Final Report

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Determining the order of limiting amino acids in practical Australian reduced protein diets for laying hens

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In submitting this report, the researcher has agreed to Poultry Hub Australia publishing this material in an edited form.

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Project Summary

Project Title	Determining the order of limiting amino acids in practical Australian reduced protein diets for laying hens
Project No.	21-306
Date	Start: 01/01/2022 End: 01/07/2023
Project Leader(s)	Dr Amy Moss
Organisation	The University of New England
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Project Aim	This study determined the limiting order of essential amino acids for laying hens fed practical Australian reduced protein diets based on wheat and sorghum.
Background	Reducing the dietary crude protein level has received increasing interest in poultry production due to its potential benefits for improving feed efficiency, gut health and litter quality while reducing water intake, nitrogen and ammonia emission, and reliance on protein meals (Hilliar et al. 2020). In laying hen production, feeding reduced protein diets may reduce the amount of undigested protein reaching the hindgut that may result in reduced harmful bacterial population and a greater number of clean eggs. However, the decreases in total dietary amino acid levels as dietary protein levels decrease may consequently reduce requirements for the individual amino acid in birds. This may be associated with the growth retardation resulting from the deficiency of single or multiple amino acids (Hurwitz et al. 1998). Determination of the relative importance of amino acids or limiting amino acid order is important in such situations when reduced protein diets are used. To our knowledge, the limiting order of essential amino acids after lysine, methionine and threonine in reduced protein wheat-sorghum-based diets for laying hens has not been reported in the literature. Thus, this study aimed to determine the limiting order of essential amino acids for laying hens fed practical Australian reduced protein diets based on wheat and sorghum.
Research Outcome	The results of this study showed that isoleucine may be considered the fourth and valine the fifth limiting amino acid after lysine, methionine and threonine in laying hens fed reduced protein diets if the amino acid order is ranked based on the egg mass. Tryptophan, arginine and phenylalanine may be considered as co-sixth limiting amino acids based on egg mass. Whereas leucine, histidine and glycine among others may be considered non-essential amino acids as the egg mass of hens fed these diets were similar to those fed the standard protein diet and reduced protein diet with sufficient levels of all essential amino acids. However, if the amino acid order is ranked based on feed conversion ratio, valine may be considered the fourth limiting amino acid, and tryptophan, isoleucine, arginine and histidine may be considered as co-fifth limiting amino acids. Leucine, phenylalanine, and glycine may be considered as non-essential amino acids after lysine, methionine and threonine for laying hens fed reduced protein diets.

Impacts and Outcomes	The outcomes of this study are directly relevant and beneficial to the Australian poultry industry. The findings may help to extend the adoption of reduced protein diets in layer hen production, resulting in improved economic and environmental outcomes, and reduction in industry reliance on imported ingredients such as soybean meal.
Publications	Manuscripts are in preparation, and a conference abstract has been presented: A.A. Jahan, T.H. Dao, N. Akter, S. Sukirno, N.K. Sharma, R.A. Swick and A.F. Moss (2023). Order of limiting amino acids in wheat-sorghum based reduced protein diets for laying hens. Proceedings of 34th Annual Australian Poultry Science Symposium, Sydney, Australia, 6–8th February 2023. A.A. Jahan, H.T. Dao, N. Akter, S. Sukirno, N.K. Sharma, D.J. Cadogan, E.J. Bradbury, P.V. Chrystal, R.A. Swick, N. Morgan, T.M. Crowley, A.F. Moss (2023). Order of limiting amino acids in wheat-sorghum based reduced protein diet for laying hens. *Animal Production Science* (in preparation).

Executive Summary

Understanding the order of limiting amino acids (AA) in reduced protein diets is important to ensure AA requirements are met cost effectively. Only the order of the first three limiting AA − lysine (Lys), methionine (Met), and threonine (Thr) − have been well established for laying hens. It is necessary to determine the next limiting AA after Lys, Met and Thr to be given priority during formulation of reduced protein diets. Therefore, this study was conducted to determine the limiting order of eight essential AA, including tryptophan (Trp), valine (Val), isoleucine (Ile), arginine (Arg), leucine (Leu), histidine (His), phenylalanine (Phe), and glycine$_{equivalent}$ (Gly) in wheat-sorghum-based reduced protein diets for laying hens.

A total of 330 Hy-Line Brown laying hens were randomly assigned to 11 dietary treatments (30 replicates of a single bird per treatment) from 20 to 39 weeks of age. Treatments were a standard protein diet (SP, 17.24% crude protein), a reduced protein diet (15% crude protein) with sufficient levels of Lys, Met and Thr but deficient in all other essential AA (RP), a reduced protein diet with sufficient levels of all essential AA (RP-EAA), and the subsequent eight dietary treatments were formulated by deletion method, where one of specific AA was removed from RP-EAA diet: tryptophan (RP-EAA-Trp), valine (RP-EAA-Val), isoleucine (RP-EAA-Ile), arginine (RP-EAA-Arg), leucine (RP-EAA-Leu), histidine (RP-EAA-His), phenylalanine (RP-EAA-Phe), and glycine$_{equivalent}$ (RP-EAA-Gly).

Eggs were collected and weighed daily, while feed intake and the feed conversion ratio (FCR) were calculated weekly. Egg quality traits were measured at 29 and 39 weeks. Serum uric acid level, eggshell cleanness score, caecal microbiota composition, and apparent nutrient digestibility were measured at week 40. The results showed that hens fed the RP and RP-EAA-Ile had significantly lower egg mass compared to hens offered the SP, RP-EAA-Leu, RP-EAA-His and RP-EAA-Gly diets ($P < 0.001$). Also, hens fed the RP-EAA-Val had lower egg mass compared to those fed the SP, RP-EAA-His and RP-EAA-Gly diets ($P < 0.001$). Higher FCR were observed in hens fed the RP and RP-EAA-Val diets compared to those offered the RP-EAA, RP-EAA-Leu, RP-EAA-Phe and RP-EAA-Gly diets ($P < 0.05$). Thus, according to the results on egg mass, Ile may be considered the fourth and Val may be considered the fifth limiting AA after Lys, Met and Thr in laying hens fed reduced protein diets. Tryptophan, Arg and Phe may be considered as co-sixth limiting AA based on the egg mass. Whereas, the other AA including Leu, His, and Gly may be considered non-essential AA, as the egg mass of hens fed these diets was similar to those fed the RP-EAA and SP diets. However, if the AA order is ranked based on FCR, Val may be considered the fourth limiting AA, and Trp, Ile, Arg and His may be considered as co-fifth limiting AA. Finally, Leu, Phe and Gly may be considered as non-essential AA after Lys, Met and Thr for laying hens fed reduced protein diets. Egg quality traits, serum uric acid level, eggshell cleanness score, caecal microbiota composition and apparent nutrient digestibility were not significantly different between the dietary treatments.

