em cada ensaio (inicial: 14 a 28, cria: 56 a 70 e recria: 98 a 112 dias). Os tratamentos consistiram em seis níveis graduais de nitrogênio nas dietas (N1=8; N2=16; N3=24; N4=32; N5=40 e N6=48 g N /kg de ração), formuladas utilizando a técnica da diluição. As análises de regressão entre nitrogênio ingerido e excretado foram realizadas para ajustar a função exponencial e determinar o NMR, que foi estimado em 294, 331 e 355 mg/PC_{kg}^{0,67} para as fases inicial, cria e recria, respectivamente. Os valores determinados para NMR foram aplicados para o cálculo do NR_{maxT} como o valor limiar da função entre o nitrogênio ingerido e o retido. O NR_{maxT} foi estimado pelo algoritmo de Levenberg-Marquardt até que a soma dos quadrados dos resíduos fosse mínima. Os valores estimados de NR_{maxT} foram 3200, 2633 e 1826 mg/PC_{kg}^{0,67} para as fases inicial, cria e recria, respectivamente. A determinação desses parâmetros foram uma condição prévia para determinar as exigências dos aminoácidos com base em um modelo exponencial da utilização de nitrogênio dependente do desempenho e eficiência de utilização dos aminoácidos na dieta, desenvolvidos no estudo subsequente. No segundo estudo, objetivou-se aplicar os dados de eficiência e exigências dos aminoácidos obtidos a partir de ensaios de balanço de nitrogênio para os aminoácidos essenciais e determinar a relação ideal entre eles para frangas em crescimento. Três ensaios de balanço de nitrogênio foram conduzidos utilizando 96 frangas Hy-line W-36 em cada ensaio (inicial: 14 a 28, cria: 56 a 70 e recria: 98 a 112 dias), distribuídas em doze tratamentos com oito repetições de uma ave cada. Uma dieta balanceada (DB) foi formulada para atender a IAAR e as exigências dos demais nutrientes. As dietas limitantes foram formuladas diluindo a DB com amido de milho para atender 70% da exigência dos aminoácidos e novamente suplementadas com aminoácidos industriais e outros ingredientes para atingir os mesmos níveis da DB, exceto para o aminoácido a ser avaliado que permaneceu deficiente em 30%. Em cada ensaio, foram obtidos os dados de ingestão, excreção, deposição e retenção de nitrogênio. Os valores de b foram estimados por $b = (\ln(NR_{maxT}) - \ln(NR_{maxT} - NR)) / (NI)$. Além disso, os dados também foram utilizados para calcular as exigências pela equação: $Exig. = (EAA)_{BDX} (2 - DEL - (ND_{EAA} / ND_{BD}))$. Os valores de bc^{-1} foram obtidos dividindo b pela concentração de aminoácidos na dieta. A IAAR pelo método de Goettingen foi obtida dividindo bc^{-1} da lisina pelo valor de bc^{-1}

¹ de cada aminoácido em estudo. A relação entre a exigências dos aminoácidos testados com a exigência da lisina determina a IAAR pelo método de Louvain. A IAAR determinada pelo método de Goettingen foi Lys 100; Met+Cis 86, 88, 81; Trp 17, 22, 22; Thr 77, 67, 68; Arg 103, 111, 109; Val 75, 80, 84; Ile 64, 67, 75; Leu 101, 121, 124; Phe+Tir 112, 126, 130; Gly+Ser 81, 130, 112; His 35, 35, 36% de 14 a 28, 56 a 70 e 98 a 112 dias, respectivamente. A IAAR determinada pelo método de Louvain foi Lys 100, Met+Cys 73, 80, 67; Trp 18, 20, 22; Thr 67, 68, 69; Arg 108, 108, 108; Val 77, 80, 84; Ile 70, 75, 77; Leu 113, 117, 129; Phe+Tir 121, 125, 131; Gly+Ser 78, 133, 124; His 37, 38, 39% de 14 a 28, 56 a 70 e 98 a 112 dias, respectivamente. O terceiro estudo teve como objetivo determinar a exigência de nitrogênio para manutenção (NMR), o máximo potencial de retenção de nitrogênio (NR_{maxT}) e a relação ideal dos aminoácidos essenciais para galinhas em produção. Um ensaio de balanço de nitrogênio foi conduzido utilizando 56 galinhas Hy-line W-36 no período de 28 a 30 semanas. Os tratamentos consistiram em seis níveis graduais de nitrogênio nas dietas (L1=8; L2=16; L3=24; L4=32; L5=40 e L6=48 g N /kg de ração), formuladas utilizando a técnica da diluição. As análises de regressão entre nitrogênio ingerido e excretado foram realizadas para ajustar a função exponencial e determinar o $NMR = 292 \text{ mg/PC}_{kg}^{0,67}$, que foram aplicados para o cálculo do $NR_{maxT} = 1883 \text{ mg/PC}_{kg}^{0,67}$ como o valor limiar da função entre o nitrogênio ingerido e o retido. Um segundo ensaio de balanço de nitrogênio foi conduzido utilizando 96 galinhas Hy-line W-36 no mesmo período do ensaio anterior, distribuídas em doze tratamentos com oito repetições de uma ave cada. Uma dieta balanceada (DB) foi formulada para atender a IAAR e as exigências dos demais nutrientes. As dietas limitantes foram formuladas diluindo a DB com amido de milho para atender 45% da exigência dos aminoácidos e novamente suplementadas com aminoácidos industriais e outros ingredientes para atingir os mesmos níveis da DB, exceto para o aminoácido a ser avaliado que permaneceu deficiente em 55%. Foram obtidos os dados de ingestão, excreção, deposição e retenção de nitrogênio. Os valores de b foram estimados por $b = (\ln(NR_{maxT}) - \ln(NR)) / (NI)$. Além disso, os dados também foram utilizados para calcular as exigências pela equação: $Exig. = (EAA)_{BDX} (2 - DEL - (ND_{EAA} / ND_{BD}))$. Os valores de bc^{-1} foram obtidos dividindo b pela concentração de aminoácidos na dieta. A IAAR pelo método de Goettingen foi obtida dividindo bc^{-1} da lisina pelo valor de bc^{-1} de cada aminoácido em estudo. A relação entre a exigências dos aminoácidos testados com a exigência da lisina determina a IAAR pelo método de Louvain. A IAAR determinada pelo método de Goettingen foi Lys 100; Met+Cis 88, Trp 21, Thr 69, Arg 109, Val 90, Ile 75, Leu 127, Phe+Tir 110, Gly+Ser 73 e His 29%. A IAAR determinada pelo método de Louvain foi Lys 100, Met+Cys 88, Trp 21, Thr 69, Arg 104, Val 91, Ile 78, Leu 121, Phe+Tir 119, Gly+Ser 77 e His 29%.

THE IDEAL ESSENTIAL AMINO ACID RATIO FOR LAYING HENS IN GROWTH AND PRODUCTION

ABSTRACT – The traditional dose-response method used to determine the ideal essential amino acid ratio (IAAR) is based on the response of the bird to the gradual increase of the test amino acid concentration in the diets, since it is considered very costly, since a test is required for each amino acid. On the other hand, by the deletion method, only one assay is required to determine IAAR, so it is faster and practical. Thus, the aim of this thesis was to determine the ideal amino acids ratio for pullets and laying hens using the deletion method. The first study was conducted to estimate daily N maintenance requirements (NMR) and the genetic potential for daily N retention (NR_{maxT}) of pullets in growth phase. Three nitrogen balance trials were conducted, a total of 56 Hy-line W-36 pullets were used in each trial in age periods (starter: 14 to 28, grower: 56 to 70 and developer: 98 to 112 days). The treatments consisted of six graded levels of nitrogen in the diets ($L1=8$; $L2=16$; $L3=24$; $L4=32$; $L5=40$ and $L6=48$ g N /kg of feed), formulated using the dilution technique. The regression analyses between nitrogen intake and nitrogen excretion were performed to fit the exponential function and to determine the NMR. It was estimated the daily NMR as 294, 331 and 355 mg/BW_{kg}^{0.67} for the initial, grower and developer periods, respectively, were applied for further calculation of NR_{maxT} as the threshold value of the function between N intake and daily N balance. The NR_{maxT} was estimated by a statistical procedure following several iteration steps by the Levenberg-Marquardt algorithm until the sum of the squares of the residual was minimized. The NR_{maxT} was estimated in 3200, 2633, and 1826 mg/BW_{kg}^{0.67} for starter, grower, and developer periods, respectively. The determined model parameters were the precondition for modeling of the amino acid requirement based on an exponential N-utilization model and depended on performance and dietary amino acid efficiency. This procedure will be further developed and applied in the subsequent study. In the second study, the aim was to apply the efficiency data and amino acid requirements obtained from nitrogen balance assays for the essential amino acids and to determine the ideal ratio between them for growing pullets. Three nitrogen balance trials were conducted, a total of 96 Hy-line W-36 pullets were used in each trial (starter: 14 to 28, grower: 56 to 70 and developer: 98 to 112 days). Twelve treatments with eight replicates and one bird per cage were used. A balanced diet (BD) was formulated to meet the IAAR and the requirement of other nutrients for pullets. To formulate the deficient diets, the BD was diluted with corn starch to reduce 30% of the dietary amino acid content. The diets were again supplemented with vitamin, mineral, fiber, energy, and industrial amino acids sources to achieve the same levels of the BD, except for the amino acid to be evaluated in the treatment, which remain reduced in 30%. In each trial, the data of nitrogen intake, excretion, deposition and retention were obtained in a balance trial. The b values were estimated by $b = (\ln(NR_{maxT}) - \ln(NR_{maxT} - NR)) / (NI)$. Also, these data were used to calculate the requirements by the equation: Requirement = $(EAA)_{BD} \times (2 - DEL - (ND_{EAA} / ND_{BD}))$. The bc^{-1} values were obtained dividing b by the dietary AA concentration. The IAAR by Goettingen approach was obtained by dividing the bc^{-1} from lysine by the bc^{-1} value from each other AA in each trial. The

relation between the AA requirements with Lys requirements provided de IAAR by Louvain approach. The IAAR determined by Goettingen approach were Lys 100; Met+Cis 86, 88, 81; Trp 17, 22, 22; Thr 77, 67, 68; Arg 103, 111, 109; Val 75, 80, 84; Ile 64, 67, 75; Leu 101, 121, 124; Phe+Tir 112, 126, 130; Gly+Ser 81, 130, 112; His 35, 35, 36% from 14 to 28 days, 56 to 70 days and 98 to 112 days, respectively. The IAAR determined by Louvain approach were Lys 100, Met+Cys 73, 80, 67; Trp 18, 20, 22; Thr 67, 68, 69; Arg 108, 108, 108; Val 77, 80, 84; Ile 70, 75, 77; Leu 113, 117, 129; Phe+Tir 121, 125, 131; Gly+Ser 78, 133, 124; His 37, 38, 39% from 14 to 28 days, 56 to 70 days and 98 to 112 days, respectively. The third study aimed to determine daily N maintenance requirements (NMR), the genetic potential for daily N retention (NR_{maxT}) and ideal amino acids ratio for laying hens. One nitrogen balance trial was conducted, a total of 56 Hy-line W-36 laying hens from 28 to 30 weeks. The treatments consisted of six graded levels of nitrogen in the diets ($N_1=8$; $N_2=16$; $N_3=24$; $N_4=32$; $N_5=40$ and $N_6=48$ g N /kg of feed), formulated using the dilution technique. The regression analyses between nitrogen intake and nitrogen excretion were performed to fit the exponential function and to determine the $NMR=292 \text{ mg/BW}_{kg}^{0.67}$ that was applied for further calculation of $NR_{maxT}=1883 \text{ mg/BW}_{kg}^{0.67}$. A second nitrogen balance trial was conducted, a total of 96 Hy-line W-36 laying hens were used in the same period. Twelve treatments with eight replicates and one bird per cage were used. A balanced diet (BD) was formulated to meet the IAAR and the requirement of other nutrients for pullets. To formulate the deficient diets, the BD was diluted with corn starch to meet 45% of the amino acid requirement. The diets were again supplemented with vitamin, mineral, fiber, energy, and industrial amino acids sources to achieve the same levels of the BD, except for the amino acid to be evaluated in the treatment, which remain reduced in 55%. In each trial, the data of nitrogen intake, excretion, deposition, and retention were obtained in a balance trial. The b values were estimated by $b=(\ln(NR_{maxT})-\ln(NR_{maxT}-NR))/(NI)$. Also, these data were used to calculate the requirements by the equation: $\text{Requirement}=(EAA)_{BD} \times (2-DEL-(ND_{EAA}/ND_{BD}))$. The bc^{-1} values were obtained dividing b by the dietary AA concentration. The IAAR by Goettingen approach was obtained by dividing the bc^{-1} from lysine by the bc^{-1} value from each other AA in each trial. The relation between the AA requirements with Lys requirements provided de IAAR by Louvain approach. The IAAR determined by Goettingen approach were Lys 100; Met+Cis 88, Trp 21, Thr 69, Arg 109, Val 90, Ile 75, Leu 127, Phe+Tir 110, Gly+Ser 73 e His 29%. A IAAR determinada pelo método de Louvain foi Lys 100, Met+Cys 88, Trp 21, Thr 69, Arg 104, Val 91, Ile 78, Leu 121, Phe+Tir 119, Gly+Ser 77 e His 29%.

LISTA E ABREVIATURAS

AA – Amino acid (aminoácidos);

AAI – Amino acid intake (ingestão de aminoácidos);

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 – Efficiency of amino acid utilization (eficiência de utilização do aminoácido);

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

BD – Balanced diet (dieta balanceada);

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);

EAABD – 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);

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);

Lys – Lysine (lisina);

Met – Methionine (metionina);

N – Nitrogen (nitrogênio);

ND – Nitrogen deposition (Nitrogênio depositado);

NDBD – Nitrogen deposition in birds feed balanced diet (Nitrogênio depositado em aves alimentadas com a dieta balanceada);

NDEAA – Nitrogen deposition in birds feed limiting diets (Nitrogênio depositado em aves alimentadas com dietas limitantes);

NDmaxT - Maximum theoretical nitrogen deposition (máxima deposição teórica de nitrogênio);

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

NEX – Nitrogen excretion (Nitrogênio excretado);

NI – Nitrogen intake (nitrogênio ingerido);

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

NR – Nitrogen Retention (nitrogênio retido);

NRmaxT – Maximum theoretical nitrogen retention (máxima retenção teórica de nitrogênio);

Phe – Phenylalanine (fenilalanina);

Ser – Serine (serina);

Thr – Threonine (treonina);

Trp – Tryptophan (triptofano);

Tyr – Tyrosine (tirosina);

Val – Valine (valina);

CAPITULO 1 – Considerações gerais

Introdução

A produção avícola é atualmente um dos sistemas mais eficientes de produção de proteína animal em termos de custos, devido ao melhoramento genético feito nas últimas décadas que possibilitou o desenvolvimento de novas linhagens comerciais com maior potencial de produção e melhor aproveitamento do alimento fornecido. Consequentemente, este melhoramento genético também tornou as aves mais exigentes em termos de nutrientes (Costa et al., 2008), criando a necessidade de constantemente avaliar suas exigências afim de proporcionar o melhor programa nutricional para as linhagens modernas. A nutrição é um dos pontos críticos na produção de aves que deve ser manejado adequadamente para garantir os altos níveis de produção, e o aumento da eficiência produtiva é consequência da adoção de estratégias nutricionais que resultam em melhor aproveitamento dos nutrientes.

Na avicultura industrial de postura, os componentes proteicos das rações são os mais onerosos e representam cerca de 40 a 45% do custo das formulações. A eficiência de utilização da proteína dietética fornecida dependente da quantidade, composição e digestibilidade de seus aminoácidos, os quais são exigidos em níveis específicos pelas aves. Durante muitos anos, as formulações de rações para aves foram baseadas no conceito de proteína bruta e isso resultou em dietas com deficiência ou excesso de aminoácidos. Para minimizar esse problema, as dietas passaram a ser formuladas utilizando-se o conceito de proteína ideal conforme proposto por Baker & Han (1994), Emmert & Baker (1997) e Baker et al. (2002) que preconiza a existência de um perfil de aminoácidos intimamente relacionados. A adoção desse conceito só foi possível devido a produção industrial de aminoácidos que permitiu redução da proteína dietética e obtenção de uma ração mais econômica, além de melhorar aproveitamento dos aminoácidos e reduzir o impacto ao meio ambiente devido a redução do nitrogênio excretado.

Embora o conceito da proteína ideal esteja bastante difundido para aves em crescimento, alguns aspectos ainda necessitam ser aperfeiçoados, sobretudo, quando são adicionados outros aspectos fisiológicos como a produção de ovos em poedeiras e reprodutoras pesadas. Assim, esforços contínuos são realizados por parte

das instituições de pesquisa e da indústria, no sentido de atualizar as exigências de aminoácidos para poedeiras e suas relações ideais de acordo com a idade das aves e desempenho (Pessoa et al., 2015). Entretanto, a atualização das exigências de aminoácidos para poedeiras não segue o mesmo ritmo de desenvolvimento das aves uma vez que são determinadas por ensaios de dose resposta que necessitam de um longo período de coleta de dados e que permitem a avaliação de um único aminoácido por ensaio. Por outro lado, Baker et al. (2002) sugere que a melhor forma de estimar a relação ideal dos aminoácidos é usando apenas um ensaio onde as mesmas aves são submetidas à uma mesma dieta referência. Especificamente para esta situação, o método da deleção foi proposto por Wang & Fuller (1989) com suínos e posteriormente usado em aves (Gruber et al., 2000; Roth et al., 2001; Dorigam et al., 2015) para definir a proteína ideal para frangos de corte em apenas um ensaio.

