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Original Research Article

The influence of non-bound amino acid inclusions and starch-protein digestive dynamics on growth performance of broiler chickens offered wheat-based diets with two crude protein concentrations

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ABSTRACT

The primary objective of this study was to investigate the influence of high and low inclusions of non-bound amino acid (NBAA) in standard and reduced-crude protein (CP), wheat-based diets on growth performance in broiler chickens. Dietary treatments were formulated to either 210 or 180 g/kg CP. The 210 g/kg CP diets contained either 12.1 or 21.1 g/kg NBAA and 180 g/kg CP diets contained either 44.0 or 55.5 g/kg NBAA. The formulations also generated different dietary starch:protein ratios which impacted on starch-protein digestive dynamics. Each of the four dietary treatments were offered to 7 replicates of 15 birds housed in floor pens from 14 to 35 days post-hatch or a total of 420 male Ross 308 chickens. Growth performance, relative abdominal fat-pad weights, breast muscle and leg shank yields were determined. Ileal starch and protein (N) digestibility coefficients, disappearance rates and starch:protein disappearance rate ratios were defined. Apparent ileal digestibility coefficients and disappearance rates of 16 amino acids were determined at 35 days post-hatch and free concentrations of 20 amino acids in systemic plasma were determined at 34 days post-hatch. The transition from 210 to 180 g/kg CP diets depressed weight gain by 11.3% (1742 versus 1964 g/bird) and FCR by 10.4% (1.606 versus 1.455), although both parameters were subject to treatment interactions. The treatment interaction ($P < 0.001$) observed for FCR was because high NBAA inclusions significantly improved FCR by 4.17% (1.424 versus 1.486) in birds offered 210 g/kg CP diets, but significantly depressed FCR by 3.36% (1.632 versus 1.579) in 180 g/kg CP diets. A quadratic relationship ($r = 0.860$; $P < 0.001$) between dietary NBAA inclusions and FCR was detected, which indicated that when NBAA inclusions exceed 18.5 g/kg efficiency of feed conversion deteriorated. However, a multiple linear regression ($r = 0.913$; $P < 0.001$) was detected for FCR where both NBAA inclusions and analysed dietary starch:protein ratios were significantly ($P < 0.001$) related to FCR. This relationship indicates that growth performance of broiler chickens offered wheat-based diets is strongly influenced by dietary NBAA inclusions coupled with dietary starch:protein ratios and consideration is given to the possible underlying mechanisms.

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1. Introduction

There are compelling reasons to pursue the development of reduced-crude protein (CP) diets as their potential advantages extend to attenuated nitrogen (N) and ammonia (NH_3) pollution of the environment, improved litter quality and bird welfare, and enhanced flock health (Greenhalgh et al., 2020a). Perhaps more importantly, their successful development would reduce the

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dependence on expensive, imported soybean meal for most countries in the world, including Australia. Wheat is the dominant feed grain in Australian chicken-meat production; however, the reduction of crude protein (CP) concentrations in wheat-based diets for broiler chickens is a real challenge. Birds were better able to accommodate dietary CP reductions when offered maize-based diets in comparison to wheat-based diets in two direct comparisons (Chrystal et al., 2021; Greenhalgh et al., 2022). The relative inferiority of wheat could stem from higher dietary inclusions of non-bound amino acids (NBAA) in wheat-based diets coupled with the more rapid digestion rates of wheat starch which impacts on starch-protein digestive dynamics (Selle et al., 2023).

The forecast made by Baker (2009) was that "there are limits to how much intact protein can be replaced by free amino acids in terms of achieving maximal weight gain and feed efficiency of broiler chicks". This suggests that there are thresholds on the extent to which NBAA can substitute for protein-bound amino acids in reduced-CP broiler diets without compromising growth performance. Non-bound and protein-bound amino acids are probably not bioequivalent because intestinal uptakes of NBAA are more rapid, which has post-enteral consequences (Selle et al., 2022). The differences in intestinal uptakes generate post-enteral amino acid imbalances and amino acids surplus to requirements for protein synthesis are rapidly catabolised (Brosnan, 2003). The protein content of wheat (11.76 g/kg CP; $n = 27$) is typically higher than maize (8.0 g/kg CP; $n = 7$), as surveyed by Bryden et al. (2009). This difference dictates that higher NBAA inclusions in wheat-based, reduced-CP diets are required to meet targeted amino acid specifications combined with lower inclusions of soybean meal. Moreover, Ravindran et al. (1999) reported that the average ileal amino acid digestibility coefficient of soybean meal exceeded that of wheat by 9.68% (0.816 versus 0.774). The likelihood is that both factors are impediments to CP reductions in wheat-based diets.

Thus, there is the possibility that elevated NBAA inclusions are contributing to compromised growth performance of broiler chickens offered reduced-CP, wheat-based diets. Therefore, a 2 × 2 factorial array of dietary treatments was formulated to 210 or 180 g/kg CP with low and high NBAA regimes. The 210 CP g/kg diets contained either 12.1 or 21.1 g/kg NBAA and the 180 g/kg CP diets contained either 44.0 or 55.5 g/kg NBAA. Consequently, specified dietary starch:protein ratios expanded from 1.90 to 1.95 in 210 g/kg CP diets and from 2.74 to 2.80 in 180 g/kg CP diets as more wheat was present in the high NBAA regime diets. This permitted the additional opportunity to assess the impact of dietary starch:protein ratios on the performance of broiler chickens.

The digestion rate of wheat starch is twice that of maize starch under in vitro conditions and wheat generates more rapidly digestible starch (29.5% versus 20.9%) than maize (Giuberti et al., 2012). Moreover, there are indications that slowly digestible starch advantages broiler performance (Enting et al., 2005; Weurding et al., 2003). Slowly digestible pea starch was compared with rapidly digestible wheat starch in broiler diets by Herwig et al. (2019). The balance of 25% slowly and 75% rapidly digestible starch maximised feed efficiency and linearly improved breast meat yield. These researchers suggested that gradual glucose absorption from slowly digestible starch may better match the physiological energy requirements of broiler chickens by allowing the immediate utilisation of glucose for muscle deposition instead of energy storage. The relevance of starch and protein digestive dynamics was demonstrated by Liu et al. (2020) in which six dietary treatments were formulated based on pre-determined starch and protein digestion rates of relevant feedstuffs. In broiler chickens from 7 to 35 days post-hatch, the minimum FCR of 1.450 was supported by a starch:protein digestion rate ratio of 1.663 as predicted by a quadratic relationship ($r = 0.648$; $P < 0.001$). Thus, the premise of starch and protein digestive dynamics is that glucose and amino acids should be made available in appropriately balanced quantities at sites of protein synthesis for efficient protein deposition and growth (Liu and Selle, 2015; Selle and Liu, 2019).

2. Material and methods

2.1. Animal ethics

All experimental procedures fully complied with specific guidelines (2019/1651) issued by the Animal Ethics Committee of the University of Sydney.

