

Effects of supplemented nonessential amino acids and nonprotein nitrogen on growth and nitrogen excretion characteristics of broiler chickens fed diets with very low crude protein concentrations

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ABSTRACT Reducing dietary CP for broiler chickens below a certain threshold results in decreased growth, even when the supply of essential amino acids and glycine equivalent (**Gly_{equi}**) is adequate, probably because other nonessential amino acids (**neAA**) are growth-limiting. Nonprotein nitrogen (**NPN**) might be used for the synthesis of neAA. Therefore, the effects of specific neAA and ammonium chloride (**NH₄Cl**) supplementation on the growth and N-excretion characteristics of broiler chickens were investigated. Nine male Ross 308 broiler chickens were kept in each of 81 metabolism units from day 7 to 21 and received 1 of 9 diets in 9 replicates in a one-factorial arrangement of treatments. Two diets with different neAA concentrations, except for Gly_{equi}, were mixed resulting in CP levels of 180 (**CP180**) and 160 (**CP160**) g/kg. In six other diets, CP160 was supplemented with either L-Ala, L-Pro, L-Asp, a mix of L-Asp and L-Asn·H₂O, L-Glu, or a mix of L-Glu and L-Gln to achieve concentrations of the respective neAA as formulated in CP180. In a further

diet, NH₄Cl was added to CP160 to achieve the CP concentration of CP180. The ADG and gain:feed ratio (**G:F**) from day 7 to 21 were highest at CP180. Reduced neAA concentrations in CP160 decreased ADG and G:F. Supplementation of Asp+Asn, Glu, and Glu+Gln to CP160 increased ADG and G:F, but not to the level found for CP180. Compared with CP160, addition of Asp increased G:F but not ADG. Supplementation of Asp+Asn caused higher ADG and G:F than supplementation of Asp alone. The N-utilization efficiency was highest at CP160 and at CP160 supplemented with Ala, Pro, and Glu. Lower N-utilization efficiency was found at CP180 than at CP160, without and with supplemented neAA. The treatment containing NH₄Cl presented the lowest ADG, G:F, and N-utilization efficiency. These results showed that individual supplementation of Asp+Asn, Glu, and Glu+Gln partly compensates for the growth-reducing effects of very low CP diets. Supplementation of NH₄Cl as NPN source is not suitable for broiler chickens.

Key words: nonessential amino acid, ammonia, low crude protein, uric acid, nitrogen-utilization efficiency

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INTRODUCTION

The potential to reduce dietary CP without decreasing growth of broiler chickens is limited when only essential amino acids (**AA**) are considered because insufficient Gly and Ser concentrations limit growth (Waguespack et al., 2009; Ospina-Rojas et al., 2012; Siegert and Rodehutschord, 2019). These AA can undergo

interconversion; therefore, the glycine equivalent (**Gly_{equi}**) is calculated to investigate the response of broiler chickens to Gly and Ser (Dean et al., 2006; Siegert and Rodehutschord, 2019).

Hofmann et al. (2019) showed that dietary CP concentration of 163 g/kg resulted in high growth performance of broiler chickens when fed a minimum of 15 g Gly_{equi}/kg. This Gly_{equi} concentration was sufficient at different CP levels when concentrations of essential AA and factors influencing the response of broiler chickens to dietary Gly_{equi} were constant. This response was influenced by threonine (Ospina-Rojas et al., 2013; Siegert et al., 2015a) and choline (Siegert et al., 2015a) concentrations, as well as the Met/(Met+Cys) ratio (Siegert et al., 2015b). However, a reduction of nonessential

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amino acid (neAA) concentrations other than Gly_{equi} that resulted in 147 g CP/kg decreased growth (Hofmann et al., 2019). The authors suggested that growth was limited by at least one other neAA, in addition to Gly_{equi}.