The findings of this study may help to facilitate precision feed formulation and extend the adoption of reduced protein diets in layer hen production while reducing industry reliance on imported soybean meal.

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Introduction

Soybean meal and cereal grains are the most significant portions of poultry diets, in terms of total volume and feed cost. However, limited arable land and global climate change remain major challenges to meet the increasing demand for these crops in the near future (Chrystal et al. 2020). Reducing dietary crude protein level with supplementation of crystalline amino acids (AA) is an effective solution to satisfy at least part of the protein requirement of chickens. The increasing availability of crystalline AA has allowed a greater reduction of dietary protein levels. Each time a new AA has become commercially available, the inclusion rate of soybean meal in poultry diets has reduced (Swick & Creswell 2019).

Reducing the dietary crude protein (CP) level has received increasing interest in poultry production due to its potential benefits to improve feed efficiency, gut health, and litter quality, while reducing water intake, nitrogen and ammonia emission, and reliance on protein meals (Hilliar et al. 2020). In laying hen production, feeding reduced protein diets may reduce the amount of undigested protein reaching the hindgut and may result in reduced harmful bacterial population and thus a greater number of clean eggs. However, the reductions in total dietary AA levels as dietary protein levels decrease may consequently reduce requirements for the individual AA in birds. This may be associated with growth retardation resulting from the deficiency of single or multiple AA (Hurwitz et al. 1998). Determination of the relative importance of AA or limiting AA order is important in such situations when reduced protein diets are used. To our knowledge, the limiting order of essential AA after Met, Lys, and Thr in reduced protein wheat-sorghum-based diets for laying hens has not been reported in the literature. Thus, this study aimed to determine the limiting order of essential AA for laying hens fed practical Australian reduced protein diets based on wheat and sorghum. The results of this study may help to extend the adoption of reduced protein diets in layer hen production and reduce the industry reliance on imported ingredients such as soybean meal.

Objectives

This study aimed to determine the limiting order of essential AA for laying hens fed practical Australian reduced protein diets based on wheat and sorghum.

Methodology

Experimental design, animals and diets

This study was conducted at the Laureldale Research Station of the University of the New England, Armidale, NSW, Australia. The experimental design and all other procedures were approved by the Animal Ethics Committee of the University of New England (approval number: ARA21-096) and met the requirements of the Australian Code of Practice for the care and use of animals for scientific purposes (NHMRC 2013).

A deletion method as described by Fernandez et al. (1994) has been widely used to determine the order of limiting AA in chickens (Wang et al. 1997; Wang & Parsons 1998; Peter et al. 2000). This method was also recently used by the research group at the University of Arkansas, USA (Maynard et al. 2020) to determine the fourth limiting AA in broilers fed reduced protein based on corn and soybean meal. Thus, the deletion method was used to determine limiting AA order in this study.

Feeds for 11 dietary treatments were formulated in this study (with a target intake of 110 g/bird/day). The first diet was a standard protein diet with sufficient levels of CP and other nutrients according to the nutritional recommendations for Hy-Line Brown laying hens (treatment 1, control diet, SP). The second and third diets were reduced protein diets (20 g/kg lower CP levels compared to the SP diet) with sufficient levels of Lys, Met, and Thr and deficient (treatment 2, RP) or sufficient (treatment 3, RP-EAA) in the remaining eight essential AA including Trp, Val, Ile, Arg, Leu, His, Phe and $Gly_{equivalent}$. Each of these eight essential AA was then individually deleted from the RP-EAA diet by removing its supplemental level to generate treatments 4 to 11. The RP diet was generated by removing all added crystalline Trp, Val, Ile, Arg, Leu, His, Phe, and $Gly_{equivalent}$ from the RP-EAA diet. A description of dietary treatments is given in Table 1. The AA orders were ranked based on the egg mass and feed conversion ratio (FCR) of the respective treatments. The nutritional composition of major ingredients including dry matter, CP, crude fat, and ash content, was analysed before diet formulation. Levels of essential AA were selected based on Hy-Line Brown nutritional recommendations (Hy-Line International, 2016). Mixed diets were subjected to proximate and AA analysis to confirm the formulation objectives.

Table 1 Description of dietary treatments

Treatment number	Treatment code	Treatment description
1	SP	A standard protein diet with sufficient levels of all essential amino acids
2	RP	A reduced protein diet with sufficient levels of Lys, Met and Thr but deficient in Trp, Arg, Ile, Val, Leu, His, Phe and Gly.
3	RP-EAA	A reduced protein diet with sufficient levels of all essential amino acids including Lys, Met, Thr, Trp, Arg, Ile, Val, Leu, His, Phe and Gly.
4	RP-EAA-Trp	Treatment 3 deficient in Trp
5	RP-EAA-Val	Treatment 3 deficient in Val
6	RP-EAA-Ile	Treatment 3 deficient in Ile
7	RP-EAA-Arg	Treatment 3 deficient in Arg
8	RP-EAA-Leu	Treatment 3 deficient in Leu
9	RP-EAA-His	Treatment 3 deficient in His
10	RP-EAA-Phe	Treatment 3 deficient in Phe
11	RP-EAA-Gly	Treatment 3 deficient in Gly

Hy-Line Brown laying hens (n = 330) were assigned to the dietary treatments in a randomised design from 20 to 39 weeks of age. There were 30 replicate cages of a single bird per treatment. Feed was provided as mash. Birds had free access to the feed and water throughout the study. Egg production and feed consumption were recorded daily and weekly, respectively. Egg mass and FCR were calculated from egg production, egg weight, and feed consumption. The FCR was calculated as the ratio of feed to egg mass. Hens were weighed at the beginning (week 20), middle (week 29), and end of the study (week 39). Egg quality (shell thickness, shell reflectivity, shell breaking strength, albumen height, Haugh unit, yolk colour score) were measured at 29 and 39 weeks of age. The lighting program of 16 hours light: 8 hours dark was maintained throughout the study. An automatic timer was used to operate the lighting program in the hen house. Temperature and relative humidity inside the shed were measured at bird height and recorded daily. The temperature and relative humidity in the hen house recorded during the experiment are shown in Figure 1.