2.2. Experimental design

The experimental design comprised a 2 × 2 factorial array of treatments with two dietary crude protein concentrations (210 and 180 g/kg) and low and high regimes of NBAA inclusions. The 'low' regime diets contained 12.13 and 44.01 g/kg NBAA in the 210 and 180 g/kg CP diets, respectively, the corresponding levels in the 'high' regime diets were 21.13 and 55.45 g/kg. The composition and nutrient specifications of the experimental diets are shown in Tables 1 and 2. The four isoenergetic (13.0 MJ/kg ME) diets were formulated to contain 11.0 g/kg digestible lysine and 13.3 g/kg glycine equivalents, a dietary electrolyte balance of 250 mEq/kg and nonstarch polysaccharide (NSP)-degrading enzyme (Danisco Animal Nutrition, København, Denmark) was included across all diets. The NBAA that mainly contributed to the differences between the

Table 1
Composition of the experimental diets (as-is basis).

Item, g/kg	210 g/kg CP diets		180 g/kg CP diets	
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime
Feed ingredient				
Wheat	638.00	655.00	792.00	810.00
Soybean meal	200.00	176.00	29.60	–
Canola seed	60.00	60.00	60.00	60.00
Soy oil	33.40	29.90	6.94	3.70
L-Lysine HCl	4.24	4.96	9.19	10.10
D,L-Methionine	2.59	2.88	3.73	4.08
L-Threonine	1.78	1.99	3.92	4.21
L-Tryptophan	–	–	0.47	0.68
L-Valine	0.94	1.00	3.55	3.72
L-Arginine	0.80	2.14	5.43	6.95
L-Tyrosine	1.32	–	6.50	1.79
L-Isoleucine	0.68	0.74	3.28	3.43
L-Phenylalanine	–	–	–	1.70
L-Leucine	–	0.39	3.66	4.81
L-Histidine	–	–	0.80	1.31
Glycine	0.40	0.73	2.57	2.99
L-Serine	0.31	0.71	2.93	3.44
L-Proline	–	6.68	–	8.46
Sodium chloride	–	–	–	2.72
Sodium bicarbonate	5.54	5.53	5.40	1.43
Potassium carbonate	0.39	1.55	8.22	12.90
Limestone	11.90	12.10	12.70	12.80
Dicalcium phosphate	14.50	14.70	15.70	15.90
Xylanase	0.20	0.20	0.20	0.20
Choline chloride (60%)	0.90	0.90	0.90	0.90
Celite	20.00	20.00	20.00	20.00
Vitamin-mineral premix[1]	2.00	2.00	2.00	2.00
Total NBAA	12.1	21.1	44.0	55.5

CP = crude protein; NBAA = non-bound amino acids.
[1] The vitamin-mineral premix supplied per tonne of feed: retinol 12 MIU, cholecalciferol 5 MIU, tocopherol 50 g, menadione 3 g, thiamine 3 g, riboflavin 9 g, pyridoxine 5 g, cobalamin 0.025 g, niacin 50 g, pantothenate 18 g, folate 2 g, biotin 0.2 g, copper 20 g, iron 40 g, manganese 110 g, cobalt 0.25 g, iodine 1 g, molybdenum 2 g, zinc 90 g, selenium 0.3 g.

Table 2
Nutrient specifications of the experimental diets (as-is basis; amino acids expressed on a digestible basis).

Item, g/kg	210 g/kg CP diets		180 g/kg CP diets	
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime
Metabolisable energy, MJ/kg	13.0	13.0	13.0	13.0
Crude protein	210	210	180	180
Starch	399	409	493	504
Starch:protein ratio	1.90	1.95	2.74	2.80
Calcium	8.25	8.25	8.25	8.25
Total phosphorus	6.26	6.20	5.88	5.80
Available phosphorus	4.13	4.13	4.13	4.13
Sodium	1.80	1.80	1.80	1.80
Potassium	8.00	8.00	9.00	11.00
Chloride	1.37	1.52	2.40	4.21
Electrolyte balance, mEq/kg	250	250	250	250
Crude fat	71.5	68.0	45.6	42.3
Crude fibre	20.9	20.4	18.0	17.4
Lysine	11.00	11.00	11.00	11.00
Arginine	11.40	12.10	11.40	12.10
Histidine	4.28	4.07	3.63	3.85
Isoleucine	7.70	7.37	7.70	7.37
Leucine	12.40	12.10	11.80	12.10
Methionine	5.12	5.31	5.57	5.79
Phenylalanine	8.34	7.90	5.47	6.60
Threonine	7.37	7.26	7.37	7.26
Tryptophan	2.13	2.01	1.82	1.87
Valine	8.80	8.47	8.80	8.47
Glycine	7.26	7.26	7.26	7.26
Proline	14.00	20.20	12.30	20.20
Serine	8.49	8.49	8.49	8.49
Tyrosine	6.81	5.17	9.67	4.62
Glycine equivalents	13.30	13.30	13.30	13.30

CP = crude protein; NBAA = non-bound amino acids.

two regimes were proline, phenylalanine, arginine and leucine, but the ideal amino acid ratios of all four diets were consistent with Aviagen recommendations.

2.3. Diet preparation

The diets were formulated based on near-infrared spectroscopy (NIR) of wheat, soybean meal and canola seed via the AMINONir Advanced program (Evonik Nutrition & Care GmbH, Hanau, Germany). Acid insoluble ash (AIA; Celite World Minerals, Lompoc, CA, USA) was included at 20 g/kg in all diets as an inert marker to determine the digestibility coefficients of starch, protein (N) and amino acids. The diets were steam-pelleted at a temperature of 80 °C with a conditioner residence time of 14 s. The analysed nutrient compositions of the diets are shown in Table 3, where some discrepancies are evident. Lysine is an example, which may have arisen because extractions of both non-bound and protein-bound lysine in the analyses lacked uniformity.

2.4. Bird management

A total of 420 off-sex (parent line) male Ross 308 one-day-old chicks were procured from a commercial hatchery and offered a common starter diet from 1 to 13 days post-hatch. At 14 days post-hatch, birds were individually identified (wing-tags) and allocated into 28 floor pens (15 birds per pen) based on body weights. The mean body weight of the 28 pens was 409 ± 14.7 g/bird at 14 days post-hatch. Each of the dietary treatments were then offered to 7 replicate floor pens, holding 15 birds per pen, from 14 to 35 days post-hatch. The pen dimensions were 1.5 m in both width and depth. Birds had unrestricted access to feed and water in an environmentally controlled facility with a 23-h-on-1-h-off lighting regime for the first week and an 18-h-on-6-h-off lighting regime during weeks 1 to 5. An initial room temperature of 32 ± 1 °C was maintained for the first week, which was gradually decreased to 22 ± 1 °C by the end of the third week and maintained at this temperature for the duration of the feeding study. Body weights were determined at 14 and 35 days post-hatch and feed intakes were recorded from which feed conversion ratios were calculated. The incidence of dead or culled birds was recorded daily and their body weights used to adjust feed intake and FCR calculations.