Other studies have investigated the effect of neAA, other than Gly and Ser, in diets with growth-limiting neAA concentrations. The supplemented neAA had little or no effects on growth, probably because dietary Gly_{equi} was limiting in those studies. This included supplementation of a single neAA, such as Ala, Pro, Asp (Corzo et al., 2005; Dean et al., 2006; Awad et al., 2015), Glu (Pinchasov et al., 1990; Kerr and Kidd, 1999; Hussein et al., 2001), Asn (Bregendahl et al., 2002), and Gln (Bregendahl et al., 2002; Kriseldi et al., 2018). Moreover, combinations of Glu and Asp (Leclercq et al., 1994; Bregendahl et al., 2002) did not overcome the growth-limiting effect of diets deficient in neAA. However, the effects of dietary neAA supplementation on growth when the Gly_{equi} concentration is adequate have not yet been investigated.

In addition, supplementation of nonprotein nitrogen (NPN) in the form of diammonium citrate and triammonium citrate was observed to increase the growth of broiler chickens (Featherston et al., 1962; Lee and Blair, 1972). Furthermore, a growth-increasing effect of NPN was investigated in pigs fed a mixture of diammonium phosphate and diammonium citrate, or following a urea infusion into the cecum (Mansilla et al., 2015, 2017). It has been reported that ammonia (herein, ammonia includes both NH₃ and NH₄⁺ unless otherwise stated) from NPN can be used for neAA synthesis, particularly when neAA are limiting (Karasawa, 1989, 1999; Mansilla et al., 2015). Therefore, ammonia added to broiler diets with growth-limiting concentrations of neAA might be used to synthesize growth-limiting neAA.

The primary objective of this study was to investigate whether single neAA supplemented to Gly_{equi} adequate diets with very low CP concentration can remove or diminish the growth-reducing effect of CP reduction. The secondary objective was to investigate the effect of ammonia as a source of NPN on growth in a diet with insufficient neAA concentrations.

MATERIALS AND METHODS

Birds and Housing

The experiment was carried out at the Agricultural Experiment Station of the University of Hohenheim, Germany, and was approved by the Regierungspräsidium Tübingen, Germany (Project no. HOH 54/18TE) according to German Animal Welfare Legislation.

Day-old male Ross 308 broiler chickens were supplied by a commercial hatchery (Brüterei Süd ZN der BWE-Brüterei Weser-Ems GmbH & Co. KG, Regenstauf, Germany) and allocated to 4 floor pens (3 m × 4 m) on deep litter bedding. Birds were fed a commercial starter diet containing 215 g CP/kg and 12.5 MJ ME/kg (Deuka

Landkornstarter 315042025, Deutsche Tiernahrung Cremer GmbH & Co. KG, Heilbronn, Germany) up to day 7 of the experiment. Birds were then distributed to 81 metabolism units (1 m × 1 m × 1 m) on a mesh-wired floor. The broiler chickens were allocated to metabolism units on day 7 because the mesh-wired floor did not allow for an earlier placement. Nine birds each were assigned to the metabolism units, with an equal mean bird weight in every unit. One of 9 diets was provided until the end of the experiment on day 21. Each diet was tested in 9 metabolism units as replicates in a completely randomized block design. Feed and water were provided for *ad libitum* consumption throughout the experiment. Lighting was continuously on for the first 3 d, followed by a lighting regime of 18 h light and 6 h dark until the end of the experiment. The temperature was maintained at 34°C for the first 3 d of the experiment and was then gradually decreased to 25°C on the last day of the experiment.

Experimental Diets

The 9 diets were mixed at the certified feed mill of the University of Hohenheim and mainly comprised corn and soybean meal (Table 1). Concentrations of all nutrients reported herein were calculated on a standardized DM of 88%, unless otherwise stated. All nutrients were calculated to meet or exceed the recommendations of the Gesellschaft für Ernährungsphysiologie (1999), except for CP and the Met/(Met+Cys) ratio, which was 0.66, because this ratio was found to maximize ADG (Siegert et al., 2015b). The Gly_{equi} concentration was 15 g/kg in all diets, because this concentration was determined to optimize broiler growth when using a diet with similar composition (Hofmann et al., 2019). Concentrations of essential AA were adjusted by adding free AA in variable concentrations. Choline was used at a concentration of 1.1 g/kg to meet the recommendations of the Gesellschaft für Ernährungsphysiologie (1999) and because, as an endogenous precursor of Gly, choline can influence the response of broiler chickens to dietary Gly_{equi} (Siegert et al., 2015a). Diets were formulated to be isoenergetic by varying proportions of soybean oil.