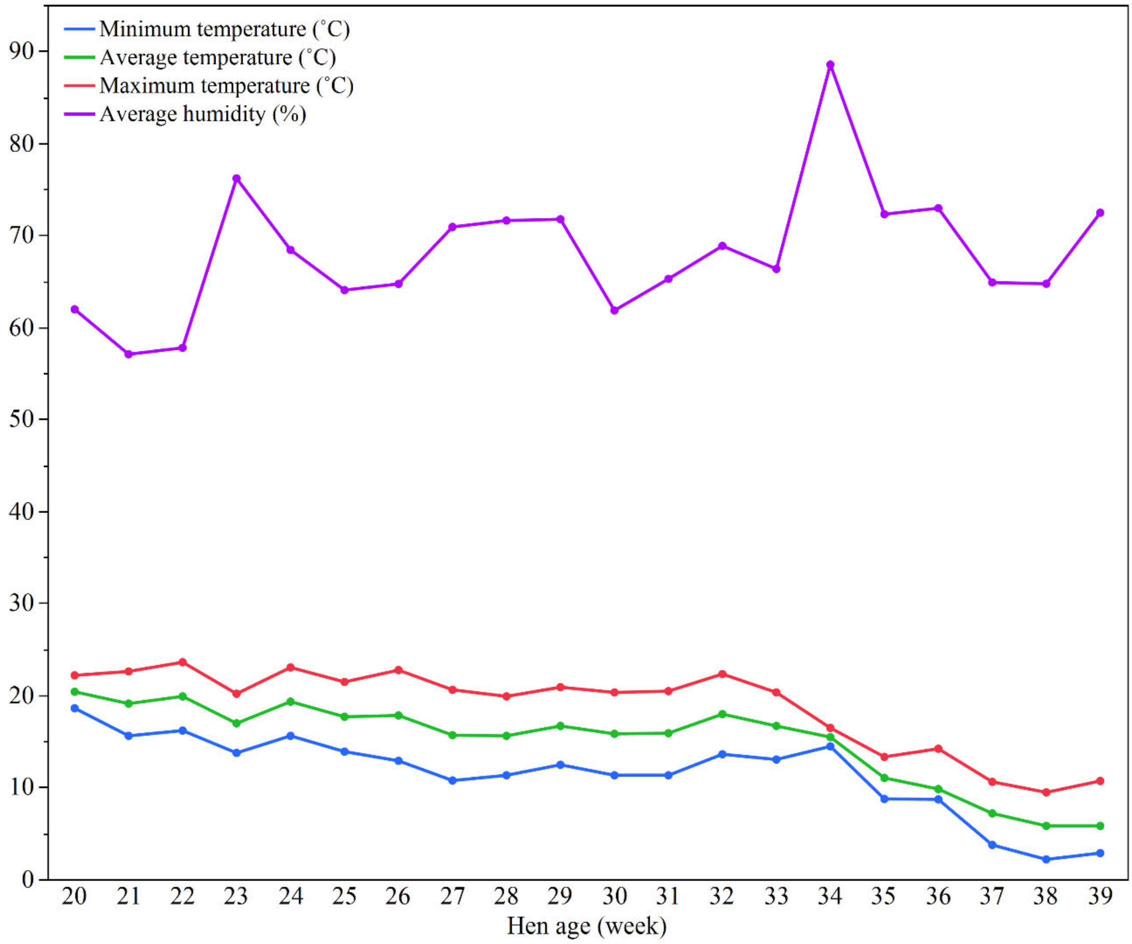

Figure 1 Temperature and relative humidity of the hen house during the study

Blood serum and caecal digesta samples (8 hens per treatment, 88 hens in total) were collected at the end of the study (week 40) to determine serum uric acid level and caecal relative total bacterial population, respectively. The relative total bacterial population in the caeca was determined by the quantitative real-time PCR (Rotor-Gene 6000 real-time PCR machine, Corbett, Sydney, Australia). The specific 16S rRNA primers were used for the quantification of different bacterial populations including *Bacillus* spp., *Bacteroides* spp., *Bifidobacterium* spp., Enterobacteriaceae, *Lactobacillus* spp., *Ruminococcus* spp., and total bacteria, expressed as $\log_{10}$ genomic DNA copies per gram of caecal digesta, were quantified as described by Kheravii et al. (2017). The percentages of dirty eggs in the dietary treatments were determined at week 40 using Australian standards (Standard 4.2.5, Australian Federal Register of Legislation 2014) to correlate the eggshell cleanness with the total bacterial load in the treatments. In addition, a total collection of excreta method (8 hens per treatment, 88 hens in total) was used to evaluate the apparent total tract energy and protein digestibility of the feeding treatments at week 40 over 3 consecutive days (72 hours). Excreta were collected from individual cages twice daily starting from 0800 and 1600 after removing feathers and feed residues and stored at 4°C. The gross energy and crude protein content of the excreta were measured for the determination of energy and protein retainment. Total feed consumption of individual hens in each treatment during the 3-day excreta collection was recorded for the determination of gross energy and crude protein intake. Apparent protein and energy digestibility were calculated following equations described by Kong and Adeola (2014). In more detail, apparent protein digestibility was calculated by dividing average protein retained by average protein intake during 3-day excreta collection and multiplying by 100. Similarly, apparent energy digestibility was calculated by dividing average energy retained by average energy intake during 3-day excreta collection and multiplying by 100. Of which, protein and energy intake were calculated by multiplying average feed intake during 3-day excreta collection by the crude protein and gross energy level of the feed, respectively. Protein retained was calculated by subtracting from the protein intake the average protein excreted during 3-day excreta collection. Energy retained was calculated by subtracting from the energy intake the average energy excreted during 3-day excreta collection. Amount of protein and energy excreted through the excreta were calculated by multiplying average excreta volume during 3-day excreta collection by the crude protein and gross energy level of the excreta, respectively. All data were calculated as per dry matter basis.

$$\text{Apparent protein digestibility (\%)} = (CP_{retained}/CP_{intake}) \times 100$$

$$\text{Apparent energy digestibility (\%)} = (GE_{retained}/GE_{intake}) \times 100$$

$$CP_{intake}\ \text{(g/day)} = CP_{feed}\ (\%) \times FI\ \text{(g/day/hen)}$$

$$GE_{intake}\ \text{(kcal/day)} = GE_{feed}\ \text{(kcal/g)} \times FI\ \text{(g/day/hen)}$$

$$CP_{retained}\ \text{(g/day)} = CP_{intake} - CP_{excreta}\ (\%) \times \text{excreta volume (g/day/hen)}$$

$$GE_{retained}\ \text{(kcal/day)} = GE_{intake} - GE_{excreta}\ \text{(kcal/g)} \times \text{excreta volume (g/day/hen)}$$

Where CP, GE and FI are crude protein, gross energy and feed intake, respectively.