No presente estudo, foi determinado a relação ideal de todos os aminoácidos essenciais em um único ensaio com base no método da deleção, o que proporcionou valores mais precisos por minimizar os efeitos do ambiente, dieta e genótipo sobre a determinação da relação ideal. Esse método se baseia na pressuposição que a remoção de um aminoácido não limitante não altera a resposta das aves e que a alteração na resposta da ave, com a redução de uma proporção de cada aminoácido, é usada para calcular o perfil ideal dos aminoácidos em cada dieta cujo o aminoácido é limitante. Os resultados obtidos contribuirão para redução do custo das pesquisas com animais, além de possibilitar informações precisas do perfil ideal da proteína dietética que deve ser fornecida para poedeiras comerciais. Desta forma, o projeto teve o objetivo de determinar o perfil ideal de aminoácidos essenciais para poedeiras comerciais da linhagem Hy-Line em diferentes fases de criação utilizando o método da deleção.

Revisão de Literatura

Proteína ideal

O conceito de proteína ideal foi definido inicialmente em 1964 por Mitchell como o balanço exato de aminoácidos que atende as exigências dos animais para manutenção, e crescimento. Parsons & Baker (1994) definem como uma mistura de aminoácidos totalmente disponíveis para digestão e metabolismo, capaz de fornecer

sem excessos e deficiências as necessidades absolutas de todos os aminoácidos exigidos para manutenção e produção da ave com máxima eficiência, evitando o seu uso como fonte de energia e reduzindo a excreção de nitrogênio a partir de uma adequada combinação de concentrados proteicos e suplementação de aminoácidos cristalinos.

Dean & Scott (1965) foram os primeiros a usar dietas experimentais nos estudos da determinação da relação ideal de aminoácidos, desde então, vários estudos tentaram determinar a relação ideal para aves e suínos (Fuller et al., 1989; Wang & Fuller, 1989; Baker & Han, 1994; Mack et al., 1999; Baker et al., 2002; Coon, 2004; Dorigam et al., 2015). A grande maioria desses estudos foram propostos para frangos de corte, de forma que existem poucas referências para as outras categorias. Tem-se utilizado diferentes aproximações metodológicas, comumente os autores estimam as necessidades de alguns aminoácidos essenciais em ensaios diferentes e calculam as exigências dos demais aminoácidos através da composição corporal e das penas.

O uso do conceito de proteína ideal consiste em eleger um aminoácido como referência e basear as exigências dos outros aminoácidos como uma proporção desse aminoácido referência. Segundo Baker & Han (1994) e Emmert & Baker (1997), a lisina é utilizada como aminoácido referência por algumas razões: (1) é o primeiro aminoácido limitante nas dietas para suínos e o segundo, depois da metionina + cistina, nas dietas para aves; (2) assim como a treonina, é um aminoácido estritamente essencial, não havendo uma via para síntese endógena; (3) diferente de outros aminoácidos, o metabolismo da lisina é voltado principalmente para a deposição de proteína corporal; (4) sua análise nos alimentos é relativamente simples, diferente do triptofano e aminoácidos sulfurados; (5) e por existir uma grande quantidade de publicações referentes às exigências de lisina para aves.

É importante salientar que a relação ideal dos aminoácidos sofra variações de acordo com a idade do animal, uma vez que a relação das exigências para manutenção, manutenção + crescimento e manutenção + produção são diferentes. Assim, uma vez que esta relação tenha sido estabelecida para certa idade ou período, os estudos para determinar a exigência destes aminoácidos precisam se concentrar em apenas um

único aminoácido referência e a exigência dos demais aminoácidos essenciais é calculada em relação a esse aminoácido referência.

A vantagem da aplicação desse conceito se baseia no fato de que as exigências quantitativas de aminoácidos são constantemente alteradas em função de diversos fatores, porém, ainda que as exigências absolutas de certos aminoácidos possam mudar, as proporções permanecem bastante estáveis (Furuya et al., 2005).

A proteína é um nutriente relativamente caro, além disso a utilização ineficaz da proteína dietética contribui para a excreção de nitrogênio, causando um grande impacto ambiental. Segundo Ferket et al. (2002), para cada 1% de proteína bruta reduzida na dieta há uma redução de até 9% da excreção de nitrogênio. No entanto, reduzir o teor de proteína na dieta mantendo o ótimo desempenho animal só é possível se conhecermos exatamente a exigência animal para todos os aminoácidos essenciais.

Métodos para determinar a relação ideal dos aminoácidos

Método dose-resposta e fatorial

Os métodos utilizados no estabelecimento das exigências nutricionais das aves são: dose resposta e fatorial. O método dose resposta consiste na determinação das exigências nutricionais com base nas respostas de desempenho dos animais submetidos a níveis crescentes do nutriente nas dietas (Sakomura & Rostagno, 2016).

Esse método tem sido utilizado na grande maioria dos estudos para definir as exigências de aminoácidos para as aves, constituindo a base para a elaboração de tabelas como o “Nutrient Requirements of Poultry” (NRC, 1994) e as Tabelas Brasileiras para Aves e Suínos (Rostagno et al., 2000; 2005; 2011 e 2017), nas quais são informadas concentrações fixas dos aminoácidos que devem estar presentes nas dietas de acordo com a fase de criação. Entretanto, como os estudos de dose resposta se baseiam na resposta da ave ao aumento gradativo da concentração do aminoácido teste nas dietas, faz-se necessário um ensaio para cada aminoácido avaliado, isso faz com que ocorra aumento nos custos para pesquisa, além de demandar maior tempo para execução (Rollin et al, 2003; Dorigam et al., 2015).

O método fatorial baseia-se no princípio que as aves necessitam dos aminoácidos para a manutenção dos processos vitais e atividades, crescimento e/ou produção de ovos, fracionando a exigência total em proporções adequadas para cada uma dessas finalidades (Sakomura & Rostagno, 2016). Contudo, a aplicação do método fatorial depende da determinação dos parâmetros ou coeficientes que expressam as exigências de manutenção, crescimento e produção, e das eficiências de utilização dos aminoácidos da dieta, a partir de informações obtidas em ensaios dose resposta.

Método da deleção

Introdução ao método da deleção

Este método é uma extensão do que foi encontrado por Bender (1965) com ratos. Segundo Wang & Fuller (1989), se baseia no conceito de que a remoção de um aminoácido não-limitante não tem efeito sobre a retenção de nitrogênio, de maneira que as mudanças da retenção de nitrogênio serão influenciadas pela remoção de uma proporção de cada aminoácido que, por sua vez, são utilizadas para calcular o perfil ideal dos aminoácidos em uma dieta na qual todos os aminoácidos são igualmente limitantes. O aminoácido reduzido se tornará o primeiro limitante na dieta, sendo possível calcular a exigência mínima para cada aminoácido sem que haja efeito sobre a retenção de nitrogênio. A relação ideal entre os aminoácidos é calculada pela relação entre o primeiro aminoácido limitante e o aminoácido presente no perfil da dieta controle.

O método da deleção necessita apenas de uma dieta controle balanceada na qual todos os aminoácidos estão em proporções adequadas, as demais dietas são obtidas a partir da redução parcial (15 a 45%) dos aminoácidos da dieta controle e então, novamente suplementadas para 100% com aminoácidos industriais, exceto para o aminoácido a ser avaliado. 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 relação entre esses dois fatores é testada na análise de regressão com os dados obtidos pelas dietas especificamente deficientes em cada aminoácido e com a dieta controle.

A vantagem em relação ao método dose resposta é a necessidade de apenas um ensaio para determinar a relação ideal entre todos os aminoácidos essenciais. Roth et al. (2001) determinaram a relação ideal entre a lisina (100%), metionina (37%), metionina + cistina (70%), treonina (66%), triptofano (14%), isoleucina (63%), leucina (108%), valina (81%), fenilalanina (62%), arginina (108%) e histidina (38%) para frangos de corte em um único ensaio. Enquanto, Mack et al. (1999) realizaram nove ensaios de dose resposta para a relação de lisina (100%), metionina + cistina (75%), treonina (63%), triptofano (19%), arginina (112%), isoleucina (71%) e valina (81%) para aves de 20 a 40 dias de idade.

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, isso permite maior grau de uniformidade e consistência, o que torna mais precisa a determinação das relações ideais dos aminoácidos (Rollin et al., 2003; Dorigam et al., 2015).

Metodologia de Goettingen para determinar a relação ideal

Esta abordagem foi desenvolvida na universidade de Goettingen, Alemanha, para determinar a relação ideal dos aminoácidos e necessita que apenas dois ensaios sejam conduzidos. No primeiro ensaio são determinados os parâmetros do modelo que caracterizam o potencial genético das aves. Esse grupo de pesquisadores preconiza que cada animal possui uma característica genética própria para a sua máxima retenção de nitrogênio (NR_{max}) e está associada à exigência de nitrogênio para manutenção (NMR). Estes parâmetros são determinados em ensaios de balanço de nitrogênio com o uso de dietas com níveis graduais de proteína e estão dispostos na equação $ND = NR_{max}T \times (1 - e^{-b \times NI}) - NMR$ (Samadi & Liebert, 2008).

De posse dessas informações, o próximo passo é determinar as eficiências de utilização dos aminoácidos a partir da equação $b = [\ln(NR_{max}T) - \ln(NR_{max}T - NR)]:NI$ e para isso é necessário outro ensaio de balanço de nitrogênio em que uma dieta controle é formulada contendo todos os aminoácidos em níveis adequados atendendo à relação ideal. Para avaliar os efeitos da deleção do aminoácido na eficiência de utilização da proteína, a dieta controle é diluída com amido para atender uma fração

dos aminoácidos e novamente suplementada com aminoácidos industriais para 100%, exceto o aminoácido estudado (Samadi & Liebert, 2008).

Para uma dada deposição de proteína, a exigência do aminoácido se torna dependente somente da eficiência de utilização determinada para cada aminoácido. Consequentemente, é possível comparar as eficiências de utilização de cada aminoácido para determinar um perfil ideal. As eficiências de utilização de cada aminoácido são relacionadas com a eficiência de utilização da lisina, e a relação ideal é obtida pela relação das eficiências bc^{-1} da lisina dividido pelo bc^{-1} dos respectivos aminoácidos (Samadi & Liebert, 2008). De acordo com Wecke & Liebert (2013) e Dorigam et al. (2015), o método de Goettingen tem sido bastante eficaz para determinar a relação ideal dos aminoácidos essenciais, sobretudo, por apresentar menor variação nos resultados em relação às outras metodologias envolvidas nos estudos de deleção.

Objetivo

Determinar os parâmetros genéticos para máxima retenção de nitrogênio (NR_{maxT}) e exigência de nitrogênio para manutenção (NMR) de poedeiras da linhagem Hy-Line nas fases de crescimento e produção (Inicial: 14 a 28; Cria: 56 a 70; Recria: 98 a 112 e Pico de produção: 196 a 220 dias). Com base nestes parâmetros, determinar a relação ideal dos aminoácidos essenciais (lisina, metionina + cistina, treonina, triptofano, isoleucina, leucina, histidina, fenilalanina + tirosina, glicina + serina, valina e arginina) para poedeiras da linhagem Hy-Line nas fases de crescimento e produção (Inicial: 14 a 28; Cria: 56 a 70; Recria: 98 a 112 e Pico de produção: 196 a 220 dias) pelo método da deleção.

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CAPÍTULO 2 - Estimation of nitrogen maintenance requirements and potential for nitrogen retention of pullets in growth phase

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

Nitrogen maintenance requirements and potential for nitrogen retention of pullets in growth phase

Running title: N maintenance requirement and the genetic potential of pullets

Letícia Soares¹, Nilva Kazue Sakomura¹, Juliano Cesar de Paula Dorigam², Frank Liebert³, Mariana Quintino do Nascimento¹, João Batista Kochenborger Fernandes¹

¹Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Jaboticabal, São Paulo, Brazil,

²Department of Animal Nutrition Research, Evonik Industries, Hanau, Wolfgang, Germany.

³Chair of Animal Nutrition, University of Göttingen, Kellnerweg, Göttingen, Germany.

Correspondence: L. Soares¹, Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Jaboticabal, São Paulo, Brazil, Via de Acesso Prof. Paulo Donato Castellane, s/n. 14884-900 - Jaboticabal, SP, Brazil. E-mail: leticia.fcav@gmail.com.

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SUMMARY

Experiments were conducted to estimate daily N maintenance requirements (NMR) and the genetic potential for daily N retention ($NR_{max}T$) of pullets in growth phase. Three nitrogen balance trials were conducted, a total of 56 Hy-line W-36 pullets were used in each trial in age periods (starter: 14 to 28, grower: 56 to 70 and developer: 98 to 112 days). The treatments consisted of six graded levels of nitrogen in the diets (L1=8; L2=16; L3=24; L4=32; L5=40 and L6=48 g N /kg of feed), formulated using the dilution technique. The regression analyses between nitrogen intake and nitrogen excretion were performed to fit the exponential function and to determine the NMR. It was estimated the daily NMR as 294, 331 and 355 mg/ $BW_{kg}^{0.67}$ for the initial, grower and developer periods, respectively, were applied for further calculation of $NR_{max}T$ as the threshold value of the function between N intake and daily N balance. The $NR_{max}T$ was estimated by a statistical procedure following several iteration steps by the Levenberg-Marquardt algorithm until the sum of the squares of the residual was minimized. The $NR_{max}T$ was estimated in 3200, 2633, and 1826 mg/ $BW_{kg}^{0.67}$ for starter, grower and developer periods, respectively. The determined model parameters were the precondition for modeling of the amino acid requirement based on an exponential N-utilization model and depended on performance and dietary amino acid efficiency. This procedure will be further developed and applied in the subsequent study.

Keywords: nitrogen utilization model, growth potential, nitrogen balance, pullets

Introduction

The nutritional conditions established during the growth period can affect egg production and egg weights of the birds during the laying period, the pullets need an adequate supply of nutrients during growth, particularly the supply of amino acids and protein for body protein synthesis and development of the reproductive organs. Besides that, the genetic improvement over the years with the laying lines hens provided birds with better productive indexes and with that, a change in the potential of nitrogen deposition.

The growth period is decisive for the productive life of the bird, any deficiency at this stage will affect the quality of the adult bird. Therefore, it is necessary to measure the protein requirement of these birds to prevent mistakes made during the growing period which can lead to poor production in lay and cannot easily be corrected during the laying period. Aiming to meet the requirement for maximum potential and improved protein utilization, it was developed a procedure called "Goettingen approach" that was developed over the last two decades (Liebert et al., 2000; Samadi & Liebert, 2006a, 2007a, 2008; Pastor et al., 2013; Dorigam, et al., 2014; Dorigam et al., 2017). This approach adopts the concepts used in the factorial modeling, dose-response and nitrogen balance studies. Therefore, this methodology is substantiated on non-linear mathematical models, using mathematical relationships between nitrogen intake (NI), excretion (NEX) and retention (NR) obtained from nitrogen balance studies.

Genetically, the growing animals have a finite potential to retain nitrogen, in addition, the genotype, body weight and age of the birds have great influence in protein deposition (Samadi & Liebert, 2006a). According to Goettingen approach, the genetic potential is defined as an unattainable "theoretical" threshold value that is not achieved in practice, even if the animals are subjected to the best nutritional program and environmental conditions (Liebert, 2015).

Within of exponential nitrogen utilization model, $NR_{max}T$ (maximum theoretical nitrogen retention) is used only as a model parameter to relate the real rate of deposition to the estimated genetic potential which is also associated to nitrogen maintenance requirement (NMR) (Thong & Liebert, 2004). The NMR parameter within the model application reflects the mandatory endogenous losses of excreta nitrogen, however, it does not correspond to the amount of protein required to restore the body's protein losses (Wecke & Liebert, 2009). In this way, it becomes possible to model the effects of higher or lower feed intake and protein deposition and predict a nutritional plan according to the goal set by the companies in their different production systems.

Many studies have already been conducted to determine the NMR and $NR_{max}T$ for broilers, broiler breeders, swine and tilapia (Liebert et al., 2006; Samadi & Liebert, 2008; Wecke & Liebert., 2009; Dorigam et al., 2014; Daulat et al., 2015; Dorigam et al., 2017) however, few studies have been carried out for pullets in the growth phase (Silva et al., 2013; Bonato, 2013). This methodology is easy to execute and allows the fractionation of the requirements, from NMR and $NR_{max}T$ it is possible to determine the efficiency of utilization and the ideal ratio of amino acids in future studies. The aim of this study was to estimate the genetic potential for daily N retention ($NR_{max}T$) and nitrogen maintenance requirement (NMR) of Hy-line W-36 pullets in growth phase based on the Goettingen approach.

Material and Methods

This study was approved by the Ethics Committee on Animal Use of the Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal (n° 9999/14).

Three nitrogen balance trials were conducted in the Laboratory of Poultry Science of the Faculty of Agriculture and Veterinary Sciences located in Jaboticabal, São Paulo, Brazil. The objective of this study was to determine the nitrogen maintenance requirement (NMR) and

the theoretical maximum nitrogen retention (NR_{maxT}) of Hy-line W-36 pullets in each growth phase. To determine the age of the birds (starter: 14 to 28, grower: 56 to 70 and developer: 98 to 112 days) was defined the intermediate period of each stage as indicated by the breeder's guidelines, so that the experimental period represented the entire growth phase. This study follows the procedure applied in earlier reports (Samadi & Liebert, 2006a; 2007; Dorigam et al., 2014; Daulat et al., 2015; Dorigam et al., 2017) performed to estimate the NMR and NR_{maxT} in nitrogen balance studies.

Animals housing and experimental design

A total of 56 Hy-line W-36 pullets were used in each trial and the birds were individually allotted in a completely randomized design consisting of six treatments and eight replicates. The birds were housed in metabolic cages with wire floors, individual galvanized feeders and nipple drinkers installed in a facility with a negative pressure ventilation system with controlled humidity and temperature. The management of the pullets and the lighting program followed the breeder's guidelines (Hy-line W-36 Management Guide, 2016).