Table 3
Analysed concentrations of the experimental diets.

Item, g/kg	210 g/kg CP diets		180 g/kg CP diets	
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime
Crude protein	201	209	175	161
Starch	361	350	432	450
Starch:protein ratio	1.80	1.67	2.47	2.80
Arginine	11.3	12.0	10.1	8.8
Histidine	4.9	4.8	4.0	3.8
Isoleucine	8.6	8.3	7.4	6.5
Leucine	13.6	13.4	12.1	11.4
Lysine	11.3	12.0	11.1	9.6
Methionine	4.3	4.1	3.8	3.2
Phenylalanine	9.0	8.8	6.5	6.9
Threonine	8.0	8.1	6.8	5.8
Valine	9.8	9.7	9.4	8.4
Alanine	7.5	7.6	5.2	4.6
Aspartic acid	14.9	14.5	8.1	6.5
Glutamic acid	46.7	45.7	40.0	38.9
Glycine	8.4	9.2	7.8	6.8
Proline	14.2	20.3	12.8	17.3
Serine	9.4	9.7	8.2	7.3
Tyrosine	5.3	4.0	6.3	3.1

CP = crude protein; NBAA = non-bound amino acids.

2.5. Sample collection and chemical analysis

At 34 days post-hatch, blood samples were collected in ethylenediaminetetraacetic acid contained vacutainer tubes (BD Vacutainer K2E, Plymouth, UK) from the brachial vein of 3 birds in each replicate pen for determining the concentrations of 20 proteinogenic amino acids in systemic plasma. Blood samples from birds within a pen were pooled and centrifuged and kept at −80 °C prior to analysis. Afterwards, decanted plasma samples were analysed using precolumn derivatisation amino acid analysis with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC; Waters AccQ-Tag Ultra, Waters Australia PL; www.waters.com) followed by separation of the derivatives and quantification by reversed phase ultra-performance liquid chromatography (RP-UPLC) as described by Cohen and Michaud (1993). All amino acids were detected by UV absorbance and quantitated using the Waters Empower software.

At day 35, 7 birds from each pen were euthanised by intravenous injections of sodium pentobarbitone, the abdominal cavity opened, and small intestines removed. Digesta samples were collected in their entirety from the distal ileum. The distal ileum was demarcated by the mid-point between Meckel's diverticulum proximally and the ileo-caecal junction proximally, and the ileo-caecal junction distally. Digesta were manually expressed from this segment and digesta samples were pooled for each pen, homogenized, freeze-dried, and ground through a 0.5-mm screen to analyse starch, protein (N) and amino acid concentrations. In addition, abdominal fat-pads were dissected out, weighed and recorded against final body weights to calculate relative abdominal fat-pad weights. Also, *Pectoralis* major, *Pectoralis* minor, and thigh

muscles were removed from the carcass and recorded against final body weights to calculate relative weights of carcass traits.

Starch concentrations in feed and digesta samples were determined by using total starch assay kits (Megazyme, Wicklow, Ireland) as described in Mahasukhonthachat et al. (2010). Nitrogen contents of diets and digesta were determined using a nitrogen determinator (Leco Corporation, St Joseph, MI) by the Dumas method and AIA concentrations were determined by the method described by Siriwan et al. (1994). Amino acid concentrations of diets and digesta were determined by 24-h liquid hydrolysis at 110 °C in 6 mol/L HCl followed by analysis of 16 amino acids using the Waters AccQ-Tag Ultra chemistry on a Waters Acquity UPLC (Waters Corporation, Milford, Massachusetts). The apparent digestibility coefficients for starch, protein (N) and amino acids sites were calculated from the following equation:

$$\text{Digestibility coefficient} = \frac{(\text{Nutrient/AIA})_{\text{diet}} - (\text{Nutrient/AIA})_{\text{digesta}}}{(\text{Nutrient/AIA})_{\text{diet}}}$$

Starch, protein (N) and amino acid disappearance rates (g/bird per day) were calculated from the following equation:

$$\text{Nutrient disappearance rate}_{(\text{g/bird per day})} = \text{feed intake}_{(\text{g/bird})} \times \text{dietary nutrient}_{(\text{g/kg})} \times \text{digestibility coefficient}$$

Ratios of starch to protein disappearance rates in the distal ileum were calculated as this eliminates the confounding influence of feed intake.

2.6. Statistical analyses

The experimental data was analysed by two-way analyses of variance using the JMP Pro 14.0 software package (JMP Software, SAS Institute Inc., Cary, NC). Linear and quadratic relationships and multiple linear regressions were established when considered appropriate. Pen means were the experimental unit and a probability level of less than 5% was considered statistically significant.

3. Results

Treatment effects on growth performance, relative abdominal fat-pad weights, breast muscle and leg quarter yields are shown in Table 4. A treatment interaction was observed for weight gain ($P = 0.039$) as the high NBAA regime numerically increased weight gain by 2.27% (1986 versus 1942 g/bird) in birds offered 210 g/kg CP diets, but numerically decreased weight gain by 4.38% (1703 versus 1781 g/bird) with 180 g/kg CP diets. Feed intake was not influenced ($P > 0.05$) by treatment. A treatment interaction was observed for FCR ($P < 0.001$). The high NBAA regime significantly improved FCR by 4.17% (1.424 versus 1.486) in 210 g/kg CP diets, but significantly compromised FCR by 3.36% (1.632 versus 1.579) in 180 g/kg CP diets. There was a treatment interaction ($P = 0.022$) for relative fat-pad weights as the high NBAA regime significantly increased fat-pad weights by 11.8% (12.30 versus 11.00 g/kg) in birds offered 180 g/kg CP diets but had no influence with 210 g/kg diets. Overlooking treatment interactions, dietary CP reductions compromised weight gain by 11.3%, FCR by 10.4% and generated 38.1% heavier fat-pads weights as main effects. The overall mortality rate of 2.63% was acceptable, although a significantly higher mortality rate of 4.29% was observed in birds offered 210 g/kg CP diets, as opposed to 0.95% for their 180 g/kg CP counterparts. The dietary CP reduction depressed breast muscle yields by 8.74% (188 versus 206 g/kg; $P < 0.001$), but leg quarter yields were not influenced by treatment ($P > 0.05$).

The effects of dietary treatments on starch and protein (N) digestive dynamics are displayed in Table 5. The dietary CP reduction depressed ileal starch digestibility coefficients by 2.12% (0.970 versus 0.991; $P = 0.013$) and protein (N) digestibility by 6.16% (0.716 versus 0.763; $P = 0.038$). Additionally, the high NBAA regime increased protein (N) digestibility by 6.99% (0.765 versus 0.715; $P = 0.028$). The dietary CP reduction increased starch disappearance rates by 18.9% (56.94 versus 47.88 g/bird per day; $P < 0.001$), but decreased protein (N) disappearance rates by 24.8% (15.98 versus 21.26 g/bird per day; $P < 0.001$). Finally, the dietary CP reduction increased ($P < 0.001$) the starch:protein disappearance rate ratio from 2.26 to 3.59 in the distal ileum.