Statistical analysis

Data were analysed using IBM SPSS statistics software (Version: 28.0.1.0, IBM Corp., Armonk, NY, USA) with α-level set at 0.05. Prior to statistical analysis, data were tested for normal distribution and approximately equal variances between the dietary treatments. For analysis of hen laying performance, hen-day egg production (%), egg mass (g), feed intake (g) and FCR were determined for the period of 20 to 29 weeks and 30 to 39 weeks separately as well as for the entire duration (20 to 39 weeks). However, both external and internal egg quality traits and egg proportions were assessed at 29 and 39 weeks. Data were subjected to ANOVA with univariate General Linear Models (GLM) fitted to each variable with treatment as fixed effect to determine the mean differences between the dietary treatment groups. Tukey's post hoc test was applied where significant differences were present to identify pairwise differences between the treatments.

Discussion of results

Diet composition and analysed nutrient content of the experimental treatments are given in Tables 2 and 3, respectively. The hen weight and laying performance over the entire study are reported in Tables 4 and 5, respectively. The hen weight was not different between the dietary treatments at the beginning (week 20), middle (week 29) or end of the study (week 39, Table 4). However, hens fed the RP and RP-EAA-Ile had significantly lower egg mass compared to hens offered the SP, RP-EAA-Leu, RP-EAA-His and RP-EAA-Gly diets ($P < 0.001$, Table 5). Also, hens fed the RP-EAA-Val had the lower egg mass compared to those fed the SP, RP-EAA-His and RP-EAA-Gly diets ($P < 0.001$, Table 5). Egg mass was not significantly different between hens fed the SP and RP-EAA diets. Egg production and feed intake were not significantly different between the dietary treatments over the entire study (Table 5). However, a higher FCR was observed in hens fed the RP and RP-EAA-Val diets compared to those offered the RP-EAA, RP-EAA-Leu, RP-EAA-Phe and RP-EAA-Gly diets ($P < 0.05$, Table 5). Hens fed the RP, RP-EAA-Val, RP-EAA-Ile, and RP-EAA-Arg had lower egg weight compared to those offered the SP, RP-EAA-His and RP-EAA-Gly diets (Table 5). Thus, according to the results on egg mass, Ile may be considered the fourth and Val the fifth limiting AA after Lys, Met and Thr in laying hens fed reduced protein diets. Tryptophan, Arg and Phe may be considered as co-sixth limiting AA based on the egg mass. Whereas, Leu, His, and Gly among others may be considered non-essential AA as the egg mass of hens fed these diets was similar to those fed the RP-EAA and SP diets. However, if the AA order is ranked based on FCR, Val may be considered fourth limiting and Trp, Ile, Arg and His may be considered as co-fifth limiting. Leucine, Phe and Gly may be considered as non-essential AA after Lys, Met and Thr for laying hens fed reduced protein diets.

Table 2 Diet composition of standard (SP) and reduced protein diets with sufficient levels of all essential amino acids (RP-EAA) as-fed basis (%, otherwise as indicated)

Dietary treatment	SP	RP-EAA
Ingredients		
Wheat	38.44	49.52
Barley	4.00	4.00
Sorghum	20.00	20.00
Soybean meal	13.77	2.68
Canola meal	10.00	9.66
Canola oil	2.59	0.80
Limestone (fine)	4.93	4.94
Limestone (coarse)	4.93	4.94
Monocalcium phosphate	0.549	0.623
Salt	0.232	0.159
Sodium bicarbonate	0.200	0.300
Choline Cl 60%	0.000	0.000
L-lysine HCl	0.078	0.410
DL-methionine	0.163	0.245
L-threonine	0.016	0.159
L-tryptophan	-	0.018
L-valine	-	0.137
L-isoleucine	-	0.175
L-arginine	-	0.237
L-leucine	-	0.287

L-histidine	-	0.101
L-phenylalanine	-	0.194
L-glycine	-	0.276
Xylanase[1] (Axtra XB TPT 201)	0.010	0.010
Phytase[2] (Quantam blue 5G 60 g/MT)	0.006	0.006
Pigment jabiru red	0.004	0.004
Pigment jabiru yellow	0.003	0.003
Vitamin-mineral premix[3]	0.100	0.100
Calculated composition		
AMEn[4], kcal/kg	2,740	2,740
Crude protein	17.24	15.00
Crude fat	4.60	2.90
Crude fibre	3.19	3.00
SID[5] arginine	0.893	0.780
SID lysine	0.740	0.740
SID methionine	0.400	0.435
SID cysteine	0.269	0.233
SID methionine + cysteine	0.670	0.670
SID tryptophan	0.192	0.160
SID histidine	0.370	0.370
SID phenylalanine	0.706	0.706
SID leucine	1.152	1.152
SID isoleucine	0.590	0.590
SID threonine	0.520	0.520
SID valine	0.692	0.650
SID valine equivalent	0.971	0.971
Calcium	4.10	4.10
Available phosphorus	0.40	0.40
Sodium	0.17	0.17
Potassium	0.64	0.46
Chloride	0.22	0.24
Choline, mg/kg	1557	1333
Linoleic acid	1.55	1.12

[1] Xylanase: Axtra XB TPT 201, Danisco Animal Nutrition (IFF).

[2] Phytase: Quantum blue 5G, 60 g/MT, AB Vista.

[3] Vitamin-mineral premix provided the following per kilogram of vitamin-mineral premix: vitamin A, 10 MIU; vitamin D, 3 MIU; vitamin E, 20 g; vitamin K, 3 g; nicotinic acid, 35 g; pantothenic acid, 12 g; folic acid, 1 g; riboflavin, 6 g; cyanocobalamin, 0.02 g; biotin, 0.1 g; pyridoxine, 5 g; thiamine, 2 g; copper, 8 g as copper sulphate pentahydrate; cobalt, 0.2 g as cobalt sulphate 21%; molybdenum, 0.5 g as sodium molybdate; iodine, 1 g as potassium iodide 68%; selenium, 0.3 g as selenium 2%; iron, 60 g as iron sulphate 30%; zinc, 60 g as zinc sulphate 35%; manganese, 90 g as manganous oxide 60%; antioxidant, 20 g.