Experimental diets

The treatments consisted of six graded levels of nitrogen in the diets (L1=8; L2=16; L3=24; L4=32; L5=40 and L6=48 g N /kg of feed), formulated using the dilution technique (Fisher & Morris, 1970). The dilution technique consisted in formulating two diets, one summit diet (L6) with high content of protein and another dilution diet (L0) without free of protein and amino acids. The diet L6 was formulated using soybean meal, corn gluten and industrial amino acids to meet the level of 48 g N /kg of feed. The L0 diet was formulated to meet the same nutritional level as the L6 diet, except for protein and amino acids. The composition of the diets is presented in Table 1. The experimental diets were obtained by diluting the L6 diet with the L0 diet, in the adequate proportion to obtain the intermediary levels of nitrogen while keeping

the same essential amino acid ratios between the diets. The proportion of high protein diet (L6) to protein-free diet used in the preparation of the experimental diets was 14:86 for L1, 31:69 for L2, 49:51 for L3, 66:34 for L4, 83:17 for L5, and 100:0 for L6. The nutritional composition of the diets is presented in Table 2.

Table 1. Composition of the basal diets used in each period of the trial

Ingredients (%)	Starter (14 to 28 d)		Grower (56 to 70 d)		Developer (98 to 112 d)	
	Summit	Dilution	Summit	Dilution	Summit	Dilution
	(L6)	(L0)	(L6)	(L0)	(L6)	(L0)
Corn	42.766		42.298		35.146	
Soybean meal	27.168		25.076		40.000	
Corn gluten meal (60%)	15.000		15.000		10.067	
Rice husk	7.638	14.973	9.980	15.152		15.152
Wheat meal					8.000	
Corn starch		63.000		65.298		65.000
Sugar		10.000		6.000		9.490
Soybean oil	0.800	2.141	0.600	2.003	2.874	1.864
Dicalcium phosphate	1.916	2.362	1.780	2.216	0.928	1.676
Limestone	0.887	0.601	0.804	0.569	1.201	0.671
L-Lysine (78%)	1.800		2.000		0.414	
DL-Methionine (99%)	1.000		1.300		0.459	
L-Threonine (99%)	0.145		0.211		0.188	
L-Valine (98%)	0.174		0.119		0.090	
L-Tryptophan (98%)	0.013		0.096			
Salt	0.394	0.412	0.175	0.135	0.333	0.336
Sodium bicarbonate			0.262	0.355		
Potassium chloride		1.011		0.973		0.954
Inert (sand)		5.200		7.000		4.558
Min. and vit. supplement ¹	0.300	0.300	0.300	0.300	0.300	0.300

¹kg of product: folic acid 2400.000 mg; pantothenic acid 30.00 g; biotin 160.00 mg; butylated hydroxytoluene 100.00 mg; niacin 84.00 g; selenium 600.00 mg; Vit A 20000000,0 UI. Vit B1 5000.00 mg; Vit B12 36000.00 mcg; Vit B2 1300 g; Vit B6 7000,00 mg; Vit D3 5000000,00 UI; Vit E 37500,00 UI; Vit K3 4000,00 mg; Mn 150,00 g; Zn 140,00 g; Fe 90,00 g; Cu 15 g; I 15,00 mg.

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

Nutrients (%)	Starter (14 to 28 d)		Grower (56 to 70 d)		Developer (98 to 112 d)	
	Summit	Dilution	Summit	Dilution	Summit	dilution
	(L6)	(L0)	(L6)	(L0)	(L6)	(L0)
Crude protein ^b	30.020		30.050		29.860	
AMEn (Mcal/kg) ^a	2900	2900	2800	2800	2900	2900
Crude fiber ^a	6.000	5.929	6.000	6.000	5.833	6.000
Calcium ^a	0.940	0.940	0.894	0.894	0.800	0.800
Sodium ^a	0.180	0.180	0.166	0.166	0.150	0.150
Potassium ^a	0.530	0.530	0.510	0.510	0.500	0.500
Av. Phosphorus ^a	0.437	0.437	0.410	0.410	0.310	0.310
SID amino acid ^b						
Lysine	2.191		1.287		1.600	
Arginine	2.249		1.390		1.640	
Histidine	0.778		0.489		0.380	
Gly+Ser	1.744		1.674		1.182	
Isoleucine	1.268		0.782		1.071	
Leucine	2.354		1.518		1.208	
Met+Cys	1.536		1.025		1.250	
Methionine	0.840		0.563		0.740	
Threonine	1.500		0.876		1.250	
Phenylalanine	1.679		1.088		0.696	
Phe+Tyr	2.700		1.700		1.300	
Tryptophan	0.379		0.258		0.310	
Valine	1.598		1.029		1.270	

^a Calculated composition using data from the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011).

^b Analyzed composition of the amino acids in the experimental diets.

Experimental procedures

Each experimental period was divided in adaptation period (5 days) and total excreta collection period (5 days) to avoid that the birds were submitted to deficient diets for to long period. The birds and leftover feed were weighed at start and end of each experimental period. The feed experimental was provided according to bird metabolic body weight which was measured during the adaptation period, whilst feed intake was determined during the excreta collection period. Each metabolic cage was equipped with a metal tray coated with a plastic bag to collect the excreta during the period of 5 days. The excreta were collected, stored, freeze and weighed at the end of each collection period to measure total excreta production.

Chemical analyses

At the end of each experimental period, the excreta samples collected from each experimental unit were homogenized and a representative amount was weighed and frozen under -20°C. Later, the samples were freeze-dried during a period of 72 hours at -80°C under 800 mbar of pressure (Edwards® 501 Modulyo freeze drier, West Sussex, United Kingdom). After the lyophilization, the dried samples were analyzed for dry matter content. Subsequently, the dried samples were milled using a micro mill (IKA® A11 Basic Analytical Mill, Staufen, Germany). The nitrogen content of the diets and excreta were analyzed in a nitrogen distiller (Kjeltec™ 8400 Foss, Foss, Hillerod, Denmark) using the Kjeldahl method (AOAC, 2005: method 2001.11). The total amino acid contents of the ingredients and the experimental diets were analyzed by Evonik Industries using near-infrared spectroscopy (NIRs) and high-performance liquid chromatography (HPLC), respectively.

Statistical analyses

Nitrogen balance data were analyzed as one-way ANOVA using the general linear model (GLM) and then fitted to an exponential model using PROC NLIN procedure in SAS (Statistic Analysis System, 9.1). The regression analyses between nitrogen intake (NI, mg/BW_{kg}^{0.67}/day) and nitrogen excretion (NEX, mg/BW_{kg}^{0.67}/day) were performed to fit the exponential function and to determine the nitrogen maintenance requirement (NMR):

$$NEX = NMR \times e^{b \times NI} \quad [1]$$

where NMR is the nitrogen maintenance requirement (mg/BW_{kg}^{0.67}/day), *b* is the slope of the exponential curve and *e* is the base number of the natural logarithm (*ln*). The NMR was estimated by considering the intercept of the curve on the y-axis (NEX) when NI=0.

The nitrogen deposition (ND, mg/BW_{kg}^{0.67}/day) was determined by the difference between NI and the NEX. The nitrogen retention (NR, mg/BW_{kg}^{0.67}/day) was obtained by the sum of ND and NMR.

According to Samadi & Liebert (2007), the regression analyses between NI and NR was used to adjust the exponential model:

$$NR = NR_{\max}T \times (1 - e^{-b \times NI}) \quad [2]$$

where NR_{max}T is the maximum theoretical nitrogen retention (mg/BW_{kg}^{0.67}/day).

Another way to express this model is by regression analysis between NI and ND:

$$ND = NR_{\max}T \times (1 - e^{-b \times NI}) - NMR \quad [3]$$

The model generated in equation (3) was used to estimate NR_{max}T for each age, where *b* is the slope of the nitrogen retention curve and *e* is the base number of the natural logarithm (ln). The NR_{max}T was estimated by a statistical procedure following several iteration steps by the Levenberg-Marquardt algorithm until the sum of the squares of the residual was minimized. The theoretical maximum nitrogen deposition (ND_{max}T, mg BW^{0.67}/kg/day) for each period was obtained by subtracting the NMR from the NR_{max}T (ND_{max}T = NR_{max}T - NMR) expression.

Results

The results obtained from the N balance trials in starter (14-28 d), grower (56-70 d) and developer (98-112 d) periods are presented in table 3.

Table 3. Summarized results of nitrogen balance trials with Hy-line W-36 pullets receiving graded levels of protein (% crude protein) in starter (14-28 d), grower (56-70 d) and developer (98-112 d) periods

Parameters	L1 (5%CP)	L2 (10%CP)	L3 (15%CP)	L4 (20%CP)	L5 (25%CP)	L6 (30%CP)	<i>P-value</i>
Starter (14 to 28 days)							
BW (g)	176±25	201±22	198±21	233±32	243±30	246±50	<i>ns</i>
FI (g/d)	18±2	17±3	18±2	20±4	20±3	21±2	<i>ns</i>
NI (mg/BW _{kg} ^{0.67} /d)	324±63	716±97	993±92	1570±114	2214±129	2519±176	***
NEX (mg/BW _{kg} ^{0.67} /d)	274±82	646±122	877±106	1215±149	1704±130	1943±125	***
ND (mg/BW _{kg} ^{0.67} /d)	50±8	71±11	116±27	354±31	509±39	575±65	***
<i>b value</i> (10 ⁶)	58 ±5	54±3	51±5	62±6	53±3	56±2	<i>ns</i>
Grower (56 to 70 days)							
BW (g)	697±49	786±42	810±45	841±41	831±39	829±46	<i>ns</i>
FI (g/d)	26±5	40±9	40±10	39±9	37±8	40±9	<i>ns</i>
NI (mg/BW _{kg} ^{0.67} /d)	267±72	638±72	1002±122	1332±88	1848±191	2289±109	***
NEX (mg/BW _{kg} ^{0.67} /d)	207±59	334±129	666±140	922±63	1235±22	1344±112	***
ND (mg/BW _{kg} ^{0.67} /d)	60±12	304±10	336±10	410±26	612±104	946±122	***
<i>b value</i> (10 ⁶)	44±4	54±5	47±5	58±7	52±2	51±4	<i>ns</i>
Developer (98 to 112 days)							
BW (g)	852±79	883±54	925±44	946±55	986±52	963±37	<i>ns</i>
FI (g/d)	66±7	54±9	54±11	61±16	53±5	55±5	<i>ns</i>
NI (mg/BW _{kg} ^{0.67} /d)	320±30	732±44	1133±107	1500±139	1914±124	2455±219	***
NEX (mg/BW _{kg} ^{0.67} /d)	205±88	388±138	682±135	951±182	1229±145	1736±114	***
ND (mg/BW _{kg} ^{0.67} /d)	115±31	344±42	452±39	550±58	685±55	719±70	***
<i>b value</i> (10 ⁶)	56±7	65±5	63±2	65±4	65±3	62±2	<i>ns</i>

BW - body weight, FI – feed intake, NI – nitrogen intake, NEX – nitrogen excretion, ND – nitrogen deposition.

ns – not significant.

*** - $p < 0.001$.

Increasing levels of protein in experimental diets did not influence BW, FI and *b value* ($P > 0.05$). Nonetheless, graded dietary protein diets elicited an increase in NI, NEX and ND regardless of the assessed period. In the starter and developer periods, it was observed an increase from L1 to L4 and a tenuous increase from L5 to L6 for ND. Conversely, in the grower period, nitrogen deposition responses exhibit a different behavior, where a stable increase in ND is observed between the levels 2 to 4, and a considerable increase from L5 to L6.

After fitting the exponential function between nitrogen intake and nitrogen excretion during a gradual increase in the protein supplied in each growth period, it was estimated the daily NMR as 294, 331 and 355 mg/BW_{kg}^{0.67} for the initial, grower and developer periods, respectively, when NI=0 as shown in figure 1.

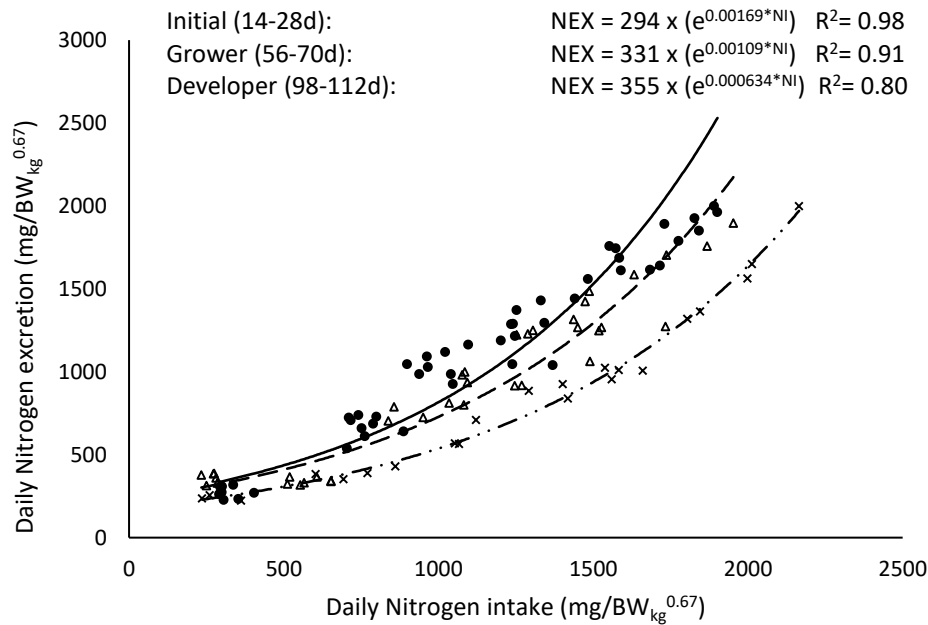


Figure 1. Estimation of the daily nitrogen requirements for maintenance (NMR) by fitting the exponential function between nitrogen intake (NI) and nitrogen excretion (NEX) for Hy-line W-36 pullets in starter (14-28 d), grower (56-70 d) and developer (98-112 d) periods. Observed (×) and predicted (—·—) values for starter (14-28 d) period. Observed (Δ) and predicted (---) values for grower (56-70 d) period. Observed (•) and predicted (—) values for developer (98-112 d) period.

The maximum theoretical nitrogen retention was estimated in 3200, 2633, and 1826 mg/BW_{kg}^{0.67} for starter, grower and developer periods, respectively, after the fitting of the exponential function between nitrogen intake and nitrogen retention, shown in figure 2.

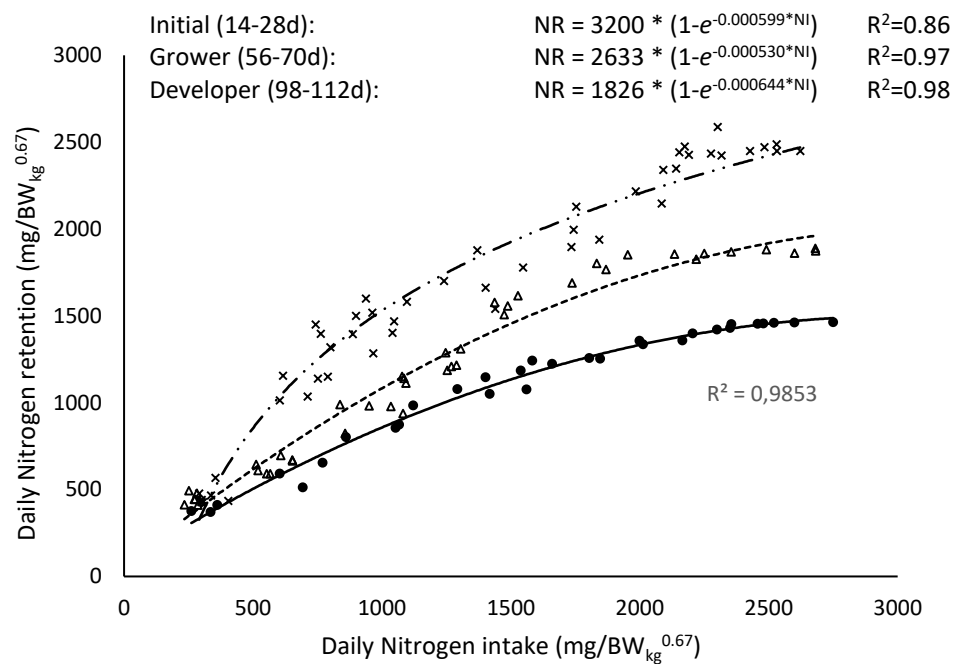


Figure 2. Estimation of the maximum theoretical nitrogen retention (NR_{maxT}) for Hy-line W-36 pullets in the starter (14-28 d), grower (56-70 d) and developer (98-112 d) periods based on the exponential fitting between nitrogen intake (NI) and nitrogen retention (NR). Observed ($\times$) and predicted ($-\cdots-$) values for starter period (14-28 d). Observed (Δ) and predicted ($---$) values for grower period (56-70 d). Observed ($\bullet$) and predicted ($---$) values for developer period (98-112 d).

The summarized results of estimating the values for NMR and threshold values for daily N retention and daily N deposition as demonstrated in the figures 1 and 2, are presented in the table 4.

Table 4. Nitrogen maintenance requirements (NMR), maximum theoretical nitrogen retention (NR_{maxT}) and maximum theoretical nitrogen deposition (ND_{maxT}) of the Hy-line W-36 pullets in starter (14-28 d), grower (56-70 d) and developer (98-112 d) periods

Period	NMR	NR_{maxT}	ND_{maxT}
		mg/BW _{kg} ^{0.67} /day	
Starter (14 to 28 d)	294	3200	2906
Grower (56 to 70 d)	331	2633	2302
Developer (98 to 112 d)	355	1826	1471

Nitrogen maintenance requirement increased in parallel to bird growth, whilst the maximum theoretical nitrogen retention and maximum theoretical nitrogen deposition decrease with increasing age.

Discussion

The aim of this study was to estimate the genetic potential for daily N retention ($NR_{max}T$) and nitrogen maintenance requirement (NMR) of pullets during growth phase using information from nitrogen balance trials and the Goettingen approach. Once determined, these parameters allows estimating efficiency of utilization and the ideal ratio of amino acids using non-linear models (Thong & Liebert, 2004a, b, c; Weck & Liebert, 2009, 2010; Pastor et al., 2013; Dorigam et al., 2017).