The effects of dietary treatments on apparent ileal amino acid digestibility coefficients are shown in Table 6. The one treatment interaction ($P = 0.033$) observed was for phenylalanine as the high NBAA regime significantly increased phenylalanine digestibility by 12.8% (0.836 versus 0.741) in 180 g/kg CP diets but had no significant influence in 210 g/kg CP diets. Tyrosine was the only amino

Table 4
Effects of dietary treatments on growth performance and relative weights of breast muscle, leg shanks and abdominal fat-pads from 14 to 35 days post-hatch.

Item	210 g/kg CP diets		180 g/kg CP diets		SEM	CP		NBAA regime		*P*-value		
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime		210	180	Low	High	CP	NBAA regime	CP × NBAA interaction
Growth performance												
Weight gain, g/bird	1942[b]	1986[b]	1781[a]	1703[a]	27.4	1964	1742	1862	1844	<0.001	0.541	0.039
Feed intake, g/bird	2887	2829	2814	2777	49.1	2858	2795	2851	2803	0.213	0.339	0.831
FCR, g/g	1.486[b]	1.424[a]	1.579[c]	1.632[b]	0.0147	1.455	1.606	1.533	1.528	<0.001	0.747	<0.001
Mortality, %	3.81	4.76	0.95	0.95	1.351	4.29[b]	0.95[a]	2.38	2.86	0.021	0.727	0.727
Relative weight												
Breast, g/kg	205	206	191	185	3.0	206[b]	188[a]	198	194	<0.001	0.407	0.314
Leg shanks, g/kg	197	194	195	198	2.0	196	197	196	196	0.629	0.999	0.241
Fat-pads, g/kg	8.94[a]	8.00[a]	11.00[b]	12.30[c]	0.453	8.47	11.74	9.96	10.51	<0.001	0.670	0.022

CP = crude protein; NBAA = non-bound amino acids.
[a,b,c] Means within a row not sharing a common superscript are significantly different at the 5% level of probability.

Table 5
Effects of dietary treatments on apparent ileal starch and protein (N) digestibility coefficients, daily starch and protein (N) disappearance rates and starch:protein disappearance rate ratios at 35 days post-hatch.

Item	210 g/kg CP diets		180 g/kg CP diets		SEM	CP		NBAA regime		*P*-value		
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime		210	180	Low	High	CP	NBAA regime	CP × NBAA interaction
Apparent ileal digestibility coefficient												
Starch	0.988	0.993	0.981	0.960	0.0078	0.991[b]	0.970[a]	0.984	0.975	0.013	0.309	0.118
Protein (N)	0.750	0.776	0.680	0.753	0.0213	0.763[b]	0.716[a]	0.715[a]	0.765[b]	0.038	0.028	0.282
Disappearance rate, g/bird per day												
Starch	48.90	46.87	56.80	57.08	1.127	47.88[a]	56.94[b]	53.15	52.37	<0.001	0.444	0.318
Protein (N)	20.66	21.86	15.91	16.05	0.500	21.26[b]	15.98[a]	18.28	18.95	<0.001	0.190	0.299
Starch:protein disappearance rate ratio	2.36	2.16	3.59	3.59	0.118	2.26[a]	3.59[b]	3.02	2.93	<0.001	0.414	0.397

CP = crude protein; NBAA = non-bound amino acids.
[a,b] Means within a row not sharing a common superscript are significantly different at the 5% level of probability.

Table 6
Effects of dietary treatments on apparent ileal amino acid digestibility coefficients at 35 days post-hatch.

Item	210 g/kg CP diets		180 g/kg CP diets		SEM	CP		NBAA regime		*P*-value		
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime		210	180	Low	High	CP	NBAA regime	CP × NBAA interaction
Arginine	0.840	0.861	0.801	0.866	0.0139	0.850	0.833	0.820[a]	0.863[b]	0.236	0.005	0.123
Histidine	0.776	0.790	0.696	0.782	0.0176	0.783[b]	0.739[a]	0.736[a]	0.786[b]	0.020	0.009	0.053
Isoleucine	0.786	0.801	0.756	0.826	0.0200	0.794	0.791	0.771[a]	0.814[b]	0.903	0.044	0.186
Leucine	0.785	0.803	0.765	0.840	0.0186	0.794	0.803	0.775[a]	0.822[b]	0.647	0.019	0.137
Lysine	0.802	0.839	0.788	0.840	0.0180	0.826	0.825	0.800[a]	0.850[b]	0.936	0.011	0.240
Methionine	0.925	0.926	0.872	0.913	0.0095	0.926[b]	0.893[a]	0.899[a]	0.920[b]	0.002	0.037	0.051
Phenylalanine	0.804[b]	0.820[b]	0.741[a]	0.836[b]	0.0176	0.812	0.789	0.773	0.828	0.203	0.004	0.033
Threonine	0.721	0.747	0.645	0.692	0.0262	0.734[b]	0.668[a]	0.683	0.719	0.019	0.182	0.682
Valine	0.765	0.783	0.742	0.808	0.0197	0.774	0.775	0.754[a]	0.796[b]	0.953	0.044	0.238
Alanine	0.707	0.740	0.536	0.676	0.0335	0.724[b]	0.606[a]	0.622[a]	0.708[b]	0.002	0.016	0.124
Aspartic acid	0.696	0.725	0.513	0.650	0.0312	0.710[b]	0.582[a]	0.605[a]	0.687[b]	<0.001	0.014	0.095
Glutamic acid	0.882	0.892	0.866	0.913	0.0098	0.887	0.889	0.874[a]	0.903[b]	0.840	0.008	0.074
Glycine	0.713	0.756	0.661	0.732	0.0233	0.735	0.697	0.687[a]	0.744[b]	0.118	0.023	0.545
Proline	0.848	0.901	0.824	0.897	0.0096	0.875	0.860	0.836[a]	0.899[b]	0.156	<0.001	0.309
Serine	0.761	0.790	0.711	0.786	0.0203	0.776	0.749	0.736[a]	0.788[b]	0.196	0.018	0.271
Tyrosine	0.811	0.774	0.833	0.816	0.0184	0.792	0.825	0.822	0.795	0.094	0.153	0.579

CP = crude protein; NBAA = non-bound amino acids.
[a,b] Means within a row not sharing a common superscript are significantly different at the 5% level of probability.