[4] AMEn: N corrected apparent metabolisable energy.

[5] Digestible amino acid coefficients for raw ingredients were determined by Near-Infra Red spectroscopy (Foss NIR 6500, Denmark) standardised with Evonik AMINONIR Advanced calibration.

Table 3 Analysed nutrient values of experimental diets (%, as-is basis)[1]

Nutrient composition	SP	RP	RP-EAA	RP-EAA-Trp	RP-EAA-Val	RP-EAA-Ile	RP-EAA-Arg	RP-EAA-Leu	RP-EAA-His	RP-EAA-Phe	RP-EAA-Gly
Dry matter	91.46	91.34	91.66	91.76	91.25	91.06	91.08	91.34	91.35	91.33	91.26
Gross energy, kcal/kg	3,712	3,523	3,638	3,589	3,584	3,569	3,571	3,536	3,571	3,583	3,509
Crude protein	18.15	14.37	15.82	15.66	15.84	15.25	15.44	15.30	15.20	14.90	14.77
Ash	15.07	15.04	13.64	14.70	14.27	14.53	14.52	14.77	14.40	13.75	15.73
Calcium	5.00	5.27	4.81	4.37	4.60	4.33	4.84	4.45	4.22	4.48	5.53
Total phosphorus	0.60	0.58	0.63	0.57	0.58	0.57	0.61	0.57	0.58	0.54	0.58
Aspartic acid	0.926	0.645	0.671	0.688	0.687	0.691	0.723	0.723	0.750	0.696	0.692
Serine	0.599	0.483	0.517	0.525	0.572	0.555	0.560	0.560	0.550	0.548	0.527
Glutamic acid	2.530	2.323	2.506	2.501	2.547	2.512	2.607	2.607	2.656	2.543	2.512
Glycine	0.500	0.406	0.671	0.684	0.778	0.721	0.715	0.715	0.686	0.701	0.463
Histidine	0.271	0.212	0.342	0.345	0.383	0.353	0.350	0.350	0.253	0.349	0.329
Arginine	0.688	0.536	0.742	0.824	0.888	0.806	0.560	0.859	0.754	0.838	0.828
Threonine	0.416	0.433	0.510	0.514	0.557	0.528	0.525	0.530	0.502	0.540	0.499
Alanine	0.566	0.465	0.496	0.512	0.533	0.522	0.528	0.537	0.546	0.521	0.508
Proline	0.843	0.768	0.885	0.809	0.937	0.914	0.916	0.914	0.937	0.960	0.944
Cysteine	0.693	0.528	0.317	0.430	0.611	0.575	0.571	0.563	0.612	0.623	0.569
Tyrosine	0.363	0.268	0.213	0.328	0.359	0.313	0.325	0.314	0.318	0.344	0.321
Valine	0.364	0.307	0.515	0.473	0.359	0.500	0.499	0.499	0.456	0.484	0.499
Methionine	0.293	0.315	0.565	0.324	0.413	0.432	0.448	0.349	0.397	0.376	0.328
Lysine	0.703	0.746	0.795	0.740	0.853	0.823	0.864	0.844	0.911	0.858	0.790
Isoleucine	0.387	0.298	0.387	0.487	0.516	0.366	0.507	0.504	0.482	0.527	0.493
Leucine	0.837	0.698	0.844	1.072	1.119	1.125	1.094	0.852	1.054	1.088	1.058
Phenylalanine	0.506	0.396	0.669	0.707	0.726	0.706	0.648	0.668	0.623	0.504	0.699

[1] Values of all the amino acids presented were total amino acids (measured on an as-is basis).

Table 4 Mean body weight of hens offered dietary treatments

Treatment	Hen age (week)		
	Week 19	Week 29	Week 39
SP	1,838	2,197	2,350
RP	1,853	2,166	2,321
RP-EAA	1,837	2,188	2,349
RP-EAA-Trp	1,836	2,179	2,338
RP-EAA-Val	1,825	2,139	2,303
RP-EAA-Ile	1,874	2,185	2,339
RP-EAA-Arg	1,855	2,176	2,342
RP-EAA-Leu	1,849	2,164	2,316
RP-EAA-His	1,881	2,240	2,409
RP-EAA-Phe	1,834	2,171	2,308
RP-EAA-Gly	1,873	2,212	2,355
SEM	23.01	31.67	37.56
P-value	0.773	0.709	0.815

Table 5 Laying performance of hens offered dietary treatments from weeks 20 to 39

Treatment	Egg weight (g)	Hen day egg production (%)	Egg mass (g)	Feed intake (g)	FCR (kg feed/kg egg)
P	60.2[bcd]	95.4	57.5[cd]	128	2.238[ab]
RP	57.7[a]	92.8	53.7[a]	127	2.378[c]
RP-EAA	59.1[abcd]	94.7	56.0[abcd]	123	2.203[a]
RP-EAA-Trp	58.5[ab]	94.6	55.4[abc]	125	2.262[abc]
RP-EAA-Val	57.7[a]	93.3	54.0[ab]	125	2.334[bc]
RP-EAA-Ile	57.6[a]	93.4	53.9[a]	124	2.305[abc]
RP-EAA-Arg	57.8[a]	95.8	55.4[abc]	125	2.263[abc]
RP-EAA-Leu	59.1[abcd]	95.6	56.5[bcd]	125	2.212[a]
RP-EAA-His	60.5[cd]	95.2	57.7[cd]	131	2.278[abc]
RP-EAA-Phe	58.7[abc]	94.8	55.7[abc]	122	2.205[a]
RP-EAA-Gly	60.7[d]	96.2	58.4[d]	128	2.193[a]
SEM	0.21	0.25	0.28	0.70	0.013
P-value	0.001	0.098	< 0.001	0.281	0.046