Goettingen approach considers that the nitrogen retention is partitioned in NMR and $NR_{max}T$. In the model, the NMR reflects the inevitable endogenous losses of nitrogen via excreta depending on the nitrogen intake. The $NR_{max}T$ expresses an estimate of the genetic potential of the animal but is given as a theoretical condition since the determined values could be not achieved in practice. Both NMR and $NR_{max}T$ are specific for each age, gender and genotype (Samadi & Liebert, 2006b).

Considering the NMR results obtained in each period, an average value of 327 mg/ $BW_{kg}^{0.67}$ /day was calculated for pullets in growing phase. Silva et al. (2013) and Bonato (2013) found average NMR of 284 mg/ $BW_{kg}^{0.67}$ /day and 307 mg/ $BW_{kg}^{0.67}$ /day for Dekalb White[®] pullets in the same period, respectively. Daulat et al. (2015) determined average NMR of 310 mg/ $BW_{kg}^{0.67}$ /day in a trial with naked neck broilers in growth phase.

According to Lamot et al. (2017) diets with different nitrogen and energy balance provides synthesis of body protein, that is, there is a minimum retention of nitrogen even though there is an energy cost. When considering that the animal does not ingest nitrogen, it is assumed

that a part of the body protein is degraded to make up the pool of free amino acids and part of the mobilized amino acids is re-synthesized to meet the metabolic priorities. The result of the inefficiency of the re-syntheses process is quantified in NMR so that the differences found in NMR can be related to protein synthesis and degradation rates of different genotypes.

Samadi & Liebert (2006a) determined NMR of $252 \text{ mg/BW}_{\text{kg}}^{0.67}/\text{day}$ for Cobb 500[®] broilers in the period from 10 to 85 days. Dorigam et al. (2017) working with broiler breeder hens of the Ross 308AP[®] genotype found NMR of $255 \text{ mg/BW}_{\text{kg}}^{0.67}/\text{day}$ in the egg production period. Wecke et al. (2016) and Samadi & Liebert (2008) determined average NMR of $221 \text{ mg/BW}_{\text{kg}}^{0.67}/\text{day}$ and $240 \text{ mg/BW}_{\text{kg}}^{0.67}/\text{day}$ for Ross 308[®] broilers in the growth and finisher phases, respectively. These results are, on average, 35% lower than those found in the study and can be attributed to differences in genotype and age of the birds. The average NMR of $164 \text{ mg/BW}_{\text{kg}}^{0.67}/\text{day}$ determined by Pastor et al. (2013) differs from the values found in the current studies.

The age of the birds influences the nutritional requirements of nitrogen due to changes occurring in protein deposition potential that rapidly increases during growth, reaches a maximum and then decreasing growth rate as the animal approximates the adult where higher proportion of protein is destined for maintenance. In general, it is assumed that the requirement of nitrogen as a percentage of the diet decreases with the increase of the age of the birds, due to the reduction in the relative rate of growth and the increase of the alimentary consumption capacity (Bertechini, 2012).

The techniques of nitrogen balance and comparative slaughter describe the potential of nitrogen retention differently, however both techniques allow similar estimates (Silva, 2012). Nitrogen balance becomes a simple and practical tool to estimate the maximum nitrogen deposition potential of the bird and the inevitable losses caused by protein turnover in growing

animals (Samadi & Liebert, 2006a; b; 2007a; b; 2008), since the experimental period of the nitrogen balance is shorter and only needs to quantify the amount of nitrogen ingested and excreted. While comparative slaughtering, besides more laborious has the fact that the animals sampled are not the same, what can influence in the determination of the coefficients.

A validation of the model parameter b as a measure of dietary protein quality, which is independent of the actual level of protein intake, has been reported in several studies (Thong & Liebert, 2004a, b, c; Wecke & Liebert, 2009; Pastor et al., 2013) and it is also can be observed in the table 3, once that the b -values not presented difference statistical insofar as increased the level of protein intake. A physiologically based control is provided in an experiment when the observed model parameter reflecting dietary protein quality independent of protein intake (b) remains unchanged. If this precondition is fulfilled, as in this study, the individual N balance data can be utilized to estimate the threshold value of $NR_{max}T$ (Liebert, 2015).

In this study, the values to $NR_{max}T$ of 3200, 2633 and 1826 mg/ $BW_{kg}^{0.67}/d$ for initial, grower and developer periods were determined, respectively. Bonato (2013) found similar values of $NR_{max}T$ of 3208, 2353 and 1739 mg/ $BW_{kg}^{0.67}/day$, whilst Silva et al. (2013) found values of 2239, 1910 and 1776 mg/ $BW_{kg}^{0.67}/day$, working with Dekalb White[®] pullets in the same period. It is difficult to compare the data with other experiments due to variations in strains, age, feed intake and dietary characteristics. However, several studies (Samadi & Liebert, 2008; Pastor, et al., 2013; Dorigam, et al., 2014; Santos, et al., 2014; Daulat, et al., 2015; Wecke, et al., 2016) presented similar characteristics where a decrease of threshold values with increasing age were observed, indicating that the genetic potential is independent of the diet (Samadi & Liebert, 2008). According to Samadi & Liebert (2007b) the decreasing potential for nitrogen deposition might be due to morphological changes in the gut, a changing fractional rate of protein synthesis and impaired efficiency of amino acid deposition in the carcass.

The estimation of $NR_{max}T$ and NMR are required for basic applications of the exponential model, as well as to define the genotype in terms of theoretical potential for N deposition, evaluate the value of the food protein quality based on the observed efficiency of the limiting amino acid, determination of amino acid requirements from the efficiency of the dietary amino acid, model the amino acid requirement according to the performance level objective and the N losses in the process of N utilization in terms of minimizing excretion of nitrogen per unit of nitrogen deposited (Liebert, 2017).

From the point of view of production, the animals are assumed to achieve a performance corresponding to until 50% of the values determined for $NR_{max}T$ due to limiting factors, such as environment and diet. Thus, knowing the genetic potential or the physiological limit of the lineage allows the adoption of strategies to optimize the performance of the birds.

The daily nitrogen maintenance requirements for Hy-line W-36 pullets were estimated as 294, 331 and 355 mg/BW_{kg}^{0.67} whereas the daily maximum theoretical nitrogen retention for Hy-line W-36 pullets was estimated as 3200, 2633, and 1826 mg/BW_{kg}^{0.67} for the initial, grower and developer periods, respectively. These values can be used later as parameters in non-linear mathematical models to estimate the efficiency of utilization and the ideal ratio of the essential amino acids for growing pullets by the Goettingen approach.

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CAPÍTULO 3 - Optimal in-feed amino acid ratio for growing pullets based on deletion method and non-linear modelling

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Optimal in-feed amino acid ratio for growing pullets based on deletion method and non-linear modelling

Running title: Optimal in-feed amino acid ratio for growing pullets

Letícia Soares¹, Nilva Kazue Sakomura¹, Juliano Cesar de Paula Dorigam², Frank Liebert³, Bruno Balbino Leme¹, João Batista Kochenborg Fernandes¹

¹Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Jaboticabal, São Paulo, Brazil,

²Department of Animal Nutrition Research, Evonik Industries, Hanau, Wolfgang, Germany.

³Chair of Animal Nutrition, University of Göttingen, Kellnerweg, Göttingen, Germany.

Correspondence: L. Soares¹, Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Jaboticabal, São Paulo, Brazil, Via de Acesso Prof. Paulo Donato Castelane, s/n. 14884-900 - Jaboticabal, SP, Brazil. E-mail: leticia.fcav@gmail.com.

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SUMMARY

To determine the ideal essential amino acid ratio (IAAR) for growing pullets, three nitrogen balance trials were conducted, a total of 96 Hy-line W-36 pullets were used in each trial in age periods. Twelve treatments with eight individual replicates. A balanced diet (BD) was formulated to meet the IAAR and the requirement of other nutrients for pullets. 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 trial, the data of nitrogen intake, excretion, deposition and retention were obtained in a balance trial. The b values were estimated. Also, these data were used to calculate the AA requirements by Louvain approach. The bc^{-1} values were obtained dividing b by the dietary AA concentration. The IAAR by Goettingen approach was obtained by dividing the bc^{-1} from lysine by the bc^{-1} value from each other AA in each trial. The relation between the AA requirements with Lys requirements provided the IAAR by Louvain approach. The IAAR determined by Goettingen approach were Lys 100%; Met+Cys 86, 88, 81%; Trp 17, 22, 22%; Thr 77, 67, 68%; Arg 103, 111, 109%; Val 75, 80, 84%; Ile 64, 67, 75%; Leu 101, 121, 124%; Phe+Tir 112, 126, 130%; Gly+Ser 81, 130, 112%; His 35, 35, 36% from 14 to 28 days, 56 to 70 days and 98 to 112 days, respectively. The IAAR determined by Louvain approach were Lys 100%, Met+Cys 73, 80, 67%; Trp 18, 20, 22%; Thr 67, 68, 69%; Arg 108, 108, 108%; Val 77, 80, 84%; Ile 70, 75, 77%; Leu 113, 117, 129%; Phe+Tir 121, 125, 131%; Gly+Ser 78, 133, 124%; His 37, 38, 39% from 14 to 28 days, 56 to 70 days and 98 to 112 days, respectively.

Introduction

Accelerated growth and high body protein deposition rate are the common characteristics of the modern poultry lines. From the nutritional point of view, it is essential to provide adequate nutrients, especially amino acids that are necessary for body protein synthesis and lean tissue deposition, as well as for other essential secondary physiological functions such as serotonin, adrenaline, nitric oxide, glutathione and carnitine synthesis.

Contrary to the past, where feeds were formulated for poultry based on crude protein values, poultry diets currently are formulated based on the ideal protein concept as proposed by Baker & Han (1994), Emmert & Baker (1997) and Baker et al. (2003), whose main assumption is the existence of a closely related amino acid profile to support proper protein synthesis roles in the organism. However, the ideal amino acid profile for laying hens and, mainly pullets is not well developed as that observed for broiler chickens and pigs.

The dose response method has been used in most of studies to define the amino acid requirements for poultry, providing the basis for nutritional tables such as the “Nutrient Requirements of Poultry” (NRC, 1994) and the Brazilian Tables for Poultry and Swine (Rostagno et al., 2000; 2005; 2011; 2017). However, since dose response studies are based on the response of the bird to the gradual increase of the test amino acid in the diets, an assay is required for each amino acid evaluated, which may cause an increase in costs for research as well as longer execution time (Rollin et al, 2003; Dorigam et al., 2015).

The deletion method described by Wang & Fuller (1989) provides more accurate values by minimizing the effects of the environment, diet and genotype on the determination of the ideal amino acid ratio. This methodology assumes that the removal of a non-limiting amino acid does not alter the response of the birds and that the change in bird response to reductions on the proportion of an amino acid, is used to calculate the ideal amino acid profile in each diet

whose amino acid is limiting. The principle of methodology was utilized in our procedure, but the effect of individual amino acid deletion was directly measures by amino acid efficiency from modelling of observed N balance data (Liebert, 2008).

Another approach can be applied from the deletion method to obtain the ideal amino acid ratio was proposed by Rollin et al. (2003) based on an equation to determine the requirements of amino acids. Although it was suggested that the utilization of a limiting EAA is well described by a non-linear model (Fatufe et al., 2004; Samadi & 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 & Liebert, 2006; 2007). In this way, it is possible to compare the efficiency of utilization of individual AA directly to evaluate the optimal AA ratio (Samadi & Liebert, 2008).

The advantage of this method over the dose response method is the need for only one assay to determine the optimal ratio of all essential amino acids. Moreover, all amino acid ratios are determined simultaneously, using the same group of animals and the same control diet, which increase the accuracy of optimal amino acid ratios estimated (Rollin et al., 2003; Dorigam et al., 2015). Thus, the aim of this study was to apply the efficiency and amino acids requirements 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 ratio for Hy-line W36 pullets.

Material and Methods

This study was approved by the Ethics Committee on Animal Use of the Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal (n° 9999/14).

Three nitrogen balance trials were conducted in the Laboratory of Poultry Science of the Faculty of Agriculture and Veterinary Sciences of Jaboticabal, São Paulo, Brazil. The nitrogen balance trials were conducted to determine the efficiency of amino acid utilization to derive an optimal amino acid ratio for Hy-line W-36 pullets in each growth phase (starter: 14 to 28, grower: 56 to 70 and developer: 98 to 112 days).

Animals housing and experimental design

In each trial, a total of 96 Hy-line W-36 pullets were individually allotted in a completely randomized design with 12 treatments and 8 replicates. The birds were housed in metabolic cages with wire floors, individual galvanized feeders and nipple drinkers installed in a facility with a negative pressure ventilation system with controlled humidity and temperature. The management of the pullets and the lighting program followed the recommendations of the lineage guidelines (Hy-line W-36 Management Guide, 2016).

Experimental diets

The treatments consisted a balance diet (BD) and eleven different diets deficient in one of the amino acid tested (lysine, methionine + cystine, threonine, valine, isoleucine, arginine, tryptophan, leucine, histidine, glycine + serine and phenylalanine + tyrosine).

The BD was formulated to properly meet the nutritional requirements and the ideal amino acid ratio (IAAR) provided by the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011) for pullets. The content of nitrogen and amino acid in the BD was supplied by corn, soybean meal, corn gluten meal and a mixture of industrial amino acids (Table 1).

To formulate the deficient diets, the BD was diluted with corn starch to reduce 30% of the dietary amino acid content. The diets were again supplemented with vitamin, mineral, fiber, energy and industrial amino acids sources to achieve the same levels of the BD, except for the amino acid to be evaluated in the treatment, which will remain reduced in 30%. Thus, the deficient diets are composed in 70% of the BD, 19% of corn starch, 5% of rice husk, 3% of L-Alanine to achieve the same protein level of BD and the others 3% is divided in industrial amino acids, limestone, dicalcium phosphate, vitamin and mineral. The nutritional composition of the diets is presented in Table 2, 3 and 4, respectively.

Table 1. Composition of balance diets used in each period of the trial

Ingredients (%)	Balanced diets (BD)		
	Starter (14 to 28 d)	Grower (56 to 70 d)	Developer (98 to 112 d)
Corn	63.257	64.344	56.979
Wheat meal	13.619	3.989	-
Soybean meal	4.115	1.800	1.000
Rice husk	5.529	14.624	14.235
Limestone	1.007	0.595	0.769
Dicalcium phosphate	1.895	1.826	1.479
Soybean oil	1.500	3.500	4.716
Sugar	-	-	7.000
Salt	0.397	0.327	0.309
Potassium chloride	0.250	0.495	0.604
L-Alanine (96%)	5.115	5.875	5.961
L-Lysine HCl (78%)	0.702	0.504	0.400
DL-Methionine (99%)	0.316	0.233	0.183
L-Arginine (99%)	0.453	0.337	0.284
L-Valine (98%)	0.257	0.178	0.144
L-Threonine (99%)	0.303	0.200	0.158
L-Isoleucine (99%)	0.283	0.219	0.178
L-Leucine (98%)	0.117	-	-
L-Glycine (96%)	-	0.237	0.139
L-Phenylalanine (99%)	0.439	0.308	0.263
L-Tryptophan (98%)	0.075	0.072	0.070
L-Histidine (99%)	0.073	0.038	0.030
Inert (sand)	-	-	4.800
Min. and vit. supplement ¹	0.300	0.300	0.300

¹kg of product: folic acid 2400.000 mg; pantothenic acid 30.00 g; biotin 160.00 mg; butylated hydroxytoluene 100.00 mg; niacin 84.00 g; selenium 600.00 mg; Vit A 20000000,0 UI; Vit B1 5000.00 mg; Vit B12 36000.00 mcg; Vit B2 1300 g; Vit B6 7000,00 mg; Vit D3 5000000,00 UI; Vit E 37500,00 UI; Vit K3 4000,00 mg; Mn 150,00 g; Zn 140,00 g; Fe 90,00 g; Cu 15 g; I 15,00 mg.

Table 2. Nutritional composition of the experimental diets with individual amino acid deletion used in starter (14 to 28 d) period

Nutrients (%)	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Calcium ^a	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940
Chloride ^a	0.404	0.502	0.502	0.502	0.502	0.502	0.502	0.502	0.502	0.502	0.502	0.502
AMEn (Mcal/kg) ^a	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900
Crude fiber ^a	4.800	4.800	4.800	4.800	4.800	4.800	4.800	4.800	4.800	4.800	4.800	4.800
Av. phosphorus ^a	0.437	0.437	0.437	0.437	0.437	0.437	0.437	0.437	0.437	0.437	0.437	0.437
Potassium ^a	0.530	0.530	0.530	0.530	0.530	0.530	0.530	0.530	0.530	0.530	0.530	0.530
Sodium ^a	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180
Crude protein ^b	18.86	17.56	17.77	18.66	18.07	17.67	18.54	17.90	17.92	18.66	18.23	18.03
SID Amino acid ^b												
Arginine	0.947	0.922	0.943	0.947	0.926	0.649	0.953	0.927	0.940	0.930	0.921	0.926
Phenylalanine	0.921	0.897	0.910	0.887	0.898	0.885	0.914	0.928	0.883	0.579	0.925	0.891
Phe+Tyr	1.069	1.067	1.080	1.057	1.068	1.055	1.084	1.068	1.053	0.749	1.065	1.061
Glycine + Serine	0.692	0.694	0.687	0.688	0.694	0.679	0.685	0.695	0.687	0.691	0.480	0.684
Histidine	0.331	0.334	0.327	0.328	0.330	0.325	0.324	0.331	0.323	0.334	0.329	0.231
Isoleucine	0.624	0.614	0.601	0.625	0.624	0.629	0.606	0.420	0.597	0.599	0.611	0.617
Leucine	0.993	0.965	0.969	0.982	0.966	0.993	0.979	0.998	0.680	0.983	0.974	0.982
Lysine	0.876	0.613	0.876	0.876	0.876	0.876	0.876	0.876	0.876	0.876	0.876	0.876
Methionine + Cystine	0.648	0.653	0.452	0.646	0.648	0.655	0.641	0.646	0.640	0.657	0.637	0.639
Methionine	0.538	0.526	0.334	0.539	0.526	0.536	0.549	0.521	0.525	0.531	0.528	0.519
Threonine	0.596	0.594	0.577	0.578	0.419	0.585	0.579	0.587	0.591	0.592	0.587	0.598
Tryptophan	0.167	0.166	0.159	0.116	0.153	0.160	0.153	0.157	0.167	0.163	0.158	0.154
Valine	0.669	0.664	0.667	0.666	0.669	0.660	0.459	0.665	0.662	0.668	0.661	0.669

BD=balanced diet, ^a Calculated composition using data from the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011), ^b Analyzed composition of the amino acids in the experimental diets.