A diet with 180 g CP/kg (CP180) and a diet with reduced neAA concentrations and 160 g CP/kg (CP160) were used. The neAA concentrations in CP180 and CP160 were formulated according to Hofmann et al. (2019), where similar neAA concentrations were shown to be sufficient for maximum growth and growth-limiting, respectively. Concentrations of neAA and CP were reduced by substituting soybean meal with corn. Six additional diets were mixed by supplementing free L-Ala (CP160+Ala), L-Pro (CP160+Pro), L-Asp (CP160+Asp), a mix of L-Asp and L-Asn·H₂O that contained equal proportions of Asp and Asn (CP160+Asp+Asn), L-Glu (CP160+Glu), and a mix of L-Glu and L-Gln that contained equal proportions of Glu and Gln (CP160+Glu+Gln). The neAA were added at the expense of cornstarch to CP160 to

Table 1. Ingredient composition of experimental diets (g/kg).

Treatment ¹	CP180	CP160	CP160+Ala	CP160+Pro	CP160+Asp	CP160+Asp+Asn	CP160+Glu	CP160+Glu+Gln	CP160+NH ₄ Cl
Corn	638.1					747.1			
Soybean meal	228					112			
Soybean oil	51.4					30			
L-Lysine·HCl	4.2					8.2			
DL-Methionine	3.0					3.6			
L-Cysteine·HCl·H ₂ O	0.4					1.3			
L-Threonine	1.7					3.6			
L-Tryptophan	-					0.6			
L-Arginine	2.3					5.9			
L-Isoleucine	1.4					3.5			
L-Leucine	-					1.6			
L-Valine	3.6					5.8			
L-Histidine	-					0.8			
L-Phenylalanine	-					2.0			
L-Tyrosine	0.7					2.2			
Glycine	3.6					7.3			
Choline chloride ²	0.9					1.7			
Sodium chloride	1.0					1.0			
Trace element premix ³	0.5					0.5			
Vitamin premix ⁴	2.0					2.0			
Monocalcium phosphate	16.2					17.2			
Limestone	14.6					15.2			
Sodium bicarbonate	3.0					3.0			
L-Proline	-	-	-	2.1	-	-	-	-	-
L-Alanine	-	-	1.7	-	-	-	-	-	-
L-Glutamic acid	-	-	-	-	-	-	8.4	4.2	-
L-Glutamine	-	-	-	-	-	-	-	4.2	-
L-Aspartic acid	-	-	-	-	5.6	2.8	-	-	-
L-Asparagine·H ₂ O	-	-	-	-	-	3.2	-	-	-
Ammonium chloride	-	-	-	-	-	-	-	-	12.6
Cornstarch	23.4	23.9	22.2	21.8	18.3	17.9	15.5	15.5	11.3

¹Nutrient composition (on a 88% DM basis): CP160 = 160 g CP/kg; CP180 = 180 g CP/kg.

²50% choline chloride, 373 g/kg choline concentration.

³Trace element premix (Gelamin Gesellschaft für Tierernährung mbH, Memmingen, Germany) provided per kg of diet: 25 mg calcium from calcium carbonate; 80 mg manganese from manganese-(II)-oxide; 60 mg zinc from zinc sulfate; 25 mg iron from ferrous-(II)-sulfate monohydrate; 7.5 mg copper from cupric-(II)-sulfate pentahydrate; 0.6 mg iodine from calcium iodate; 0.2 mg selenium from sodium selenite; 15 mg sepiolite.