[a,b,c,d] Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Feeding reduced protein diets balanced in AA is becoming more and more important in tropical and subtropical regions to reduce production cost and the adverse effects of heat stress (Keshavarz & Austic 2004; Poosuwan et al. 2010). It has been indicated that high protein diets decrease protein digestibility and increase heat production in laying hens, which may adversely affect the hens' production performance in tropical regions (Bunchasak & Silapasorn 2005). However, protein/AA deficiency may also make the birds more susceptible to diseases, as nutrient supply in sufficient amount and at suitable time is required to support birds' immune systems (Humphrey & Klasing 2004; Poosuwan et al. 2010). Thus, special attention should be given when feeding reduced protein diets during challenging conditions. The limiting orders of essential AA would play crucial roles in such circumstances. Previous research has indicated that supplementation of branched-chain AA (e.g. Ile, Val and Leu) in the reduced protein diets is necessary to maintain egg production, egg weight, and egg mass (Harms & Russell 2000; Vieira et al. 2016). Also, it has been suggested that antagonism between Ile, Val and Leu may appear at higher concentrations in higher protein diets compared to the reduced protein diets (Peganova & Eder, 2003). The results of this study were consistent with Harms and Ivey (1993) who reported that supplementation of either Ile or Val to corn-soybean meal based reduced protein diets (13.8% CP) with sufficient levels of Met, Lys, Thr, Trp, and Arg increased the egg mass of Hy-Line Brown laying hens from 31 to 38 weeks of age. Lelis et al. (2014) indicated that Val may be the next limiting AA after Met, Lys, Trp, and Thr in a 14.8% CP diet for laying hens from 42 to 54 weeks of age. Similarly, Da Silva et al. (2012) demonstrated that Trp is the fourth limiting AA, and Val and Ile are co-fifth limiting AA in corn-soybean meal based reduced protein diets. Wen et al. (2019) added that Val requirement in laying hens fed a corn-peanut based diet was highest for egg mass, followed by egg production, and lowest for FCR. This study provides the new information that Trp may be less important than Val, and the Val requirement in regard to FCR may be higher than the requirement for egg mass in wheat-sorghum-soybean meal based reduced protein diets for laying hens. Dong et al. (2016) reported that Ile supplementation from 0.1 to 0.4% to a corn-soybean meal reduced protein diet (14% CP) with sufficient levels of Met, Lys, Thr, Trp, and Val did not affect laying performance and egg quality in Lohmann Brown laying hens. The lack of response to Ile supplementation to the reduced protein diet reported by Dong et al. (2016) might be due to the insufficient levels of Arg and His in the diet while Ile, Arg and His might be equally important (co-limiting AA) in the reduced protein diets as observed in this study.

Internal and external egg quality parameters and egg components at weeks 29 and 39 are reported in Tables 6 to 11. There were no significant differences in egg component, internal and external egg quality between the dietary treatments at weeks 29 and 39. It is quite interesting that hens fed the RP-EAA-Arg diet tended to have lower egg width compared to the hens fed the SP diet at both weeks 29 ($P = 0.089$) and 39 ($P = 0.090$); however, the reasons are unclear. The current findings were supported by previous studies (Dong et al. 2016; Vieira et al. 2016). The findings of this study also demonstrated that reduced protein diets with 20 g/kg lower CP level compared to standard protein diets can be achieved without negative effects on laying performance and egg quality of the hens. Determining the orders of essential AA based on FCR may be more meaningful than the egg mass in practice as FCR could reflect the economic efficiency of the farms.

Table 6 Internal egg quality of hens offered dietary treatments at week 29

Treatment	Albumen height (mm)	Yolk colour	Haugh unit	Yolk height (mm)	Yolk diameter (mm)	Yolk index
SP	8.88	13.00	92.69	22.15	41.50	0.536
RP	9.23	11.73	94.81	22.66	40.21	0.568
RP-EAA	9.58	12.57	96.66	22.71	40.96	0.557
RP-EAA-Trp	9.50	12.33	96.11	22.57	43.30	0.522
RP-EAA-Val	9.29	13.00	94.59	22.71	42.40	0.538
RP-EAA-Ile	9.69	11.87	97.23	22.89	43.09	0.532
RP-EAA-Arg	9.21	12.21	95.30	22.15	41.95	0.529
RP-EAA-Leu	9.02	12.71	93.93	22.37	41.98	0.537
RP-EAA-His	8.87	12.53	92.55	23.03	42.65	0.541
RP-EAA-Phe	9.97	11.67	98.73	22.53	42.38	0.532
RP-EAA-Gly	9.15	12.87	94.12	22.58	41.97	0.539
SEM	0.10	0.12	0.50	0.07	0.21	0.003
P-value	0.365	0.338	0.179	0.236	0.125	0.250

Table 7 External egg quality of hens offered dietary treatments at week 29

Treatment	Eggshell breaking strength (Kgf)	Shell thickness (mm)	Egg Length (mm)	Egg width (mm)	Egg shape index	Reflectivity (%)
SP	5.08	0.434	57.68	44.25	0.768	22.87
RP	5.00	0.431	57.00	43.57	0.765	24.47
RP-EAA	4.98	0.434	56.77	44.08	0.777	24.57
RP-EAA-Trp	4.76	0.443	56.59	44.19	0.781	23.71
RP-EAA-Val	4.95	0.445	57.14	43.84	0.768	24.59
RP-EAA-Ile	4.99	0.439	56.73	43.89	0.774	23.43
RP-EAA-Arg	5.21	0.444	56.68	43.07	0.760	24.79
RP-EAA-Leu	4.90	0.432	57.09	43.62	0.764	23.23
RP-EAA-His	5.24	0.438	57.59	44.10	0.766	23.72
RP-EAA-Phe	4.70	0.433	56.55	43.64	0.772	24.41
RP-EAA-Gly	4.56	0.437	57.07	44.14	0.774	23.32
SEM	0.06	0.002	0.12	0.08	0.002	0.22
P-value	0.299	0.653	0.613	0.089	0.354	0.626

Table 8 Egg proportion of hens offered dietary treatments at week 29

Treatment	Albumen weight (g)	Yolk weight (g)	Shell weight (g)	Albumen (%)	Yolk (%)	Shell (%)
SP	42.24	16.08	6.28	65.35	24.92	9.89
RP	40.05	16.08	6.21	64.26	25.78	9.96
RP-EAA	41.13	16.02	6.21	64.90	25.29	9.81
RP-EAA-Trp	40.91	16.45	6.37	64.18	25.83	10.00
RP-EAA-Val	40.48	16.60	6.39	63.77	26.17	10.07
RP-EAA-Ile	39.83	16.34	6.32	63.74	26.13	10.13
RP-EAA-Arg	38.98	15.58	6.20	64.12	25.67	10.21
RP-EAA-Leu	40.18	16.09	6.19	64.29	25.79	9.92
RP-EAA-His	41.38	16.79	6.48	64.29	25.76	9.95
RP-EAA-Phe	40.19	15.50	6.05	65.06	25.14	9.80
RP-EAA-Gly	41.61	15.83	6.32	65.27	24.81	9.92
SEM	0.23	0.11	0.04	0.15	0.14	0.04
P-value	0.157	0.230	0.420	0.196	0.386	0.687