Table 3. Nutritional composition of the experimental diets with individual amino acid deletion used in grower (56 to 70 d) period

Nutrients (%)	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Calcium ^a	0.832	0.832	0.832	0.832	0.832	0.832	0.832	0.832	0.832	0.832	0.832	0.832
Chloride ^a	0.472	0.487	0.487	0.487	0.487	0.487	0.487	0.487	0.487	0.487	0.487	0.487
AMEn (Mcal/kg) ^a	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900
Crude fiber ^a	6.538	6.538	6.538	6.538	6.538	6.538	6.538	6.538	6.538	6.538	6.538	6.538
Av. phosphorus ^a	0.392	0.392	0.392	0.392	0.392	0.392	0.392	0.392	0.392	0.392	0.392	0.392
Potassium ^a	0.520	0.520	0.520	0.520	0.520	0.520	0.520	0.520	0.520	0.520	0.520	0.520
Sodium ^a	0.160	0.160	0.160	0.160	0.160	0.160	0.160	0.160	0.160	0.160	0.160	0.160
Crude protein ^b	16.22	16.10	15.72	15.88	16.18	16.26	16.01	16.45	16.86	16.77	16.03	16.77
SID Amino acid ^b												
Arginine	0.699	0.689	0.667	0.632	0.653	0.493	0.673	0.660	0.679	0.660	0.662	0.687
Phenylalanine	0.652	0.647	0.648	0.632	0.639	0.639	0.620	0.637	0.635	0.407	0.639	0.648
Phe+Tyr	0.776	0.776	0.776	0.776	0.776	0.776	0.776	0.776	0.776	0.543	0.776	0.776
Glycine + Serine	0.838	0.787	0.776	0.784	0.825	0.823	0.775	0.828	0.825	0.785	0.543	0.761
Histidine	0.246	0.237	0.237	0.240	0.237	0.254	0.243	0.254	0.253	0.240	0.224	0.170
Isoleucine	0.484	0.470	0.467	0.491	0.457	0.424	0.488	0.324	0.467	0.479	0.485	0.458
Leucine	0.766	0.741	0.726	0.701	0.717	0.741	0.730	0.732	0.454	0.732	0.743	0.749
Lysine	0.684	0.423	0.588	0.577	0.637	0.636	0.618	0.624	0.593	0.590	0.625	0.586
Methionine + Cystine	0.507	0.459	0.337	0.496	0.485	0.506	0.489	0.524	0.506	0.475	0.519	0.463
Methionine	0.351	0.343	0.222	0.365	0.366	0.425	0.373	0.404	0.413	0.359	0.409	0.350
Threonine	0.435	0.417	0.414	0.406	0.290	0.448	0.437	0.451	0.423	0.412	0.430	0.423
Tryptophan	0.124	0.124	0.124	0.087	0.124	0.124	0.124	0.124	0.124	0.124	0.124	0.124
Valine	0.510	0.502	0.494	0.497	0.490	0.502	0.367	0.492	0.509	0.503	0.509	0.503

BD=balanced diet, ^a Calculated composition using data from the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011), ^b Analyzed composition of the amino acids in the experimental diets.

Table 4. Nutritional composition of the experimental diets with individual deletion used in developer (98 to 112 d) period

Nutrients (%)	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Calcium ^a	0.800	0.800	0.800	0.800	0.800	0.800	0.800	0.800	0.800	0.800	0.800	0.800
Chloride ^a	0.506	0.550	0.550	0.550	0.550	0.550	0.550	0.550	0.550	0.550	0.550	0.550
AMEn (Mcal/kg) ^a	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900
Crude fiber ^a	6.657	6.572	6.572	6.572	6.572	6.572	6.572	6.572	6.572	6.572	6.572	6.572
Av. phosphorus ^a	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.310	0.310
Potassium ^a	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500
Sodium ^a	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150
Crude protein ^b	14.51	14.34	13.72	14.14	14.17	13.68	14.42	14.00	14.52	13.62	14.10	14.50
SID Amino acid ^b												
Arginine	0.538	0.510	0.528	0.526	0.501	0.342	0.507	0.524	0.509	0.522	0.514	0.525
Phenylalanine	0.556	0.529	0.571	0.552	0.553	0.566	0.557	0.554	0.548	0.383	0.559	0.539
Phe+Tyr	0.663	0.634	0.678	0.659	0.658	0.671	0.664	0.661	0.655	0.490	0.697	0.644
Glycine + Serine	0.634	0.622	0.650	0.631	0.612	0.649	0.612	0.643	0.621	0.621	0.476	0.629
Histidine	0.198	0.195	0.197	0.197	0.185	0.202	0.188	0.200	0.195	0.184	0.198	0.147
Isoleucine	0.399	0.366	0.391	0.392	0.395	0.388	0.379	0.286	0.375	0.392	0.383	0.391
Leucine	0.613	0.602	0.608	0.600	0.603	0.606	0.603	0.609	0.404	0.595	0.603	0.606
Lysine	0.496	0.334	0.484	0.463	0.453	0.466	0.451	0.465	0.469	0.463	0.465	0.466
Methionine + Cystine	0.377	0.357	0.299	0.352	0.373	0.371	0.362	0.357	0.355	0.350	0.358	0.367
Methionine	0.300	0.261	0.201	0.257	0.286	0.274	0.270	0.261	0.262	0.255	0.267	0.275
Threonine	0.357	0.317	0.354	0.349	0.257	0.337	0.322	0.348	0.342	0.343	0.338	0.357
Tryptophan	0.106	0.106	0.106	0.074	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106
Valine	0.427	0.400	0.426	0.428	0.418	0.416	0.293	0.426	0.410	0.406	0.399	0.422

BD=balanced diet, ^a Calculated composition using data from the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011), ^b Analyzed composition of the amino acids in the experimental diets.

Experimental procedures

Each experimental period was divided in adaptation (5 days) and total excreta collection periods (5 days) to avoid that the birds were submitted to deficient diets for an extended period. The birds and feed leftovers were weighed at start and end of each experimental period. The feed supply was corrected according to the metabolic body weight of the birds measured during the adaptation period and the feed intake was determined during the collection period. Each metabolic cage was equipped with a metal tray coated with a plastic bag to collect the excreta during the period of 5 days. The excreta were collected, stored in plastic pots and weighed at the end of each collection period to measure total excreta production.

Chemical analyses

At the end of each experimental period, the excreta samples collected from each experimental unit were homogenized and a representative amount was weighed in little plastic pots to freeze at -20°C. Later, the samples were freeze-dried during a period of 72 hours at -80°C under 800 mbar of pressure (Edwards® 501 Modulyo freeze drier, West Sussex, United Kingdom). After the lyophilization, the dried samples were weighed to quantify the dry matter content. Subsequently, the dried samples were milled using a micro mill (IKA® A11 Basic Analytical Mill, Staufen, Germany). The nitrogen content of the diets and excreta were analyzed in a nitrogen distiller (Kjeltec™ 8400 Foss, Foss, Hillerød, Denmark) using the Kjeldahl method (AOAC, 2005: method 2001.11). The total amino acid contents of the ingredients and the experimental diets were analyzed by Evonik Industries using near-infrared spectroscopy (NIRs) and high-performance liquid chromatography (HPLC), respectively.

Statistical analyses

The nitrogen balance data were analyzed by a one-way ANOVA using a GLM procedure in SAS (Statistical Analysis System, version 9.1). Significant differences between

efficiency of protein utilization were tested using Dunnett's test and the p-values <0.05 were deemed statistically significant. Once it is confirmed the difference between the efficiency of protein utilization obtained by the test diets and the efficiencies obtained by the BD, the ideal amino acid ratios can be further calculated.

Modelling the ideal amino acid ratios as proposed by the Goettingen approach

The nitrogen deposition (ND, mg/BW_{kg}^{0.67}/day) was determined by the difference between nitrogen intake (NI, mg/BW_{kg}^{0.67}/day) and nitrogen excretion (NEX, mg/BW_{kg}^{0.67}/day). The nitrogen retention (NR, mg/BW_{kg}^{0.67}/day) was obtained by the sum of ND and nitrogen maintenance requirement (NMR, mg/BW_{kg}^{0.67}/day). The NMR value considered were 294, 331 and 355 mg/BW_{kg}^{0.67}/day determined in the previous study (data not published) for starter, grower and developer periods, respectively.

In the limiting diets where the test amino acid was reduced in 30%, the protein quality (*b*) in each treatment was obtained by the equation proposed by Samadi & Liebert (2008):

$$b = \ln(NR_{max}T) - \ln(NR_{max}T - NR)/NI \quad (1)$$

Where NR_{max}T is the maximum theoretical nitrogen retention (mg/BW_{kg}^{0.67}/day). The values of NR_{max}T for Hy-line W36 used in the calculations were 3200 mg/BW_{kg}^{0.67}/day from 14 to 28 d, 2633 mg/BW_{kg}^{0.67}/day from 56 to 70 d and 1826 mg/BW_{kg}^{0.67}/day from 98 to 112 d. These values were estimated in a previous study (data not published).

The dietary protein quality (*b*) is linearly dependent on the concentration of the limiting amino acid in the dietary protein (*c*). The proposed calculation of *c* was:

$$c = 16 \times LAI/NI \quad (2)$$

Where LAAI is the limiting amino acid intake and NI is the nitrogen intake. The slope of the linear function (bc^{-1}) is an expression of dietary efficiency of the limiting AA under study and is utilized as a model parameter. However, the amino acid efficiency parameter (bc^{-1}) summarizes absorption and post-absorptive utilization of the limiting amino acid. This application is only valid when the first limiting position of the AA under study is ensured.

According to Samadi & Liebert (2008), the model parameter bc^{-1} is directly suited for assessing optimal dietary AA ratios, because the order of observed bc^{-1} data from individual AA is indirectly related to the physiological requirement per unit protein deposition as one important factor. In addition, AA utilization during digestion, absorption and post-absorptive utilization processing is the second important factor influencing the order of bc^{-1} data.

The ratio between lysine efficiency (reference) and the efficiency of the limiting amino acid under study is utilized to derive the ideal amino acid ratios (IAAR), according to the calculation (Wecke & Liebert, 2013):

$$IAAR = bc^{-1}_{Lys}/bc^{-1}_{AA} \quad (3)$$

Modelling the ideal amino acid ratios as proposed by the Louvain approach (Rollin et al., 2003)

The amino acid requirement (g/kg DM) was determined using the following equation proposed by Rollin et al. (2003):

$$\text{Requirement} = (EAA)_{BD} \times \left(2 - DEL \left(\frac{ND_{EAA}}{ND_{BD}} \right) \right) \quad (1)$$

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 ($\text{mg N/BW}_{\text{kg}}^{0.67}/\text{d}$) corresponding to the EAA diet and ND_{BD} is the N deposition observed on the BD ($\text{mg N/BW}_{\text{kg}}^{0.67}/\text{d}$). 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).

Results

Summarized results of the nitrogen balance trials in starter, grower and developer periods are presented in Table 6. The diets limiting in amino acid test had lower nitrogen balance data compared to BD in all periods evaluated ($p < 0.05$). The deletion of essential amino acids reduced nitrogen retention and protein quality, but in different proportions depending on the deleted amino acid.

Only in grower period, there was marked depression in nitrogen retention when there was a deletion of tryptophan, however, it did not affect body weight and feed intake when compared to other limiting diets ($p > 0.05$).

As expected, amino acid deficient diets have lower protein quality, characterized by the decrease in the value of parameter b , in relation to BD ($p < 0.05$). In starter period, the leucine-deficient diet presented higher b -value compared to the other limiting diets, but still lower than the BD protein quality. The deletion of tryptophan in grower period and of leucine in developer period significantly reduced the quality of dietary protein in relation to the other limiting diets evaluated.

When evaluating the efficiency of amino acids utilization, there is a gradual increase in bc^{-1} values from one period to the next period. From the utilization efficiencies and amino acid requirements presented in Table 5 were determine the ideal ratios of the essential amino acid for Hy-line W-36 pullets by Goettingen and Louvain approaches presented in Table 6.

Table 5. Effect of deleting a single amino acid from the diet on protein quality and nitrogen retention of pullets in nitrogen balance trials

Diets	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Starter (14 to 28 d)												
BW	396±87	379±74	364±78	390±67	370±66	357±61	356±68	370±61	376±51	365±69	385±71	368±45
FI	20±4	19±4	21±5	21±6	20±5	18±3	20±5	18±3	19±3	21±6	21±5	18±3
NI	1177±127	1170±117	1257±53	1247±142	1209±93	1142±139	1222±128	1127±66	1140±120	1228±76	1263±138	1131±58
ND	566±126	390±68	387±122	442±99	390±72	345±64	432±146	560±133	511±134	516±121	498±92	487±137
NR	860±126	684±68	681±122	763±99	684±72	639±64	726±146	854±133	805±134	810±121	792±92	781±137
<i>b</i> (10⁶)	36 ^a ±4.9	19 ^b ±2.3	19 ^b ±4.0	25 ^b ±4.8	20 ^b ±2.7	21 ^b ±4.1	20 ^b ±4.7	28 ^b ±4.6	29 ^b ±6.5	27 ^b ±3.8	25 ^b ±4.6	25 ^b ±4.0
<i>bc</i>⁻¹		6.0±0.7	7.9±1.6	35.3±7.8	7.9±1.2	5.9±1.1	8.0±1.8	9.2±1.9	5.7±1.9	5.2±0.9	7.1±1.8	17.1±3.2
<i>c</i>		3.17	2.40	0.71	2.53	3.60	2.50	3.03	5.12	5.17	3.50	1.63
AAR		0.60	0.44	0.11	0.41	0.65	0.46	0.42	0.68	0.73	0.47	0.22
Grower (56 to 70 d)												
BW	894±38	848±51	780±70	866±56	856±31	826±30	816±30	802±69	819±58	870±30	867±29	870±28
FI	44±6	43±4	44±2	40±5	41±5	39±4	42±3	35±4	41±5	40±3	39±4	39±5
NI	1039±121	1039±74	1166±138	951±99	978±95	972±104	1057±91	908±176	1035±181	940±63	937±92	912±104
ND	370±51	284±49	342±86	232±19	232±98	230±60	289±99	260±73	219±67	223±64	363±58	296±72
NR	701±51	615±49	673±86	423±99	563±98	561±60	620±99	591±73	518±67	554±64	694±58	627±72
<i>b</i> (10⁶)	38 ^a ±2.8	26 ^b ±1.5	27 ^b ±5.4	18 ^b ±3.7	25 ^b ±4.5	23 ^b ±1.8	25 ^b ±3.7	27 ^b ±5.2	23 ^b ±3.3	25 ^b ±4	32 ^b ±4.8	30 ^b ±3.1
<i>bc</i>⁻¹		9.0±0.5	10.6±2.5	40.9±6.8	13.6±2.4	8.3±0.6	11.1±1.7	13.2±2.6	7.4±1.0	7.3±1.2	6.9±1.3	25.7±3.0
<i>c</i>		2.89	2.55	0.44	1.84	2.77	2.25	2.04	3.09	3.4	4.66	1.17
AAR		0.43	0.34	0.09	0.29	0.47	0.34	0.32	0.51	0.54	0.58	0.16
Developer (98 to 112 d)												
BW	983±37	960±52	830±43	951±61	943±62	910±52	891±80	918±30	915±55	960±48	948±63	961±46
FI	43±3	41±5	39±4	40±5	39±3	39±4	40±4	39±4	39±4	41±4	40±5	38±4
NI	760±70	708±103	816±79	764±101	725±58	744±90	803±70	737±79	732±60	732±91	725±77	682±81
ND	374±40	271±86	273±93	319±87	332±91	309±83	374±68	321±78	305±99	324±93	345±84	282±100
NR	729±40	626±86	628±93	674±87	687±91	664±83	709±68	676±78	660±99	679±93	700±84	637±100

<i>b</i> (10⁶)	77 ^a ±5.6	60 ^b ±4.9	59 ^b ±6.5	61 ^b ±12.7	63 ^b ±6.8	59 ^b ±8.0	62 ^b ±7.7	63 ^b ±4.7	35 ^c ±7.1	70 ^b ±7.4	71 ^b ±3.2	66 ^b ±7.9
<i>bc</i>⁻¹		24.7±2.0	29.8±3.99	117.6±22.3	35.8±4.1	23.1±3.0	29.8±3.9	32.5±2.5	20.2±2.3	19.1±2.4	22.3±1.1	70.6±8.4
<i>c</i>		2.43	1.98	0.52	1.76	2.6	2.08	1.94	1.73	3.66	3.19	0.94
AAR		0.33	0.22	0.07	0.23	0.36	0.28	0.26	0.43	0.44	0.41	0.13

*significant at 5% by Dunnett test. BD=balanced diet, BW=Body weight (g), FI=feed intake (g/d), NI=nitrogen intake (mg/BW_{kg}^{0.67}/d), ND=nitrogen deposition (mg/BW_{kg}^{0.67}/d), NR=nitrogen retention (mg/BW_{kg}^{0.67}/d), b= slope of the exponential function (efficiency of protein), bc⁻¹= efficiency of AA utilization, c= concentration of the amino acid in the dietary protein (g/100gCP).

Discussion

The aim of this study was to predict the dietary IAAR for pullets in growth phase, considering the quantitative performance data, AA efficiencies and AA requirements observed with the individual dilutions of the dietary amino acids based on the combination of the deletion method, Goettingen approach and Louvain approach. The Goettingen approach allows to determine the amino acid requirement using non-linear models considering the sex, age and genotype (Samadi & Liebert, 2006b; 2007a, b; Wecke & Liebert, 2009), while the Louvain method can be used to determine the IAAR between the essential amino acids in one set of experiments using the same birds and same reference diet.