acid not influenced by treatment. The dietary CP reduction depressed digestibilities of histidine by 5.62% (0.739 versus 0.783; $P = 0.020$), methionine by 3.56% (0.893 versus 0.926; $P = 0.002$), threonine by 8.99% (0.688 versus 0.734; $P = 0.019$), alanine by 16.3% (0.606 versus 0.724; $P = 0.002$) and aspartic acid by 18.0% (0.582 versus 0.710; $P < 0.001$). Again, the high NBAA regime significantly increased digestibilities of 13 of the 16 amino acids assessed as a main effect with no treatment interaction, where threonine and tyrosine were the exceptions. Collectively, the high NBAA regime increased average digestibility coefficients of these 13 amino acids by 6.68% (0.814 versus 0.763). Across the essential amino acids, the high NBAA regime increased arginine by 5.24% (0.863 versus 0.820; $P = 0.005$), histidine by 6.79% (0.786 versus 0.736; $P = 0.009$), isoleucine by 5.58% (0.814 versus 0.771; $P = 0.044$), leucine by 6.25% (0.822 versus 0.775; $P = 0.019$), lysine by 6.25% (0.850 versus 0.800; $P = 0.011$), methionine by 2.34% (0.920 versus 0.899; $P = 0.037$) and valine 5.57% (0.796 versus 0.754; $P = 0.044$). Across the non-essential amino acids, the high NBAA regime increased alanine by 13.8% (0.708 versus 0.622; $P = 0.016$), aspartic acid by 13.6% (0.687 versus 0.605; $P = 0.014$), glutamic acid by 3.32% (0.903 versus 0.874; $P = 0.008$), glycine 8.30% (0.744 versus 0.687; $P = 0.023$), proline by 7.54% (0.899 versus 0.836; $P < 0.001$) and serine by 7.07% (0.788 versus 0.736; $P = 0.018$).

The responses of distal ileal amino acid disappearance rates to dietary treatments are shown in Table 7 where significant treatment interactions were observed for 6 amino acids. With both arginine and lysine the transition from low to high NBAA regimes significantly accelerated disappearance rates in 210 g/kg CP diets, but significantly retarded disappearance rates in birds offered 180 g/kg CP diets. Disappearance rates of phenylalanine significantly increased following the transition from low to high NBAA regimes but only in 180 g/kg CP diets. Disappearance rates of glycine significantly increased following the transition from low to high NBAA regimes but only in 210 g/kg CP diets. Proline disappearance rates significantly accelerated following the low to high NBAA transition at both dietary CP levels but this was more pronounced in birds offered 210 g/kg CP diets. Tyrosine disappearance rates were significantly retarded from the low to high NBAA transition at both dietary CP levels but this was more pronounced in 180 g/kg CP diets. As main effects, the dietary CP reduction significantly depressed the disappearance rates of 10 amino acids with no treatment interaction. Collectively, average disappearance rates were depressed by 24.3% (1.06 versus 1.40 g/bird per day) and depressions ranged from 9.80% for valine to 60.6% for aspartic acid across the 10 amino acids. The transition from low to high NBAA regimes decreased methionine disappearance rates by 10.2% (0.44 versus 0.49 g/bird per day; $P < 0.001$).

Treatment effects on concentrations of 20 free amino acids in systemic plasma are displayed in Table 8. Treatment interactions were observed for histidine ($P = 0.036$), phenylalanine ($P = 0.004$), tryptophan ($P = 0.022$) and tyrosine ($P = 0.011$). The

Table 7
Effects of dietary treatments on apparent amino acid disappearance rates (g/bird per day) in distal ileum at 35 days post-hatch.

Item	210 g/kg CP diets		180 g/kg CP diets		SEM	CP		NBAA regime		*P*-value		
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime		210	180	Low	High	CP	NBAA regime	CP × NBAA interaction
Arginine	1.30^{c}	1.39^{d}	1.08^{b}	1.01^{a}	0.022	1.35	1.05	1.19	1.20	<0.001	0.749	0.001
Histidine	0.52	0.51	0.37	0.39	0.010	0.52^{b}	0.38^{a}	0.45	0.45	<0.001	0.618	0.131
Isoleucine	0.93	0.90	0.75	0.71	0.020	0.91^{b}	0.72^{a}	0.84	0.80	<0.001	0.093	0.858
Leucine	1.46	1.45	1.24	1.27	0.032	1.46^{b}	1.25^{a}	1.35	1.36	<0.001	0.814	0.498
Lysine	1.26^{c}	1.37^{d}	1.17^{b}	1.09^{a}	0.026	1.31	1.13	1.21	1.22	<0.001	0.680	0.002
Methionine	0.55	0.51	0.44	0.39	0.007	0.53^{b}	0.41^{a}	0.49^{b}	0.44^{a}	<0.001	<0.001	0.105
Phenylalanine	0.99^{c}	0.97^{c}	0.64^{a}	0.76^{b}	0.019	0.98	0.70	0.82	0.87	<0.001	0.015	0.001
Threonine	0.79	0.81	0.59	0.53	0.022	0.80^{b}	0.56^{a}	0.69	0.67	<0.001	0.492	0.086
Valine	1.03	1.02	0.93	0.90	0.024	1.02^{b}	0.92^{a}	0.98	0.96	<0.001	0.414	0.531
Alanine	0.73	0.76	0.37	0.41	0.023	0.74^{b}	0.39^{a}	0.55	0.58	<0.001	0.134	0.859
Aspartic acid	1.42	1.42	0.55	0.56	0.037	1.42^{b}	0.56^{a}	0.99	0.99	<0.001	0.993	0.894
Glutamic acid	5.66	5.49	4.64	4.70	0.082	5.58^{b}	4.67^{a}	5.15	5.10	<0.001	0.528	0.185
Glycine	0.82^{b}	0.94^{c}	0.69^{a}	0.66^{a}	0.022	0.88	0.67	0.76	0.80	<0.001	0.069	0.003
Proline	1.65^{b}	2.46^{d}	1.41^{a}	2.05^{c}	0.032	2.06	1.73	1.53	2.26	<0.001	<0.001	0.014
Serine	0.98	1.03	0.78	0.75	0.022	1.01^{b}	0.77^{a}	0.88	0.90	<0.001	0.480	0.111
Tyrosine	0.59^{c}	0.42^{b}	0.70^{d}	0.33^{a}	0.011	0.50	0.52	0.65	0.38	0.181	<0.001	<0.001

CP = crude protein; NBAA = non-bound amino acids.
[a,b,c,d] Means within a row not sharing a common superscript are significantly different at the 5% level of probability.

Table 8
Effects of dietary treatments on free amino acid concentrations (μg/mL) in systemic plasma at 34 days post-hatch.