Table 9 Internal egg quality of hens offered dietary treatments at week 39

Treatment	Albumen height (mm)	Yolk colour	Haugh unit	Yolk height (mm)	Yolk diameter (mm)	Yolk index
SP	8.80	13.82	91.83	22.45	39.53	0.577
RP	8.84	12.71	93.07	22.17	39.96	0.557
RP-EAA	9.50	13.00	95.68	22.66	40.01	0.568
RP-EAA-Trp	9.32	13.00	95.38	22.49	40.03	0.568
RP-EAA-Val	9.35	13.00	95.17	22.39	41.47	0.541
RP-EAA-Ile	9.34	12.79	95.09	22.62	40.01	0.568
RP-EAA-Arg	8.95	13.50	93.95	22.06	38.72	0.575
RP-EAA-Leu	9.09	13.07	94.49	22.03	39.98	0.556
RP-EAA-His	9.11	13.64	94.19	22.30	38.49	0.592
RP-EAA-Phe	9.98	12.86	98.68	22.69	38.99	0.588
RP-EAA-Gly	8.72	12.60	91.64	22.41	39.00	0.578
SEM	0.12	0.10	0.60	0.08	0.30	0.005
P-value	0.520	0.120	0.474	0.740	0.733	0.475

Table 10 External egg quality of hens offered dietary treatments at week 39

Treatment	Eggshell breaking strength (Kgf)	Shell thickness (mm)	Egg Length (mm)	Egg width (mm)	Egg shape index	Reflectivity (%)
SP	4.91	0.428	57.10	44.57	0.781	23.38
RP	4.95	0.435	56.45	43.60	0.773	25.25
RP-EAA	4.58	0.432	57.28	44.29	0.773	25.27
RP-EAA-Trp	4.73	0.435	56.66	44.08	0.779	24.12
RP-EAA-Val	4.80	0.435	57.02	44.14	0.775	24.59
RP-EAA-Ile	4.74	0.443	56.20	43.92	0.782	23.54
RP-EAA-Arg	4.65	0.444	56.86	43.35	0.763	25.95
RP-EAA-Leu	4.32	0.438	56.61	43.81	0.774	24.23
RP-EAA-His	4.70	0.443	57.60	43.98	0.764	23.83
RP-EAA-Phe	4.95	0.438	56.72	43.68	0.770	24.57
RP-EAA-Gly	4.75	0.446	56.92	44.49	0.782	23.95
SEM	0.05	0.002	0.13	0.09	0.002	0.23
P-value	0.409	0.627	0.639	0.090	0.164	0.417

Table 11 Egg proportion of hens offered dietary treatments at week 39

Treatment	Albumen weight (g)	Yolk weight (g)	Shell weight (g)	Albumen (%)	Yolk (%)	Shell (%)
SP	42.20	16.28	6.18	65.22	25.21	9.57
RP	39.29	15.66	6.01	64.44	25.69	9.87
RP-EAA	41.26	16.85	6.27	64.07	26.19	9.74
RP-EAA-Trp	40.04	16.30	6.09	64.16	26.07	9.77
RP-EAA-Val	39.83	16.57	6.18	63.65	26.47	9.89
RP-EAA-Ile	39.38	15.97	6.20	63.98	25.94	10.08
RP-EAA-Arg	38.84	16.08	6.11	63.61	26.38	10.01
RP-EAA-Leu	39.81	15.82	6.17	64.41	25.60	9.99
RP-EAA-His	40.87	16.15	6.32	64.50	25.52	9.99
RP-EAA-Phe	39.74	15.76	6.13	64.44	25.61	9.95
RP-EAA-Gly	41.39	16.17	6.42	64.63	25.31	10.05
SEM	0.25	0.11	0.04	0.14	0.14	0.04
P-value	0.128	0.511	0.522	0.531	0.594	0.292

The serum uric acid level, eggshell cleanness score, caecal microbiota counts and nutrient digestibility of hens offered the dietary treatments at week 40 are reported in Tables 12, 13, 14 and 15, respectively. The results showed that serum uric acid levels and eggshell cleanness scores were not different between the dietary treatments at week 40 (Tables 12 and 13). The eggshell cleanness score was also not correlated with caecal total bacteria count at week 40 (P = 0.249, R^2 = 0.125). Hens fed the RP-EAA-Phe diet tended to have higher caecal *Bacteroides* spp. count compared to those fed the SP diet (P = 0.067, Table 14). Whereas hens offered the RP-EAA-Arg diet tended to have higher caecal *Ruminococcus* spp. count than hens offered the RP diet (P = 0.082, Table 14). The numbers of the other microbiota populations including *Lactobacillus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Enterobacteriaceae*, and total bacteria were not different between the dietary treatments at week 40 (Table 14). Dry matter, protein and energy digestibility were not different between the dietary treatments at week 40 (Table 15). However, hens offered almost all reduced protein diets except the RP-EAA-Val diet had lower protein intake (P = 0.001) and hens offered RP-EAA-Arg, RP-EAA-Leu and RP-EAA-Gly diets had lower protein excretion (P = 0.018) compared to hens fed the SP diets (Table 15).

Table 12 Serum uric acid level of hens offered dietary treatments at week 40

Treatment	Serum uric acid level (mg/dL)
SP	5.58
RP	4.41
RP-EAA	5.20
RP-EAA-Trp	4.63
RP-EAA-Val	4.94
RP-EAA-Ile	5.31
RP-EAA-Arg	4.67
RP-EAA-Leu	4.98
RP-EAA-His	5.00
RP-EAA-Phe	4.37
RP-EAA-Gly	4.88
SEM	0.32
P-value	0.234

Table 13 Mean eggshell cleanness scores of hens offered dietary treatments at week 40

Treatment	Eggshell cleanness score
SP	0.125
RP	0.125
RP-EAA	0.250
RP-EAA-Trp	0.125
RP-EAA-Val	0.250
RP-EAA-Ile	0.250
RP-EAA-Arg	0.250
RP-EAA-Leu	0.250
RP-EAA-His	0.125
RP-EAA-Phe	0.000
RP-EAA-Gly	0.125
SEM	0.040
P-value	0.949

Table 14 Caecal microbiota population of hens offered dietary treatments at week 40 (log_{10} [genomic DNA copies/g of caecal contents])