The birds in developer period presented lower feed intake in relation to the indicated by the lineage guideline. Cadirci et al. (2009) attributed the lower intake to the increase in heat production resulting from the deamination process of other amino acids that are not being used for protein synthesis due to amino acid deletion and this could be one of the explanations for the reduced feed intake observed in the present study.

On the other hand, the use of a semi-purified diet with an ideal profile of amino acids and highly digestible ingredients resulted in higher protein efficiency in all evaluated periods. The reduction in *b*-values of the limiting diets relative to the balanced diet validates the study, indicating that there was a limitation of the amino acids tested (Wecke & Liebert, 2013).

The ideal ratio obtained from the Goettingen approach and Louvain approach are very close to the profile recommended in the Brazilian Tables for Poultry and Swine (Rostagno et al., 2017) and the guideline of Hy-line W36 (2016), except for Met + Cys, Thr, Ile, Gly + Ser and Phe + Tyr in the starter and grower phases by Goettingen approach and Met + Cys in the developer phases and Gly + Ser in all phases by Louvain approach (Table 6). However, there is few information on the amino acid ideal profile for pullets.

Table 6. Ideal amino acid ratio for pullets observed in the literature

AA	Starter				Grower				Developer			
	GA ¹	LA ²	BTPS ³	W36 ⁴	GA	LA	BTPS	W36	GA	LA	BTPS	W36
Lysine	100	100	100	100	100	100	100	100	100	100	100	100
Methionine+Cystine	76	74	74	75	85	80	80	76	83	67	82	85
Tryptophan	17	18	18	18	22	20	20	19	21	22	22	20
Threonine	76	66	67	67	66	68	68	68	69	69	69	68
Arginine	102	109	107	107	109	109	107	107	107	110	108	107
Valine	75	77	76	74	81	79	79	78	83	83	80	80
Isoleucine	65	69	71	72	68	75	76	74	76	77	77	75
Leucine	106	112	112	-	122	115	118	-	122	126	125	-
Phenylalanina+Tyrosine	115	119	121	-	124	125	125	-	129	129	130	-
Glycine+Serine	84	78	125	-	131	134	115	-	111	123	106	-
Histidine	35	37	37	-	35	38	38	-	35	39	39	-

¹GA=Goettingen approach; ²LA=Louvain approach; ³BTPS=Brazilian Tables for Poultry and Swine; ⁴W36=Guideline of Hy-line W36.

The ideal ratio determined by the Goettingen approach of 77% for Thr:Lys for starter phase and 85% for Met + Cys:Lys for grower phase were on average 15% higher than the recommendation in the literature. By the Louvain approach was determined the ratio of 67 for Met + Cys:Lys for developer phase, this result is on average 24% lower than found by other authors. In a typical poultry diet based on corn and soybean meal, methionine is the first limiting amino acid. The methionine requirements are usually considered in combination with cystine, however, it is important to highlight that cystine was not provided in the diets in the present study. According to Leeson & Summers (2008), 25% of cystine and 2% of dietary methionine are exclusively used for the synthesis of keratin, while cystine directly participates in this process and most part of methionine acts indirectly through the endogenous conversion into cystine. Methionine and cystine present a high requirement during periods of higher feather formation, such as in the initial stage of bird's growth and in molting times (Wecke et al., 2018) which may explain a higher Met + Cys:Lys. One molecule of serine is required to synthesize one molecule of cysteine from one molecule of methionine when L-homocysteine is

metabolized to L, L-cystathione (Siegert et al., 2015) justifying the values found to Gly+Ser:Lys.

Differences can be observed for Gly + Ser:Lys determined by the Goettingen approach for starter and grower periods in relation to the recommendation made by Rostagno et al (2017), which presented opposite pattern in the periods studied. In the starter period the ideal ratio was 81% for Gly + Ser:Lys, being 35% smaller than the ratio recommended by Rostagno et al (2017), while in the grower phase the ideal ratio obtained for Gly + Ser:Lys was 14% higher. The same behavior was observed in determining the ideal ratio by Louvain approach, including in the developer phase. Glycine participates in the synthesis of many physiologically important molecules, including purine nucleotides and glutathione (Kim et al. 2007). Thus, glycine is essential for the proliferation and antioxidative defense of leucocyte and plays a role in regulating the production of cytokines by leucocytes and immune function (Zhong et al. 2003). The requirement of glycine is an age-dependent factor and adult birds can synthesize all the glycine necessary for tissue synthesis, whereas young birds only do so partially (Graber & Baker, 1973). Lack of glycine compromises the formation of uric acid, creatinine, and tissue synthesis (Ngo et al., 1977). As with Met + Cys and Phe + Tyr, the requirement of glycine is considered in combination with serine, however, no serine was given in diets. In the initial phases, in diets based on vegetable sources, the Gly + Ser plays an important role in the order of limitation, since these amino acids are hardly provided in sufficient quantities (Wang et al., 2013).

As lysine, threonine is an indispensable amino acid for body protein deposition and growth. Thus, deficiency in threonine affects the utilization of dietary lysine and consequently animal growth. The higher Thr:Lys ratio found in the present study for starter phase may be explained by the higher protein deposition, since the animals are growing. Besides its utilization

for protein synthesis, threonine is involved in other biological functions such maintenance of the body protein turnover, formation of uric acid, contributes in the formation of collagen and elastin and acts in the production of antibodies and mucins. Therefore, threonine requirement varies depending on the relative importance of these different functions, additionally, endogenous losses are very rich in threonine (Donato, 2013).

The ideal ratio determined in this study of the 64% for Ile: Lys, 101% for Leu:Lys for starter phase and 67% for Ile: Lys for grower phase are on average 11% lower than the recommendations of the Hy-line W-36 guideline and Rostagno et al. (2017). The antagonism between the branched chain amino acids (BCAAs) is known and the excess of them, mainly of the leucine, can cause reduction of consumption and performance (Rocha et al., 2013). Although there was unbalance between valine, leucine and isoleucine deficient diets, the feed intake of bird did not change. As the sulfur amino acids, the BCAAs are also pointed out as essential to the process of feather formation. Wilson et al. (2009) reported that BCAAs are the main agents involved in the stimulation of protein synthesis in muscles, leucine is the most effective (Vary & Lynch, 2007). Considering the different results in this study in comparison to the literature for the ideal ratio of Ile:Lys and Leu:Lys and the importance of the antagonism between BCAAs, it is necessary further studies.

The differences between the ideal amino acid ratio recommendations reflects the differences in how they were determined, the requirements available in the literature were estimated from data compiled from a variety of experiments and therefore influenced by genetic, environmental, bird age, and different experimental conditions and basal diets. Thus, the ideal amino acid profile recommended by Rostagno et al. (2005, 2011, 2017) and by the breeder guidelines (Hy-line W36, 2016) is subject to criticism. Baker (2003) suggests that the

ideal amino acid profile should be determined in just one experiment using the same basal diet and poultry in the same period to ensure a valid measure.

In this study, the ideal ratio of essential amino acids was determined in a single assay based on the deletion method by two different approaches, providing more accurate values by minimizing the effects of the environment, diet and genotype. The recommendations given were very similar to those presented in the literature, the use of semi-purified diets and high digestibility ingredients with the ideal balanced ratio provided higher protein efficiency. Due to limited information on the ideal profile of essential amino acids for growing pullets, further studies are necessary to validate the determined IAAR with performance assays.

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CAPÍTULO 4 - Optimal in-feed amino acid ratio for laying hens based on deletion method

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Optimal in-feed amino acid ratio for laying hens based on deletion method

Running title: Optimal in-feed amino acid ratio for laying hens

Letícia Soares¹, Nilva Kazue Sakomura¹, Juliano Cesar de Paula Dorigam², Frank Liebert³, Mariana Quintino do Nascimento¹, Bruno Balbino Leme¹.

¹Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Jaboticabal, São Paulo, Brazil,

²Department of Animal Nutrition Research, Evonik Industries, Hanau, Wolfgang, Germany.

³Chair of Animal Nutrition, University of Göttingen, Kellnerweg, Göttingen, Germany.

Correspondence: L. Soares¹, Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Jaboticabal, São Paulo, Brazil, Via de Acesso Prof. Paulo Donato Castelane, s/n. 14884-900 - Jaboticabal, SP, Brazil. E-mail: leticia.fcav@gmail.com.

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SUMMARY

A total of 56 Hy-line W-36 hens from 28 to 30 weeks were used on nitrogen balance (NB) trial to estimate daily N maintenance requirements (NMR) and the genetic potential for daily N retention ($NR_{max}T$). The treatments consisted of six graded levels of nitrogen in the diets (N1=8; N2=16; N3=24; N4=32; N5=40 and N6=48 g N /kg of feed), formulated using the dilution technique. The regression analyses between nitrogen intake and excretion were performed to fit the exponential function and to determine the $NMR=292 \text{ mg/BW}_{kg}^{0.67}$, was applied for further calculation of $NR_{max}T=1883 \text{ mg/BW}_{kg}^{0.67}$. A second NB trial was conducted, a total of 96 Hy-line W-36 hens were used in the same period to estimate the ideal amino acid ratio (IAAR). Twelve treatments with eight replicates and one bird per cage were used. A balanced diet (BD) was formulated to meet the IAAR and the requirement of other nutrients for pullets. The limiting diets were formulated diluting BD with corn starch and refilled with synthetic AAs and other feed ingredients, except for the AA under study. In each trial, the data of nitrogen intake, excretion, deposition and retention were obtained in a NB trial. The IAAR determined by Goettingen approach were Lys 100, Met+Cys 88, Trp 21, Thr 69, Arg 109, Val 90, Ile 75, Leu 127, Phe+Tir 110, Gly+Ser 73 and His 29%. The IAAR determined by Louvain approach were Lys 100, Met+Cys 88, Trp 21, Thr 69, Arg 104, Val 91, Ile 78, Leu 121, Phe+Tir 119, Gly+Ser 77 and His 29%.

Introduction

During the last decades, the productivity of laying hens has improved significantly; with higher number of eggs produced over the laying cycle and higher efficiency to utilize feed nutrients. This increase in productivity implies in a change in the requirements and consequently these hens become more demanding in nutrients, mainly, amino acids (Elliot, 2008). Optimization of protein supply by understanding the amino acid needs of these hens requires a thorough understanding of the metabolic utilization of proteins and amino acids to provide a better feeding program that suits better various environmental conditions and health status.

The efficiency of utilization of the dietary protein provided is dependent on the amount, composition and digestibility of its amino acids, which are required at specific levels by hens. According to the physiological needs, the absorbed amino acids (AA) should be available for protein synthesis of tissues, due to variation of individual tissue growth, age and performance the ideal ratio required for maintenance and performance undergoes changes (Wecke & Liebert, 2013). In the case of hens, this becomes more important since these birds will prioritize egg production rather than the synthesis of other body protein compounds.

For the purpose of modelling the protein utilization in birds, some researchers have been using non-linear models (Samadi & Liebert, 2007a). In these studies, the asymptote of the exponential equation is frequently associated to the maximum nitrogen retention ($NR_{max}T$) while the intercept is associated with the nitrogen requirement for maintenance (NMR). As $NR_{max}T$ is determined by the asymptotic value of the exponential function, the concept of "theoretical" is assigned to this parameter, since this value is not observed in practical conditions of production (Samadi & Liebert, 2007a). The NMR is obtained by the value that intercepts the y-axis of an exponential function between nitrogen excreted and ingested when

considering a nitrogen intake equal to zero. Once these two parameters have been estimated for a given genotype, it can be used in mathematical models to determine the efficiency of utilization and requirement of amino acids.

The same researchers developed a way to determine the ideal amino acid ratios using the efficiency of utilization of the amino acids studied (Wecke & Liebert, 2013; Pastor et al., 2013; Dorigam et al., 2015; Dorigam et al., 2017). In addition, this procedure combines the concepts involved in the deletion method proposed by Wang & Fuller (1989) in which is possible to determine the ideal ratio of amino acids through the changes in nitrogen retention with the individual amino acid deletions. Another approach can be applied from the deletion method to obtain the ideal amino acid ratio was proposed by Rollin et al. (2003) based on an equation to determine the requirements of amino acids. 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).

Although the concept of ideal protein is widespread, some aspects still need to be improved. Updating the amino acid requirements for laying hens does not follow the same rate of development as those observed for broiler, and therefore, there is not so much information available to recommend or to conclude about the most appropriate ratio of amino acids for egg production in laying hens. Thus, the aim of this study was to determine the IAAR of essential amino acid in diets (Methionine + Cystine, Threonine, Tryptophan, Arginine, Valine, Isoleucine, Leucine, Phenylalanine + Tyrosine, Glycine + Serine and Histidine) for laying hens in peak of egg production (Hy-line W36) making use of efficiency data calculated by the model parameters NMR and $NR_{max}T$ and the amino acid requirement proposed by Rollin et al, 2003.

Material and Methods

This study was approved by the Ethics Committee on Animal Use of the Faculty of Agriculture and Veterinary Sciences, UNESP, Jaboticabal (n° 9999/14).

Two nitrogen balance assays were conducted at the Laboratory of Poultry Science of the Faculty of Agriculture and Veterinary Sciences of Jaboticabal, São Paulo, Brazil. The assay 1 was performed to determine the nitrogen maintenance requirement (NMR) and the theoretical maximum nitrogen retention (NR_{maxT}), and the assay 2 was performed to determine the efficiency of amino acid utilization and an ideal amino acid ratio (IAAR) for Hy-line W-36 laying hens.

Assay 1: Determination of the NMR and NR_{maxT}

Bird housing and experimental design

Fifty-Six Hy-line W36 laying hens were individually allotted in a completely randomized design with six treatments and eight replicates. The birds were housed in metabolic cages with wire floors, individual galvanized feeders and nipple drinkers installed in a facility with a negative-pressure ventilation system with controlled humidity and temperature. The management of the laying hens and the lighting program followed the breeder's guidelines (Hy-line W-36 Management Guide, 2016).

Experimental diets

The treatments consisted of six graded levels of nitrogen in the diets (L1=8; L2=16; L3=24; L4=32; L5=40 and L6=48 g N /kg of feed), formulated using the dilution technique (Fisher & Morris, 1970). The dilution technique consisted in formulating two diets, one summit diet (L6) with high content of protein and another dilution diet (L0) free of protein and amino acids. The diet L6 was formulated using soybean meal, corn gluten meal and industrial amino

acids to meet the dietary concentration of 48 g N /kg of feed. The L0 diet was formulated to meet the same nutritional level as the N6 diet, except for protein and amino acids. The composition of the diets is presented in Table 1. The experimental diets were obtained by diluting the L6 diet with the L0 diet, in the adequate proportion to obtain the intermediary levels of nitrogen while keeping the same essential amino acid ratios between the diets. The proportions of the high protein diet (L6) to protein-free diet used in the preparation of the experimental diets were 15:85 for L1, 32:68 for L2, 49:51 for L3, 66:34 for L4, 83:17 for L5, and 100:0 for L6. The nutritional composition of the diets is presented in Table 2.

Table 1. Composition of the basal diets used for laying hens from 28 to 30 weeks of age

Ingredients (%)	Summit (L6)	Dilution (L0)
Corn	43.811	-
Soybean meal 45%	23.544	-
Corn gluten meal (60%)	17.000	-
Rice husk	-	10.951
Corn starch	-	64.206
Sugar	-	10.000
Soybean oil	2.167	2.131
Dicalcium phosphate	1.144	1.622
Limestone	9.721	9.348
L-Lysine HCl (78%)	0.968	-
DL-Methionine (99%)	0.599	-
L-Threonine (99%)	0.381	-
Salt	0.450	0.536
Sodium bicarbonate	0.115	-
Potassium chloride	-	1.106
Min. and vit. supplement ¹	0.100	0.100

¹kg of product: folic acid 2400.000 mg; pantothenic acid 30.00 g; biotin 160.00 mg; butylated hydroxytoluene 100.00 mg; niacin 84.00 g; selenium 600.00 mg; Vit A 20000000,0 UI. Vit B1 5000.00 mg; Vit B12 36000.00 mcg; Vit B2 1300 g; Vit B6 7000,00 mg; Vit D3 5000000,00 UI; Vit E 37500,00 UI; Vit K3 4000,00 mg; Mn 150,00 g; Zn 140,00 g; Fe 90,00 g; Cu 15 g; I 15,00 mg.

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

Nutrients (%)	Summit (L6)	Dilution (L0)
Crude protein	29.873	
AMEn (Mcal/kg)	2.900	2.900
Crude fiber ^a	2.207	4.337
Calcium ^a	4.020	4.020
Sodium ^a	0.225	0.225
Potassium ^a	0.580	0.580
Av. Phosphorus ^a	0.300	0.300
SID amino acid ^b		
Lysine	1.610	
Arginine	1.722	
Histidine	0.544	
Gly+Ser	2.141	
Isoleucine	1.145	
Leucine	2.578	
Met+Cys	1.352	
Methionine	0.788	
Threonine	1.128	
Phenylalanine	1.193	
Phe+Tyr	2.314	
Tryptophan	0.338	
Valine	1.450	

^a Calculated composition using data from the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011), ^b Analyzed composition of the amino acids in the experimental diets. were calculated using Rostagno et al. (2011) true digestibility coefficients.

Assay 2: Determination of the efficiency of amino acid utilization and ideal amino acid ratio

Bird housing and experimental design

Ninety-Six Hy-line W-36 laying hens were individually allotted in a completely randomized design with 12 treatments and eight replicates. The hens were housed in same conditions described for assay 1.

Experimental diets

The treatments consisted of a balanced diet (BD) and eleven different diets deficient in one of the amino acid test (lysine, methionine + cystine, threonine, valine, isoleucine, arginine, tryptophan, leucine, histidine, glycine + serine and phenylalanine + tyrosine).

The BD was formulated to properly meet the nutritional requirements and the ideal amino acid ratio (IAAR) provided by the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011) for laying 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 amino acids (Table 3).