Item	210 g/kg CP diets		180 g/kg CP diets		SEM	CP		NBAA regime		*P*-value		
	Low NBAA regime	High NBAA regime	Low NBAA regime	High NBAA regime		210	180	Low	High	CP	NBAA regime	CP × NBAA interaction
Arginine	47.1	46.6	26.7	32.0	4.80	46.8^{b}	29.3^{a}	36.9	39.3	0.001	0.622	0.542
Histidine	7.2^{b}	4.8^{a}	5.2^{a}	4.9^{a}	0.45	6.0	5.1	6.2	4.9	0.051	0.008	0.036
Isoleucine	18.0	13.1	12.9	9.9	1.11	15.6^{b}	11.4^{a}	15.4^{b}	11.5^{a}	0.001	0.002	0.398
Leucine	23.5	19.2	20.8	21.1	1.61	21.3	21.0	22.1	20.1	0.836	0.224	0.164
Lysine	29.2	24.5	55.0	31.6	5.88	26.9^{a}	43.3^{b}	42.1^{b}	28.1^{a}	0.001	0.025	0.125
Methionine	16.8	14.1	17.1	17.4	1.33	15.5	17.3	17.0	15.8	0.182	0.359	0.274
Phenylalanine	18.9^{c}	16.0^{b}	13.4^{a}	15.9^{b}	0.83	17.5	14.6	16.2	16.0	0.002	0.802	0.004
Threonine	78.6	59.9	70.0	50.0	12.93	69.3	59.8	74.2	55.0	0.473	0.151	0.969
Tryptophan	6.5^{b}	4.7^{a}	5.1^{a}	4.8^{a}	0.29	5.6	5.0	5.8	4.8	0.037	0.001	0.022
Valine	34.4	26.3	39.1	31.7	2.41	30.3^{a}	35.4^{b}	36.8^{b}	29.0^{a}	0.047	0.004	0.901
Alanine	81.4	82.9	76.3	72.8	5.35	82.2	74.5	78.9	77.9	0.168	0.854	0.655
Asparagine	24.4	16.0	13.6	10.9	1.53	20.2^{b}	12.4^{a}	19.0^{b}	13.7^{a}	<0.001	0.002	0.072
Aspartate	12.4	16.1	11.6	12.8	1.38	14.2	12.2	12.0	14.4	0.152	0.090	0.371
Cysteine	16.7	15.0	15.7	15.2	0.59	15.8	15.4	16.2	15.1	0.513	0.075	0.350
Glutamine	224.6	229.4	281.8	267.2	17.10	227.0^{a}	274.5^{b}	253.2	248.3	0.011	0.774	0.577
Glutamate	23.2	25.4	23.5	22.8	1.19	24.3	23.1	23.4	24.1	0.345	0.576	0.234
Glycine	63.4	59.1	69.7	65.6	3.86	61.3	67.6	66.6	62.4	0.114	0.286	0.985
Proline	69.1	105.4	69.7	133.1	7.90	87.2	101.4	69.4^{a}	119.2^{b}	0.085	<0.001	0.099
Serine	64.4	58.7	77.7	67.4	3.33	61.5^{a}	72.6^{b}	71.0^{b}	63.1^{a}	0.003	0.025	0.499
Tyrosine	49.3^{b}	29.8^{a}	64.8^{c}	30.7^{a}	2.61	39.6	47.8	57.0	30.3	0.005	<0.001	0.011

CP = crude protein; NBAA = non-bound amino acids.
[a,b,c] Means within a row not sharing a common superscript are significantly different at the 5% level of probability.

histidine, phenylalanine and tryptophan interactions were driven by the high NBAA regime depressing their free concentrations in birds offered 210 g/kg CP diets. With tyrosine, the high NBAA regime depressed concentrations at both dietary CP levels but this was more pronounced in the 180 g/kg CP diets. As a main effect, reducing dietary CP levels significantly increased plasma concentrations of lysine (61.0%), valine (16.8%), glutamine (20.9%) and serine (18.0%), but significantly decreased free plasma concentrations of arginine (37.4%), isoleucine (26.9%) and asparagine (38.6%). As a main effect, the high NBAA regime significantly increased proline plasma concentrations by 71.8%, but significantly decreased free plasma concentrations of isoleucine (25.3%), lysine (33.3%), valine (21.2%), asparagine (27.9%) and serine (11.1%). The balance of 7 amino acids (leucine, methionine, alanine, aspartic acid, cysteine, glutamic acid, glycine) was not significantly influenced by treatment.

4. Discussion

The growth performance of birds offered 210 g/kg CP diets in the present study exceeded the 2022 Aviagen performance objectives for male Ross 308 broilers from 14 to 35 days post-hatch by 3.37% (1964 versus 1900 g/bird) in weight gain and by 3.06% (1.455 versus 1.501) in FCR. The mortality rate of birds offered 210 g/kg CP diets (4.29%) was statistically higher than their 180 g/kg CP counterparts (0.95%), but this difference may not carry any biological significance. Thus, growth performance was satisfactory; nevertheless, the reasonably moderate 30 g/kg dietary CP reduction compromised weight gain by 11.3% and FCR by 10.4% as main effects. These depressions in growth performance pursuant to dietary CP reductions illustrate the challenge of developing reduced-CP, wheat-based diets. This outcome is consistent with several locally completed feeding studies with wheat-based, reduced-CP diets

(Greenhalgh et al., 2020b; Hilliar et al., 2019, 2020; Yin et al., 2020) and as mentioned, two direct comparisons between wheat- and maize-based diets (Chrystal et al., 2021; Greenhalgh et al., 2022). Obviously, for chicken-meat production in Australia, and other countries where wheat is used extensively in broiler diets, there is a real need to identify the causative factors so that the poor performance of birds offered reduced-CP, wheat-based diets can be addressed.

The striking outcomes of the present study are the treatment interactions observed for weight gain and FCR where higher NBAA inclusions in 210 g/kg CP diets were beneficial, but detrimental in 180 g/kg CP diets. It was proposed that lysine requirements should be expressed as a proportion of dietary CP rather than in absolute terms (Morris et al., 1999). Extending this to NBAA inclusions, then NBAA represented approximately 7.90% of CP in 210 g/kg diets as opposed to a 27.6% proportion in 180 g/kg CP diets, which is a 3.5-fold increase. There is a quadratic relationship ($r = 0.860$; $P < 0.001$) between dietary NBAA inclusions and FCR as shown in Fig. 1. It may be deduced from the regression equation that an NBAA inclusion of 18.5 g/kg would support the minimum FCR, but higher NBAA inclusions would compromise FCR in a quadratic manner. Thus, it appears that poultry can utilise relatively small proportions of dietary NBAA to advantage but are not able to accommodate high NBAA inclusion levels.

A similar pattern of responses was observed in Chrystal et al. (2020) in birds offered reduced-CP, maize-based diets. The transition from 195 g/kg CP diets (7.9 g/kg NBAA) to 180 g/kg CP diets (14.0 g/kg NBAA) numerically improved FCR by 1.35% (1.538 versus 1.559); however, the further transition from 180 to 165 g/kg CP diets (23.5 g/kg NBAA) significantly compromised FCR by 4.55% (1.608 versus 1.538). It appears that the NBAA inclusion increase from 14.0 to 23.5 g/kg significantly compromised FCR in part by generating post-enteral amino acid imbalances, as discussed below. The beneficial impact of the NBAA inclusion increase from 7.9 to 14.0 g/kg possibly may be attributed to an acceleration in protein digestion rates which has been reported to be advantageous in broiler chickens (Liu et al., 2020).