Treatment	Lactobacillus spp.	Ruminococcus spp.	Bacteroides spp.	Bacillus spp.	Bifidobacterium spp.	Enterobacteriaceae	Total Bacteria
SP	8.98	9.45	10.88	7.84	9.65	7.27	12.59
RP	8.70	9.34	11.05	7.58	9.76	7.61	12.48
RP-EAA	8.65	9.53	10.95	7.85	9.85	7.40	12.52
RP-EAA-Trp	8.67	9.41	11.01	7.84	9.80	8.07	12.55
RP-EAA-Val	8.86	9.42	11.04	7.68	9.79	7.18	12.51
RP-EAA-Ile	8.69	9.40	11.09	7.77	9.83	7.30	12.52
RP-EAA-Arg	8.72	9.59	10.96	7.86	9.91	7.32	12.57
RP-EAA-Leu	8.71	9.50	10.94	7.77	9.79	7.14	12.59
RP-EAA-His	8.69	9.48	10.95	7.84	9.83	7.60	12.53
RP-EAA-Phe	8.68	9.52	11.10	7.93	9.78	7.52	12.56
RP-EAA-Gly	8.81	9.50	11.06	7.76	9.80	7.88	12.56
SEM	0.11	0.05	0.05	0.14	0.06	0.59	0.05
P-value	0.744	0.082	0.067	0.908	0.373	0.991	0.903

Table 15 Dry matter, protein and energy digestibility of hens offered dietary treatments at week 40

Treatment	Dry matter digestibility (%)	Protein intake per day (g/day)	Protein excreted per day (g/day)	Retained protein per day (g/day)	Protein digestibility (%)	Energy intake per day (Kcal/day)	Energy excreted per day (Kcal/day)	Retained energy per day (Kcal/day)	Energy digestibility (%)
SP	75.24	27.74[a]	13.05[a]	14.68	51.72	567.25	109.8	457.45	80.05
RP	75.65	20.97[b]	10.33[ab]	10.64	49.99	514.19	102.48	411.7	79.82
RP-EAA	76.09	21.28[b]	10.87[ab]	10.4	49.05	489.38	95.24	394.14	80.65
RP-EAA-Trp	76.14	21.75[b]	10.52[ab]	11.23	51.53	498.58	96.07	402.51	80.65
RP-EAA-Val	77.22	22.62[ab]	10.04[ab]	12.58	54.72	511.92	93.84	418.07	81.37
RP-EAA-Ile	75.52	21.63[b]	11.38[ab]	10.25	46.93	506.36	102.27	404.09	79.65
RP-EAA-Arg	77.18	20.58[b]	9.70[b]	10.87	52.57	476.09	86.83	389.26	81.72
RP-EAA-Leu	75.88	19.64[b]	9.61[b]	10.03	50.79	454.01	89.07	364.94	80.25
RP-EAA-His	75.41	20.8[b]	10.74[ab]	10.11	48.64	490.05	96.26	393.78	80.42
RP-EAA-Phe	76.26	20.4[b]	10.19[ab]	10.25	48.56	491.73	94	397.72	80.46
RP-EAA-Gly	76.7	20.9[b]	9.70[b]	11.28	53.44	498.73	97.36	401.37	80.46
SEM	1.43	1.18	0.64	1.13	3.18	26.66	6.01	24.82	1.16
P-value	0.993	0.001	0.018	0.155	0.854	0.367	0.347	0.603	0.984

[a,b] Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

The serum uric acid level is inversely correlated to the net protein utilisation in birds reflecting the relative equivalence between protein intake, utilisation, and degradation (Robin et al. 1987). Previous studies have shown that reducing dietary protein levels from 17% to 13% decreased serum uric acid levels and increased protein digestibility in laying hens (Dao et al. 2021). Thus, the similar serum uric acid levels, eggshell cleanness scores, caecal microbiota counts and nutrient digestibility between hens fed the SP and reduced protein diets in this study might be attributed to the moderate differences in CP levels between the SP and reduced protein diets (20 g/kg). However, it may be worth noting that the laying performance and egg quality of hens fed the SP and reduced protein diets with sufficient essential AA levels were not significantly different. The findings of this study demonstrated the environmental benefits of feeding reduced protein diets by reducing nitrogenous waste in hen excreta compared to hens fed the SP diets. Therefore, reduced protein diets may be used to promote environmental sustainability in the poultry industry, at least in terms of reducing nitrogen output into the environment.

Implications

Based on the current findings, we have demonstrated that Val and Ile are the most important AA while Leu and Gly are the least important AA after Lys, Met and Thr for laying hens fed reduced protein diets. Additionally, hens offered the reduced protein diets had lower protein intake and excretion and similar serum uric acid levels, eggshell cleanness scores, caecal microbiota counts and nutrient digestibility compared to hens fed the SP diet. This study demonstrated the environmental benefits of feeding reduced protein diets by reducing nitrogenous waste released to the environment through birds' excreta, compared to birds fed the SP diets. The outcomes of this study are directly relevant and beneficial to the Australian poultry industry.

Recommendations

Key essential AA including Met, Lys and Thr are widely used in the poultry industry but others including Val, Ile, Arg and Trp, while commercially available, are still quite expensive for widespread use. As more is known regarding how best to use these AA in feed formulation, demand will increase and cost of production will decrease. The decrease in prices of crystalline AA is crucial to extend the adoption of reduced protein diets in the future. In this respect, economic analysis indicating the points at which the next limiting amino acid may be included into diets may be beneficial for industry.

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Media and Publications

Manuscripts are in preparation, and the following conference abstract has arisen from this project:

- A.A. Jahan, T.H. Dao, N. Akter, S. Sukirno, N.K. Sharma, R.A. Swick and A.F. Moss (2023). Order of limiting amino acids in wheat-sorghum based reduced protein diets for laying hens. Proceedings of 34th Annual Australian Poultry Science Symposium, Sydney, Australia, 6-8th February 2023.
- A.A. Jahan, H.T. Dao, N. Akter, S. Sukirno, N.K. Sharma, D.J. Cadogan, E.J. Bradbury, P.V. Chrystal, R.A. Swick, N. Morgan, T.M. Crowley, A.F. Moss (2023). Order of limiting amino acids in wheat-sorghum based reduced protein diet for laying hens. *Animal Production Science* (in preparation).

Intellectual Property Arising

Not applicable. IP generated pertains to the know-how of formulating reduced protein diets and the knowledge described within the report.

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