To formulate the deficient diets, the BD was diluted with corn starch to meet 45% of amino acid requirement. The diets were supplemented with vitamin, mineral, fiber, energy and industrial amino acids to achieve the same levels of the BD, except for the amino acid to be evaluated in the treatment, which was deficient in 55% (Table 4). In the growth phases, the diets were formulated to meet 70% of amino acid requirements while in the egg production to meet 45%, this difference is since laying hens are bird that are not in developing and have a great body fat reserve, these factors influence in the metabolism of the birds and a greater deficiency of amino acids is required to express response in a short period.

The nutritional composition of the diets is presented in Table 5.

Table 3. Composition of balance diets used for laying hens from 28 to 30 weeks of age

Ingredients (%)	Balanced diet
Corn	58.000
Soybean meal	1.192
Corn gluten meal (60%)	3.000
Rice husk	5.250
Limestone	8.810
Dicalcium phosphate	2.491
Soybean oil	1.608
Sugar	10.138
Salt	0.408
Potassium chloride	0.736
L-Alanine (96%)	4.790
L-Lysine (78%)	0.766
DL-Methionine (99%)	0.399
L-Arginine (99%)	0.554
L-Valine (98%)	0.399
L-Threonine (99%)	0.339
L-Isoleucine (99%)	0.376
L-Leucine (98%)	0.115
L-Phenylalanine (99%)	0.364
L-Tryptophan (98%)	0.126
L-Histidine (99%)	0.038
Min. and vit. supplement ¹	0.100

¹kg of product: folic acid 2400.000 mg; pantothenic acid 30.00 g; biotin 160.00 mg; butylated hydroxytoluene 100.00 mg; niacin 84.00 g; selenium 600.00 mg; Vit A 20000000,0 UI. Vit B1 5000.00 mg; Vit B12 36000.00 mcg; Vit B2 1300 g; Vit B6 7000,00 mg; Vit D3 5000000,00 UI; Vit E 37500,00 UI; Vit K3 4000,00 mg; Mn 150,00 g; Zn 140,00 g; Fe 90,00 g; Cu 15 g; I 15,00 mg.

Table 4. Composition of the experimental diets with individual amino acid deletion used for laying hens from 28 to 30 weeks of age

Ingredients (%)	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Balanced diet	44.969	45.000	44.970	45.000	45.006	45.007	45.000	45.000	45.000	45.000	44.978
Corn starch	31.869	31.872	31.820	31.912	31.399	31.775	31.775	31.737	31.806	31.870	31.800
Rice husk	6.115	6.111	6.114	6.111	6.111	6.110	6.111	6.111	6.111	6.111	6.114
Limestone	4.726	4.724	4.726	4.724	4.723	4.723	4.724	4.724	4.724	4.724	4.726
Dicalcium phosphate	1.487	1.487	1.487	1.487	1.486	1.486	1.486	1.486	1.486	1.486	1.487
Potassium chloride	0.609	0.608	0.609	0.608	0.608	0.608	0.608	0.608	0.608	0.608	0.608
Soybean oil	1.249	1.295	1.221	1.173	1.210	1.323	1.349	1.452	1.466	1.081	1.162
Salt	0.233	0.232	0.233	0.232	0.232	0.232	0.232	0.232	0.232	0.232	0.232
L-Alanine (96%)	5.043	4.789	4.652	4.816	5.443	4.882	4.807	4.940	4.834	4.981	4.760
L-Leucine (98%)	0.551	0.551	0.551	0.551	0.551	0.551	0.551	0.000	0.551	0.551	0.551
L-Phenylalanine (99%)	0.528	0.528	0.528	0.528	0.528	0.528	0.528	0.528	0.000	0.528	0.528
L-Arginine (99%)	0.479	0.479	0.479	0.479	0.000	0.478	0.478	0.478	0.478	0.478	0.478
L-Valine (98%)	0.407	0.407	0.407	0.407	0.407	0.000	0.407	0.407	0.407	0.407	0.407
DL-Methionine (99%)	0.379	0.000	0.379	0.379	0.379	0.379	0.379	0.379	0.379	0.379	0.379
L-Lysine (78%)	0.000	0.562	0.562	0.562	0.562	0.562	0.562	0.562	0.562	0.562	0.562
L-Threonine (99%)	0.323	0.323	0.323	0.000	0.323	0.323	0.323	0.323	0.323	0.323	0.323
L-Glycine (96%)	0.355	0.355	0.355	0.355	0.355	0.355	0.355	0.355	0.355	0.000	0.355
L-Isoleucine (99%)	0.353	0.353	0.353	0.353	0.353	0.353	0.000	0.353	0.353	0.353	0.353
L-Histidine (99%)	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.000
L-Tryptophan (98%)	0.095	0.095	0.000	0.095	0.095	0.095	0.095	0.095	0.095	0.095	0.095
Min. and vit. supplement ¹	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100

¹kg of product: folic acid 2400.000 mg; pantothenic acid 30.00 g; biotin 160.00 mg; butylated hydroxytoluene 100.00 mg; niacin 84.00 g; selenium 600.00 mg; Vit A 20000000,0 UI. Vit B1 5000.00 mg; Vit B12 36000.00 mcg; Vit B2 1300 g; Vit B6 7000,00 mg; Vit D3 5000000,00 UI; Vit E 37500,00 UI; Vit K3 4000,00 mg; Mn 150,00 g; Zn 140,00 g; Fe 90,00 g; Cu 15 g; I 15,00 mg.

Table 5. Nutritional composition of the experimental diets with individual amino acid deletions used from 28 to 30 weeks

Nutrients (%)	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
Calcium ^a	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
Chloride ^a	0.709	0.709	0.709	0.709	0.709	0.709	0.709	0.709	0.709	0.709	0.709	0.709
AMEn (Mcal/kg)	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900	2.900
Crude fiber ^a	4.400	4.400	4.400	4.400	4.400	4.400	4.400	4.400	4.400	4.400	4.400	4.400
Av. phosphorus ^a	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500
Potassium ^a	0.580	0.580	0.580	0.580	0.580	0.580	0.580	0.580	0.580	0.580	0.580	0.580
Sodium ^a	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180
Crude protein	15.92	15.71	15.64	15.96	16.06	16.12	15.75	16.10	15.79	15.88	16.09	16.17
SID amino acid ^b												
Arginine	0.876	0.875	0.863	0.863	0.876	0.381	0.878	0.870	0.872	0.868	0.871	0.886
Phenylalanine	0.847	0.840	0.836	0.832	0.835	0.843	0.840	0.844	0.835	0.315	0.837	0.833
Phe+Tyr	0.954	0.945	0.948	0.946	0.959	0.947	0.940	0.951	0.948	0.424	0.947	0.959
Glycine + Serine	0.627	0.627	0.628	0.622	0.628	0.625	0.623	0.620	0.620	0.615	0.283	0.617
Histidine	0.237	0.229	0.239	0.237	0.230	0.237	0.222	0.239	0.237	0.239	0.223	0.103
Isoleucine	0.638	0.634	0.640	0.636	0.639	0.628	0.635	0.290	0.639	0.634	0.629	0.631
Leucine	0.988	0.997	0.981	0.985	0.979	0.985	0.993	0.990	0.441	0.972	0.990	0.988
Lysine	0.808	0.363	0.817	0.812	0.800	0.804	0.811	0.805	0.800	0.801	0.813	0.810
Methionine + Cystine	0.683	0.671	0.307	0.674	0.681	0.689	0.679	0.676	0.668	0.683	0.670	0.672
Methionine	0.625	0.621	0.247	0.635	0.626	0.623	0.631	0.637	0.623	0.627	0.618	0.622
Threonine	0.576	0.555	0.568	0.566	0.257	0.565	0.556	0.552	0.574	0.572	0.565	0.570
Tryptophan	0.171	0.164	0.169	0.077	0.165	0.165	0.164	0.171	0.174	0.169	0.166	0.165
Valine	0.731	0.733	0.730	0.722	0.736	0.718	0.328	0.725	0.730	0.717	0.732	0.719

BD=balanced diet, ^a Calculated composition using data from the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011), ^b Analyzed composition of the amino acids in the experimental diets were calculated using Rostagno et al. (2011) true digestibility coefficients.

Experimental procedures

The selection and distribution of the laying hens in each experimental unit were performed according to body weight and egg production, based on recommendations of Sakomura & Rostagno (2016). To control the differences in the egg production and to confirm that all hens were laying eggs regularly, it was performed an individual control of the egg production during 14 days before starting the trial.

Each experimental period was divided into an adaptation period (5 days) and a period with total excreta and egg collection (10 days) to avoid that the birds were submitted to deficient diets for an extended period. The hens were weighed at the start and at the end of the experiment to measure body weight. The leftovers were weighed to measure daily feed intake. The excreta were collected, stored in plastic pots and weighed at the end of collection period to measure total excreta production. The egg production and egg weight were measured daily to calculate egg mass production. The egg mass was calculated by multiplying the egg weight by the egg production and then multiplied by the nitrogen content in the egg to calculate the amount of nitrogen deposited in egg mass (NEM). The NEM value is an important part of total N retention that should be considered in further estimation of the genetic potential as well as the utilization efficiency of dietary protein and amino acids for commercial laying hens. (Dorigam et al., 2016 and Dorigam et al., 2017)

Chemical analyses

The excreta and egg samples collected from each experimental unit were homogenized; an aliquot was removed and weighed in small plastic pots to freeze at -20°C. Later, the samples were freeze-dried during a period of 72 hours at -80°C under 800 mbar of pressure (Edwards® 501 Modulyo freeze drier, West Sussex, United Kingdom). After the lyophilization, the dried samples were weighed to quantify the dry matter content. Subsequently, the dried samples were

milled using a micro mill (IKA® A11 Basic Analytical Mill, Staufen, Germany). The nitrogen content of the diets, excreta and eggs were analyzed in a nitrogen distiller (Kjeltec™ 8400 Foss, Foss, Hillerod, Denmark) using the Kjeldahl method (AOAC, 2005: method 2001.11). The total amino acid contents of the ingredients and the experimental diets were analyzed by Evonik Industries using near-infrared spectroscopy (NIRs) and high-performance liquid chromatography (HPLC), respectively.

Statistical analyses

Assay 1: Determination of the NMR and $NR_{max}T$

The nitrogen balance data was submitted to ANOVA using the general linear model (GLM) and then adjusted to an exponential model using PROC NLIN procedure in SAS (Statistic Analysis System, 9.1). The regression analyses between nitrogen intake (NI, mg/BW_{kg}^{0.67}/day) and nitrogen excretion (NEX, mg/BW_{kg}^{0.67}/day) were performed to fit the exponential function and to determine the nitrogen maintenance requirement (NMR):

$$NEX = NMR \times e^{b \times NI} \quad [1]$$

where NMR is the nitrogen maintenance requirement (mg/BW_{kg}^{0.67}/day), b is the slope of the exponential curve and e is the base number of the natural logarithm ($\ln$). The NMR was estimated by considering the intercept of the curve on the y-axis (NEX) when NI=0.

The nitrogen deposition (ND, mg/BW_{kg}^{0.67}/day) was determined by the difference between NI and the NEX. The nitrogen retention (NR, mg/BW_{kg}^{0.67}/day) represents the total nitrogen utilization by the hen it was obtained by the sum of ND, NEM and NMR (Sünder et al., 2010).

According to Samadi & Liebert (2007), the regression analyses between NI and NR was used to adjust the exponential model:

$$NR = NR_{max}T \times (1 - e^{-b \times NI}) \quad [2]$$

where $NR_{max}T$ is the maximum theoretical nitrogen retention ($mg/BW_{kg}^{0.67}/day$). The $NR_{max}T$ 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. This is an important parameter within the modeling procedure to derive AA requirements for defined graded levels to make use of the theoretical maximum within the scope of practical data.

Assay 2: Determination of the efficiency of amino acid utilization and ideal amino acid ratio

The nitrogen balance data were analyzed by a one-way ANOVA using a GLM procedure in SAS (Statistical Analysis System, version 9.1). Significant differences were tested using Dunnett's test and the p-values <0.05 were deemed statistically significant. The ideal amino acid ratio was determined from the limiting diets where the test amino acid was reduced in 55%; the protein quality (b) in each treatment was obtained by the equation of Samadi & Liebert (2008):

$$b = \ln(NR_{max}T) - \ln(NR_{max}T - NR)/NI \quad [3]$$

where NR is the nitrogen retention ($mg/BW_{kg}^{0.67}/day$), NI is nitrogen intake ($mg/BW_{kg}^{0.67}/day$) and $NR_{max}T$ is the maximum theoretical nitrogen retention ($mg/BW_{kg}^{0.67}/day$) determined in assay 1.

The dietary protein quality (b) is linearly dependent on the concentration of the limiting amino acid in the feed protein (c). The calculation of c was made by the equation:

$$c = 16 \times LAAI/NI \quad [4]$$

where LAAI is the amino acid test intake and NI is the nitrogen intake. It is possible to compare the model parameters bc^{-1} of an individual AA directly. The ratio between lysine efficiency (reference) and the efficiency of the limiting amino acid under study is utilized to derive IAAR, according to the calculation (Wecke & Liebert, 2013, Dorigam, 2016 and Dorigam et al., 2016):

$$IAAR = bc^{-1}_{Lys}/bc^{-1}_{AA} \quad [5]$$

Determination of the amino acid requirement and ideal amino acid ratio (Rollin et al., 2003)

The amino acid requirement (g/kg DM) was determined using the following equation proposed by Rollin et al. (2003):

$$\text{Requirement} = (EAA)_{BD} \times \left(2 - DEL \left(\frac{ND_{EAA}}{ND_{BD}} \right) \right) \quad (1)$$

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).

Results

Assay 1: Determination of the NMR and $NR_{max}T$

The results obtained from the N balance trials with Hy-line W-36 laying hens from 28 to 30 weeks are presented in Table 6.

Table 6. Summarized results of nitrogen balance trial with Hy-line W-36 laying hens receiving graded levels of protein (% crude protein) in peak of egg production

Parameters	L1 (5%CP)	L2 (10%CP)	L3 (15%CP)	L4 (20%CP)	L5 (25%CP)	L6 (30%CP)	<i>P-value</i>
BW (g)	1296±77	1308±84	1314±65	1305±75	1324±61	1308±48	<i>ns</i>
FI (g/d)	67±12	63±14	62±12	79±11	71±10	72±3	<i>ns</i>
NI (mg/BW _{kg} ^{0.67} /d)	348±55	725±87	1303±67	2108±108	2405±98	2730±130	***
NEX (mg/BW _{kg} ^{0.67} /d)	352±79	543±61	972±115	1377±65	1800±97	2256±99	***
NEM (mg/BW _{kg} ^{0.67} /d)	48±13	194±33	565±54	720±41	738±39	675±50	***
ND (mg/BW _{kg} ^{0.67} /d)	44±11	376±53	897±57	1451±93	1343±105	1065±28	***
<i>b value</i> (10 ⁶)	621±79	627±75	652±99	595±17	634±23	617±51	<i>ns</i>

BW - body weight, FI – feed intake, NI – nitrogen intake, NEX – nitrogen excretion, NEM – nitrogen deposited in egg mass, ND – nitrogen deposition.

ns – not significant.

*** - $p < 0.001$.

Increasing levels of protein in experimental diets did not influence BW, FI and *b value* ($P > 0.05$), however, as the dietary protein diets becomes higher, there was increase in NI, NEX, NEM and ND in evaluated period. It was observed a marked increase from L1 to L5 for NEM and from L1 to L4 for ND, after these levels a decrease in values could be observed.

After fitting the exponential function between nitrogen intake and nitrogen excretion during a gradual increase in the protein supplied in peak of egg production, it was estimated the NMR of 292 mg/BW_{kg}^{0.67} from 28 to 30 weeks, as shown in figure 1.

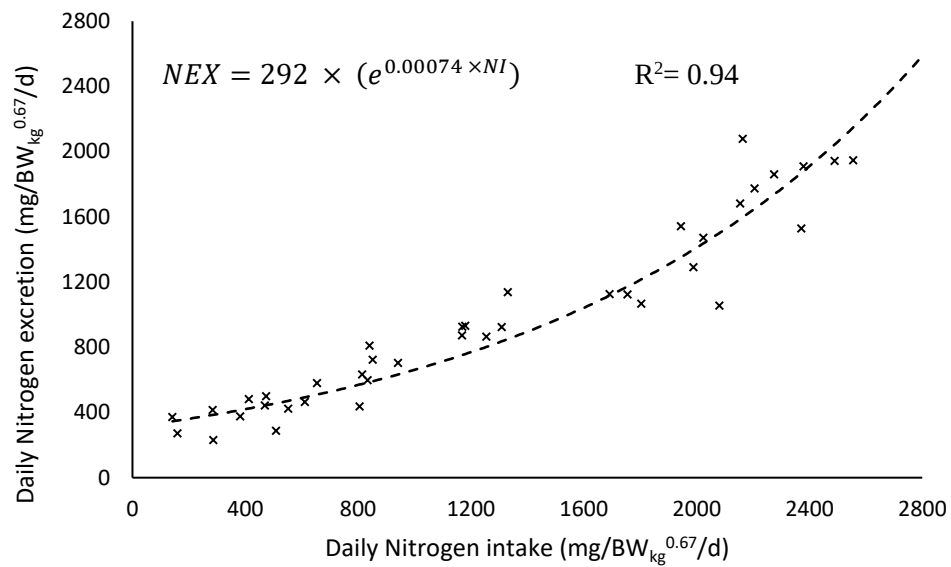


Figure 3. Estimation of the daily nitrogen requirement for maintenance (NMR) by fitting the exponential function between nitrogen intake (NI) and nitrogen excretion (NEX) for Hy-line W-36 laying hens. Observed (×) and predicted (---) values from 28 to 30 weeks.

The maximum theoretical nitrogen retention was estimated in $1883 \text{ mg/BW}_{\text{kg}}^{0.67}$ for the period evaluated after the fitting of the exponential function between nitrogen intake and nitrogen retention, shown in figure 2.