Intestinal uptakes of NBAA are more rapid than protein-bound amino acids (Wu, 2009); therefore, the likelihood is that the two amino acid sources are not totally bioequivalent (Selle et al., 2022). It follows that elevating NBAA inclusions promotes post-enteral amino acid imbalances and in Macelline et al. (2022), maximum weight gain and minimum FCR were supported by dietary inclusions of 13.1 g/kg NBAA and higher inclusions were detrimental to growth performance. Importantly, amino acids surplus to requirements for protein synthesis are rapidly catabolised (Brosnan, 2003) and NBAA are more likely to be catabolised, or undergo post-prandial oxidation (Nolles et al., 2009). Also, post-enteral amino acid imbalances triggered by high dietary NBAA inclusions invite the 'costs of deamination' (Selle et al., 2022), including NH_3 detoxification which demands inputs of glycine (and serine) and energy so that NH_3 is eliminated as uric acid (Salway, 2018). Moreover, the synthesis and excretion of 1 g of uric acid requires a 64.7 kJ energy input (Van Milgen, 2021). Instructively, escalating NBAA inclusions in reduced-CP broiler diets were quadratically related to increases in NH_3 concentrations in excreta in Hofmann et al. (2019), which is consistent with deamination of NBAA generating excess NH_3. Similarly, we have found escalating NBAA inclusions linearly increased NH_3 plasma concentrations in as yet unpublished data.

However, due to the tangible differences in the composition of standard and reduced-CP broiler diets, additional factors are involved including the higher inclusions of wheat and 'rapid' starch in wheat-based diets. The digestive dynamics of starch and protein should be considered in tandem as Moughan (2003) concluded that the efficiency of protein synthesis is markedly influenced by the harmonisation of the provision of amino acids and energy to sites of protein synthesis where the bulk of the energy is supplied from glucose derived from dietary starch. Therefore, the quadratic relationship ($r = 0.863$; $P < 0.001$) between starch:protein disappearance rate ratios and FCR detected in the present study is an instructive outcome, as shown in Fig. 2. From the regression equation it may be predicted that condensing starch:protein disappearance rate ratios from 3.87 will generate FCR improvements in a quadratic manner. The pertinent point is that dietary CP reductions will increase wheat, and thus starch, inclusion rates and amplify dietary starch:protein ratios which are transferred to expanded starch:protein disappearance rate ratios in broiler

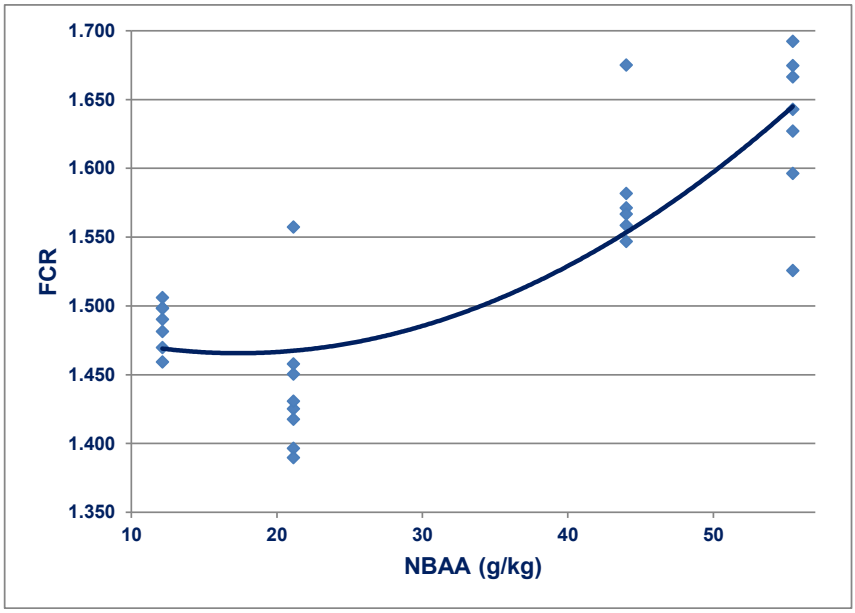

Fig. 1. Quadratic relationship ($r = 0.860$; $P < 0.0001$) between dietary concentrations of non-bound amino acids (NBAA) and FCR, FCR = 1.507 − 0.005074 × NBAA + 0.000137 × $NBAA^2$.

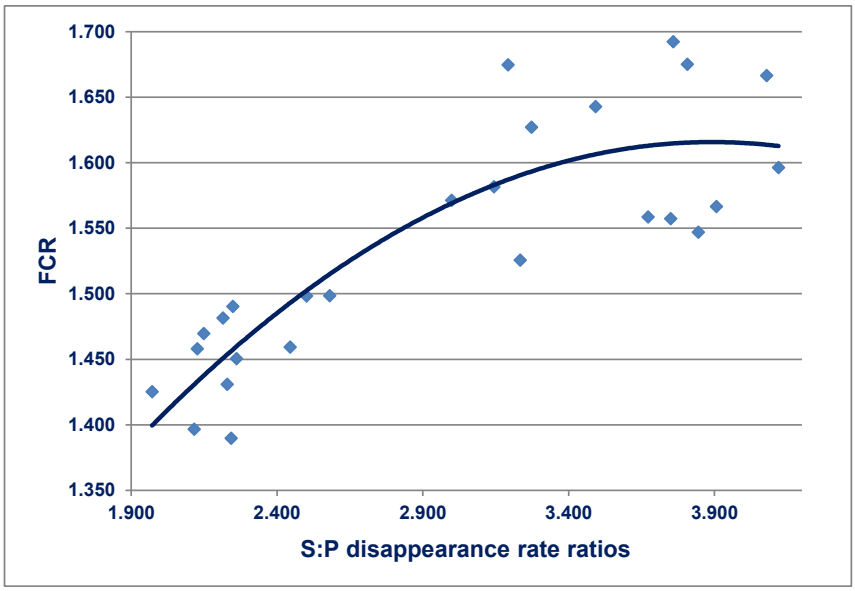

Fig. 2. Quadratic relationship ($r = 0.863$; $P < 0.0001$) between starch:protein disappearance rate ratios (S:P) and FCR, FCR = 0.727 + 0.457 × S:P − 0.059 × $(S:P)^2$.

chickens (Liu et al., 2021). Therefore, the average increase in wheat (and starch) inclusions of 23.8% from 647 to 801 g/kg from dietary CP reductions is counter to the edicts of starch-protein digestive dynamics.