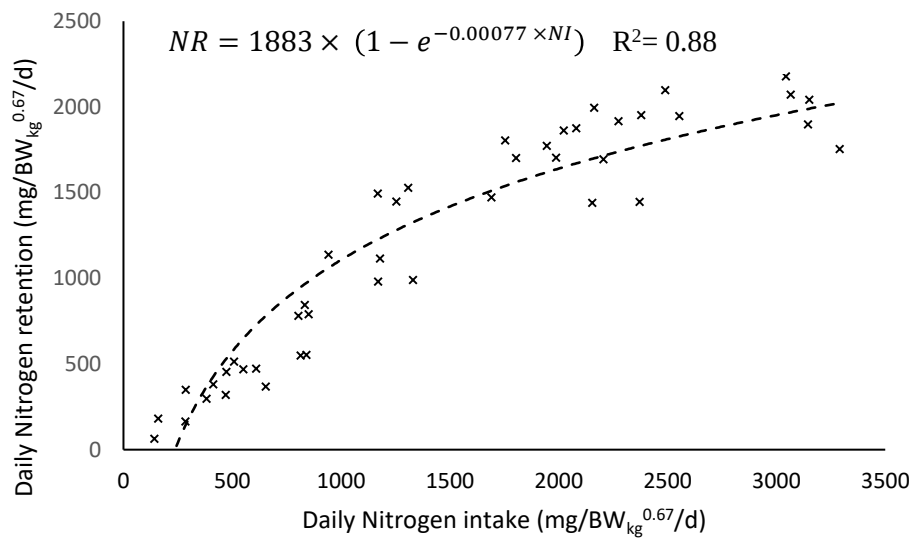


Figure 4. Estimation of the maximum theoretical nitrogen retention ($NR_{\max T}$) for Hy-line W-36 laying hens based on the exponential fitting between nitrogen intake (NI) and nitrogen retention (NR). Observed (×) and predicted (---) values from 28 to 30 weeks.

As demonstrated in the figures 1 and 2, the values to NMR of $292 \text{ mg/BW}_{\text{kg}}^{0.67}$, $NR_{\max T}$ of $1883 \text{ mg/BW}_{\text{kg}}^{0.67}$ and maximum theoretical nitrogen deposition ($ND_{\max T}$) of $1591 \text{ mg/BW}_{\text{kg}}^{0.67}$ for Hy-line W-36 laying hens from 28 to 30 weeks were estimated.

Assay 2: Determination of the efficiency of amino acid utilization, amino acid requirements and ideal amino acid ratio

Summarized results of the N balance experiments are displayed in Table 7. The amino acid deficient diets had lower nitrogen balance data compared to BD. The deletion of essential amino acids reduced nitrogen retention and protein quality in different proportions depending on the deleted amino acid, however did not influenced in the BW, FI and NI.

As expected, deficient diets have lower protein quality, characterized by the decrease in the value of parameter b in relation to BD ($p < 0.05$), thus, confirming the limiting position of the amino acids under study. The leucine, phenylalanine + tyrosine and histidine deficient diet

presented higher *b*-value compared to the other limiting diets, but still lower than the *b*-value of BD. The deletion of arginine and isoleucine significantly reduced the quality of dietary protein in relation to the other limiting diets evaluated.

When evaluating the efficiency of use of amino acids, there is a gradual increase in bc^{-1} values from one period to the next. From the utilization efficiencies and amino acid requirements presented in Table 7 were determine the ideal ratios of the essential amino acid for Hy-line W-36 laying hens from 28 to 30 weeks by Goettingen and Louvain approaches presented in Table 8.

Table 7. Effect of deleting a single amino acid from the diet on protein quality and nitrogen retention of laying hens in N balance trials

Diets	BD	Lys	Met+Cys	Trp	Thr	Arg	Val	Ile	Leu	Phe+Tyr	Gly+Ser	His
28 to 30 weeks												
BW	1329±122	1311±176	1343±138	1346±121	1310±108	1355±152	1351±110	1347±131	1323±149	1312±118	1344±115	1335±150
FI	62±9	61±9	63±11	70±15	63±11	53±6	57±12	58±9	67±13	69±8	60±11	58±9
NI	1266±227	1176±221	1160±149	1261±142	1288±105	1142±139	1219±143	1127±153	1140±128	1280±182	1213±138	1125±163
ND	770±76	593±66	529±36	544±87	572±62	612±45	599±46	639±57	591±22	598±53	591±49	592±45
NR	1062±76	885±66	821±36	836±87	864±62	904±45	891±46	931±57	883±22	890±53	883±49	884±45
<i>b</i> (10⁶)	812 ^a ±59	548 ^b ±44	507 ^b ±19	509 ^b ±36	598 ^b ±57	488 ^b ±70	587 ^b ±42	402 ^b ±33	622 ^b ±52	627 ^b ±31	516 ^b ±48	651 ^b ±65
<i>bc</i>⁻¹		321±23	363±74	1529±74	465±44	294±38	357±29	428±23	253±18	292±39	440±50	1107±98
<i>c</i>		1.71	1.40	0.33	1.29	1.66	1.64	0.94	2.46	2.15	1.17	0.59
AAR		0.673	0.592	0.141	0.464	0.700	0.612	0.525	0.814	0.801	0.518	0.195

*significant at 5% by Dunnett test. BD=balanced diet, BW=Body weight, FI=feed intake, NI=nitrogen intake, ND=nitrogen deposition, NR=nitrogen retention, *b*= slope of the exponential function (efficiency of protein), *bc*⁻¹= efficiency of AA utilization, *c*= concentration of the amino acid in the dietary protein, AAR= amino acid requirement.

Discussion

The aim of this study was to estimate the genetic potential for daily N retention ($NR_{\max T}$) and nitrogen maintenance requirement (NMR) of laying hens in peak of egg production (Hy-line W-36), to estimate the efficiency of utilization and to determine the ideal ratio of amino acids based on the combination of the deletion method and the Goettingen and Louvain approaches.

During the experimental periods (Assay 1 and 2), it was observed that the hens presented lower feed intake in relation to indicated by the Hy-line W36 guideline, probably, due of the severity in the protein and amino acids reduction. This is a common characteristic of this type of study due to the formulation technique used for the experimental diets (Bendezu et al., 2015).

The levels of N used in this study provided responses for NI, NEX and NR that allowed estimation of the coefficients of Goettingen approach. The model parameter b as a measure of dietary protein quality is independent of the level of protein intake (Wecke & Liebert, 2009; Pastor et al., 2013). Thus, when the dietary protein quality remains unchanged, as observed in this study (Table 6), the individual N balance data can be utilized to estimate the threshold value of $NR_{\max T}$ (Liebert, 2015).

The value of $292 \text{ mg/BW}_{\text{kg}}^{0.67}$ for NMR represents the amount N that the birds should minimally consume to compensate for the inevitable metabolic losses that independent of the intake (Samadi & Liebert, 2006a). The maximum theoretical nitrogen retention was estimated in $1883 \text{ mg/BW}_{\text{kg}}^{0.67}$ 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 & Liebert 2006a, b). Studies with the Goettingen approach for laying hens are few (Sünder et al., 2010), but there are already studies with broiler breeder hens (Dorigam et al.,

2017), pullets (Bonato, 2013; Silva et al., 2013) and broilers in growth phase (Samadi & Liebert, 2008; Pastor et al., 2013; 2014; Santos et al., 2014; Daulat et al., 2015 and Wecke et al., 2016).

Dorigam et al. (2017) working with broiler breeder hens of the Ross 308AP[®] genotype in production determined average NMR of 255 mg/BW_{kg}^{0.67}/day and NR_{maxT} of 1597 mg/BW_{kg}^{0.67}/day, these values are, on average, 14% lower than those found in the study and can be attributed to differences in genotype, age, period of production and mainly by the physiological differences of the birds. The broiler breeders differ from commercial laying hens in terms of body growth, voracity, lipid reserves and, above all, the egg laying frequency. This cycle normally completes with the laying of nine eggs (Nonis & Gous, 2013), on the other hand, the broiler breeders do not complete this cycle and present irregular laying, consequently a longer pause period and shorter cycles.

Samadi and Liebert (2006a) determined NMR of 252 mg/BW_{kg}^{0.67}/day for Cobb 500[®] broilers in the period from 10 to 85 days.

Silva et al. (2013) and Bonato (2013) found average NMR of 284 mg/BW_{kg}^{0.67}/day and 307 mg/BW_{kg}^{0.67}/day for Dekalb White[®] pullets in growth phase, respectively. Daulat et al. (2015) determined average NMR of 310 mg/BW_{kg}^{0.67}/day in a trial with naked neck broilers in growth phase, these values are similar to found in this study. However, Wecke et al. (2016), Pastor et al. (2013) and Samadi & Liebert (2008) determined average NMR of 221 mg/BW_{kg}^{0.67}/day, 164 mg/BW_{kg}^{0.67}/day and 240 mg/BW_{kg}^{0.67}/day for Ross 308[®] broilers in the growth and finisher phases, respectively. This variation between the values found can be justified by the use of different ingredients as protein sources.

The values determined to NR_{maxT} it is difficult to compare with the data of other experiments due to variations in strains, age, feed intake and dietary characteristics. However, several studies with animals in growth (Samadi & Liebert, 2008; Pastor, et al. 2013; Dorigam,

et al. 2014; Santos, et al. 2014; Daulat, et al. 2015 and Weck, et al. 2016) presented similar characteristics where a decrease of threshold values with increasing age were observed, indicating that animals at maturity tend to have lower values and that the genetic potential is independent of the diet (Samadi & Liebert, 2008).

The estimation of $NR_{max}T$ and NMR are required for basic applications of the exponential model, as well as to define the genotype in terms of theoretical potential for N deposition, evaluate the value of the dietary protein quality based on the observed efficiency of the limiting amino acid, and determination of amino acid ideal ratio from the efficiency of the dietary amino acid (Liebert, 2017).

The ideal amino acid profile is based on the concept that while amino acid requirements change due to genetic or environmental factors, the relationships between them are slightly affected. Thus, once the amino acid ideal profile is determined, it is sufficient to determine the requirement of a single amino acid and the others can be calculated (Baker, 2003; Dari et al., 2005). The requirements determined by NRC (1994), Leeson & Summers (2005) e Rostagno et al. (2000, 2005, 2011 and 2017) were estimated from a compilation of tests conducted with different diets, periods of production of the birds, strains and environmental conditions and then determined the amino acid ideal profile. Similarly, Coon & Zang (1999) conducted five experiments to determine the amino acid requirements of laying hens and the ideal profile recommended by them is based on the average requirements determined in the five assays.

Bregendahl et al. (2008), followed the recommendations by Baker (2003) that, to ensure an accurate measure of the amino acid ideal profile, it is necessary that the experimental conditions be the same for all amino acid requirement assays. The same authors performed seven experiments, simultaneously, to determine the ideal ratio of methionine, methionine + cystine, threonine, tryptophan, valine, isoleucine e arginine. As in this study, a single basal diet

was used, however the synthetic amino acids were added to ensure that the amino acid tested was the first limiting. The Goettingen approach allows modeling the amino acid requirement considering the sex, age and genotype (Samadi & Liebert, 2006b; 2007a, b; Wecke & Liebert, 2009), so an adequate IAAR can be established from the corrected amino acid requirement. It is important to highlight that the "requirement" determined by the Louvain approach is not used to make recommendations for nutritional levels in the diet, since it should only be used as a reference for the calculation of the ideal amino acid ratio.

The ideal ratio determined by NRC (1994), Coon & Zhang (1999), Leeson & Summers (2005), Bregendahl et al. (2008), Guideline of Hy-line W-36 (2016), Rostagno et al. (2017) and the obtained from the Goettingen and Louvain approaches are presented in Table 8.

Table 8. Ideal amino acid ratio for laying hens based on literature

AA	GA ¹	LA ²	BTPS ³	W36 ⁴	NRC (1994)	Coon & Zhang (1999)	Leeson & Summers (2005)	Bregendahl et al. (2008)
Lysine	100	100	100	100	100	100	100	100
Methionine+Cystine	89	85	98	84	84	81	88	94
Tryptophan	23	21	23	21	23	20	21	22
Threonine	69	71	77	70	68	73	80	77
Arginine	109	105	100	104	101	130	103	107
Valine	90	90	93	88	101	102	89	93
Isoleucine	75	80	78	78	94	86	79	79
Leucine	120	122	122	-	-	-	-	-
Phen+Tyr	110	116	118	-	-	-	-	-
Glycine+Serine	76	79	77	-	-	-	-	-
Histidine	28	28	29	-	-	-	-	-

¹GA= Goettingen approach; ²LA= Louvain approach, ³BTPS= Brazilian Tables for Poultry and Swine; ⁴W36= Guideline of Hy-line W36.

The ideal ratio for Met+Cys:Lys presented larger divergence, the recommendation obtained by Goettingen and Louvain approaches was the same as that determined by Leeson & Summers (2005) and similar recommendations of the Guideline for Hy-line W-36 (2016),

NRC (1994) and Coon & Zhang (1999). The ideal ratios determined by Bregendahl et al. (2008) and Rostagno et al. (2017) are much higher than the other findings in the literature.

The results for Tryptophan:Lys and Arginine:Lys are similar to literature, except the value found by Coon & Zhang (1999) for Arginine:Lys that was on average 25% higher than the others recommendations. The ideal ratio by the Goettingen and Louvain approaches for branched chain amino acids, valine and isoleucine, was similar to the other studies. Highlights the recommendations of the NRC (1994) and Coon & Zhang (1999) that are on average 15% higher. The natural and periodic event of loss and replacement of feathers have different patterns and occur in response to a series of hormonal changes due to the photoperiod, which may justify the differences between the recommendations in the literature. The feathers are keratinized structures, rich in cystine, arginine and branched-chain amino acids, whose main function is to cover the body of the birds by protecting them and aiding in body thermoregulation (Leeson & Summers, 2005). According to Stilborn et al. (1997), there is a change in the content of arginine, isoleucine, leucine and cystine throughout the life of the bird.

The Thr:Lys ratios found in the literature suffers small variations, except to the recommendations of the Guideline of Hy-line W-36 (2016), NRC (1994) and the results obtained by Goettingen and Louvain approaches in this study that are lower compared to the recommendations of the other authors. Threonine is a hydroxyl amino acid that plays an important role with glycine and serine in the metabolism of porphyrin, acetyl CoA, pyruvate and uric acid. It is essential for the synthesis and catabolism of protein, is fundamental importance in maintaining intestinal health and integrity, and an important component in the development of feathers, participating in 4 to 5% of its crude protein content (Kidd, 2000). It is found in concentrations close to 4.8% of egg protein and its requirements depend on the age and crude protein level of the diet (Kidd, 2001).

The differences between the ideal amino acid ratio recommendations reflects the differences in how they were determined, the requirements available in the literature were estimated from data compiled from a variety of experiments and therefore influenced by genetic, environmental, bird age, and different experimental conditions and basal diets.

In this study NMR and $NR_{max}T$ values were determined, making possible to know the genetic potential or the physiological limit of the Hy-line W36 strain, allowing to use strategies to optimize the performance of the birds. These values were used as parameters in non-linear mathematical models to estimate the efficiency of utilization and the ideal ratio of the essential amino acids in a single assay based on the deletion method, providing more accurate values by minimizing the effects of the environment, diet and genotype.

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CAPÍTULO 5 – Considerações finais

As aves possuem uma exigência diária de proteínas, ou seja, necessitam de uma determinada quantidade de aminoácidos para manterem suas funções vitais, reprodutivas e produtivas. As deficiências ou desbalanços nutricionais, especialmente na fase de cria e recria podem ocasionar problemas na fase adulta, refletindo na produção de ovos. Assim, o manejo adequado nas fases que antecedem a postura corresponde com precisão o potencial produtivo desses animais na fase onde há retorno econômico da criação. Na fase de produção, o ajuste no nível de aminoácidos da dieta é de grande importância, uma vez que há demanda desses nutrientes para a síntese de ovo.

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 os impactos da produção comercial no meio ambiente. Dessa forma, foram padronizadas duas metodologias para aves em crescimento e em produção. Ambas metodologias utilizam os mesmos princípios do método da deleção apresentados no primeiro capítulo, entretanto, utilizam abordagens diferentes para os cálculos e, também, diferentes interpretações biológicas para os parâmetros utilizados.

Enquanto o método de Goettingen considera os principais fatores que determinam a resposta animal, como genótipo e idade para determinar a eficiência de utilização dos aminoácidos e a partir dela obter a relação ideal, o método de Louvain determina as exigências por uma equação de fácil aplicação e relaciona com a exigência de lisina.

Embora a metodologia de Goettingen tenha sido padronizada para frangos de corte e matrizes pesadas, ainda estava em fase de estudo o uso desta metodologia em poedeiras em crescimento e produção. Inicialmente, para as fases de crescimento, foi necessário determinar o máximo potencial teórico de retenção de nitrogênio (NR_{maxT}) e suas exigências de manutenção no segundo capítulo. É importante lembrar que o valor estimado do NR_{maxT} é um valor teórico, usado apenas com referência do máximo potencial aplicado nas estimativas das exigências de aminoácidos para estas aves.

Os valores de máximo potencial teórico de retenção de nitrogênio (NR_{maxT}) e exigências de manutenção determinados para cada fase de crescimento no capítulo 2 foram utilizados para os cálculos de eficiência de utilização dos aminoácidos e determinação do perfil ideal dos aminoácidos essenciais para frangas em crescimento no capítulo 3. Um ensaio semelhante aos executados nos capítulos 2 e 3 foi desenvolvido no capítulo 4, porém com o fator “ovo” que precisa ser considerado.

Nos capítulos 3 e 4, foi desenvolvida a metodologia de Louvain simultaneamente a metodologia de Goettingen, utilizando os dados obtidos nos ensaios de balanço de nitrogênio para o cálculo das exigências dos aminoácidos essenciais para obter a relação ideal. 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.

A grande vantagem dessas duas metodologias é a redução no tempo e custo da pesquisa para estimar as exigências dos aminoácidos essenciais e suas relações com a lisina quando comparadas com o método dose-resposta que tem sido utilizado atualmente para a determinação da relação ideal dos aminoácidos. Assim, neste estudo foi proposto um novo perfil ideal de aminoácidos que representa melhor o estado fisiológico das aves.