Different dietary CP contents and NBAA inclusions generated perturbations in amino acid digestibility coefficients, this is not without precedent (Liu et al., 2021), and was evident in the present study. The dietary CP reduction significantly decreased apparent ileal digestibilities of 5 amino acids (histidine, methionine, threonine, alanine, aspartic acid) collectively by 9.42% (0.702 versus 0.775). This can be attributed to the dilution of dietary amino acid concentrations in ileal digesta by amino acids of endogenous and microbial origins following the dietary CP reduction (Ravindran, 2021). Alternatively, higher NBAA inclusions significantly increased apparent ileal digestibilities of 13 amino acids collectively by 7.32% (0.814 versus 0.758). This can be attributed in part to the ostensibly complete digestion of NBAA (Lemme et al., 2005); however, 3 of 13 amino acids (alanine, aspartic acid, glutamic acid) were present in the experimental diets as only protein-bound entities. This outcome may suggest that competition between amino acids for intestinal uptakes (Gous et al., 1977) may have been the cause.

Free plasma amino acid concentrations reflect a dynamic balance between dietary supply, post-enteral availability and their incorporation into body proteins and protein degradation through oxidative catabolism, which is further complicated by the biosynthesis of non-essential amino acids (Ng and Pascaud, 1990). Consequently, the interpretation of plasma amino acid data in relation to broiler performance is problematic, despite the fact they have been evaluated for decades (Smith and Scott, 1965). Nevertheless, free amino acid concentrations of serine ($r = 0.506$; $P = 0.006$), lysine ($r = 0.414$; $P = 0.028$) and valine ($r = 0.414$; $P = 0.028$) were positively correlated with FCR, in contrast, arginine ($r = -0.577$; $P = 0.001$) and asparagine ($r = -0.470$; $P = 0.013$) were

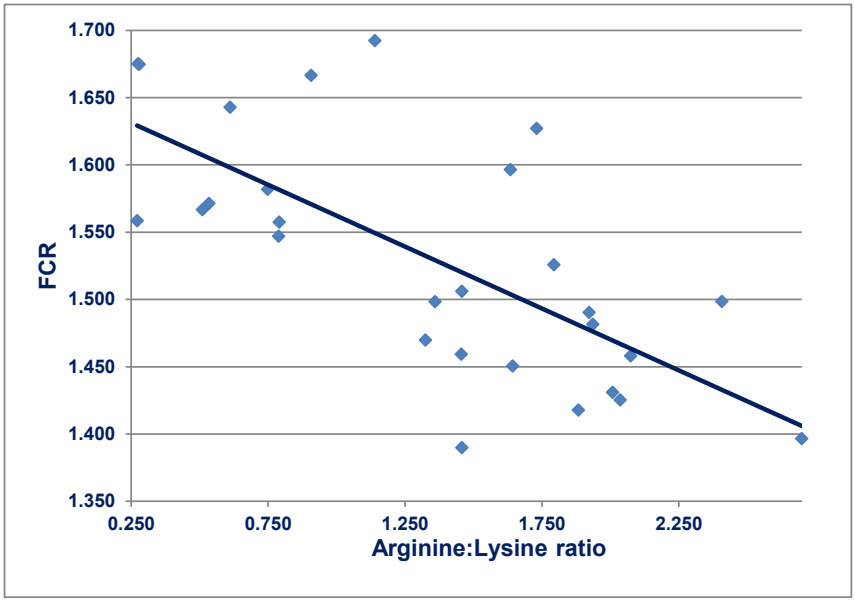

Fig. 3. Linear relationship ($r = -0.694$; $P < 0.0001$) between plasma concentration ratios of arginine:lysine and FCR.

negatively correlated with FCR. Given the recognised antagonism between arginine and lysine (Austic and Scott, 1975; D'Mello and Lewis, 1970), the contrasting outcomes in the present study, where increasing free arginine plasma concentrations were associated with improvements in FCR but lysine had the reverse effect, are of interest. This is reflected the relevant multiple regression equation ($r = 0.667$; $P = 0.001$) where:

$$y_{(FCR)} = 1.595 - 0.003*arg_{(\mu g/mL)} + 0.002*lys_{(\mu g/mL)}$$

Moreover, increasing free arginine:lysine plasma concentration ratios were linearly ($r = -0.694$; $P < 0.001$) associated with improvements in FCR as shown in Fig. 3. Thus, it appears that antagonistic interactions between the two amino acids were compromising FCR. The genesis of the arginine-lysine antagonism may involve competition between the two amino acids for renal reabsorption (Dao and Swick, 2021). The non-bound proportion of arginine relative to analysed dietary levels increased on average from 12.5% to 66.4% following the transition from 210 to 180 g/kg CP diets and the corresponding increase for lysine was from 30.8% to 73.3%. One possible implication is that arginine-lysine interactions are more likely to be observed in reduced-CP diets as they contain greater proportions of non-bound arginine and non-bound lysine.

5. Conclusions

In conclusion, it is noteworthy that a multiple linear regression ($r = 0.862$; $P < 0.001$) was detected between weight gain with dietary NBAA inclusions and analysed dietary starch:protein ratios where Weight gain = 2440.9 + 0.998 × NBAA − 284.4 × S:P ratio. Moreover, both independent variables, NBAA inclusions ($r = -0.819$; $P < 0.001$) and starch:protein ratios starch:protein ratios ($r = -0.861$; $P < 0.001$), were significantly related to weight gain. Similarly, a multiple linear regression ($r = 0.913$; $P < 0.001$) was detected for FCR where FCR = 1.018 − 0.0.003 × NBAA + 0.282 × S:P ratio. Again, both NBAA inclusions ($r = 0.818$; $P < 0.001$) and starch:protein ratios ($r = 0.898$; $P < 0.001$) were significantly related to FCR. These relationships indicate that growth performance of broiler chickens offered standard or reduced-CP, wheat-based diets is strongly influenced by both dietary NBAA inclusions coupled with dietary starch:protein ratios and their impact on starch-protein digestive dynamics. Axiomatically, dietary NBAA inclusions increase and dietary starch:protein ratios expand pursuant to reductions in CP concentrations in broiler diets.

Author contributions

Sonia Y. Liu was the principal investigator of the relevant project and is the corresponding author. All co-authors were variously involved in completion of this paper. **Peter V. Chrystal**, **Michael T. Kidd**, **Peter H. Selle** and **Sonia Y. Liu** contributed to experimental design. **Peter V. Chrystal** formulated the diets, **Shemil P. Macelline** supervised the feeding study and contributed to starch analyses, data collection and analyses. **Mehdi Toghyani** assisted data and sample collection. **Peter H. Selle** and **Shemil P. Macelline** completed the statistical analyses. The original manuscript was prepared by **Shemil P. Macelline**, **Peter H. Selle** and **Sonia Y. Liu**. All authors contributed to reviewing and editing of the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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