⁴Vitamin premix (Miavit GmbH, Essen, Germany) provided per kg of diet: 10,000 IU vitamin A as retinyl acetate (3a672a); 3,000 IU vitamin D3 as cholecalciferol (E671); 30 IU vitamin E as all rac- α -tocopherol (3a700); 2.4 mg vitamin K3 as menadione (3a711); 100 μ g biotin (3a880); 1 mg folic acid (3a316); 3 mg thiamine (3a821); 6 mg riboflavin; 6 mg pyridoxine (3a831); 30 μ g vitamin B12; 50 mg nicotinamide (3a315); 14 mg calcium D-pantothenate (3a841).

achieve the respective neAA concentration of CP180. All diets contained an unknown concentration of Asn and Gln, because dietary Asn and Gln can only be analyzed together with Asp and Glu (Fontaine, 2003). Therefore, mixtures of L-Asp and L-Asn·H₂O, and L-Glu and L-Gln were used to investigate the response of broiler chickens to Asn and Gln. Another diet was mixed by adding ammonium chloride (NH₄Cl) in place of cornstarch to CP160 (CP160+NH₄Cl) to contain a CP concentration of CP180.

The diets were produced by mixing a basal mixture that represented approximately 84% of the diets. The CP160 mixtures were obtained by combining the basal mixture with a premix, which contained the feed ingredients that differed between CP160 and CP180, and the respective neAA that varied within the CP160 mixtures or NH₄Cl. Treatment CP180 was obtained by mixing the basal mixture with soybean meal, soybean oil, and cornstarch. The diets were pelleted without using steam through a 3 mm die.

There was low variation in the analyzed concentrations of essential AA among treatments (Table 2). The highest deviations between analyzed and calculated dietary AA concentrations at CP180 and CP160 were found for dietary Cys concentration (0.07 g/kg lower concentration in CP180) and dietary Arg concentration (0.3 g/kg higher concentration in CP160). Thus, the analyzed dietary AA concentrations confirmed the formulated values.

Measurements and Sampling

Total bird weight in each metabolism unit was determined on day 7 and 21 of the experiment, and feed was weighed on the same days to calculate mortality-corrected ADG, ADFI, and gain:feed ratio (G:F). Excreta were collected quantitatively from the trays of each metabolism unit at 12 h intervals for 3 consecutive days from day 18 to 21 of the experiment. Excreta of each metabolism unit collected in this period were pooled before being analyzed. Feathers, skin scales, and spilled feed pellets were removed before excreta collection. Excreta were immediately frozen at -20°C after collection. Weight gain and feed intake were calculated by weighing birds and feed at the beginning and end of the collection period. The animals were inspected at least twice daily during the experiment. Dead birds were removed, weighed, and the feed intake of birds in the respective metabolism unit was recorded. Spilled feed was collected and frozen at -20°C. The feed residues were dried and weighed, and feed intake was corrected for these residues. The DM content of each diet was determined by taking samples at the start and at the end of the experimental phase to determine feed intake on a DM basis.

Chemical Analyses

A centrifugal mill (ZM 200; Retsch GmbH, Haan, Germany) was used to grind the feed through a 0.5 mm sieve

for analysis of crude ash, crude fat, crude fiber, and starch. Diets were ground with a vibrating cup mill (Pulverisette 9; Fritsch GmbH, Idar-Oberstein, Germany) before AA and CP analyses. Freeze-dried excreta samples were ground with the same mill before uric acid (UA) analysis.

Crude ash (method no. 8.1), crude fat (method no. 5.1.1.), crude fiber (method no. 6.1.1), DM (method no. 3.1), and starch (method no. 7.2.1) were determined in accordance with the official methods for nutrient analyses in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2007).

Dietary CP concentration was analyzed by the Dumas method (method 990.03 of the Association of Official Analytical Chemists, 1995). In short, samples were combusted, thereby releasing CO₂, H₂O, NO₂, and N₂. Subsequently, NO₂ was reduced to N₂, CO₂ and H₂O were removed, and N₂ was measured volumetrically. Dietary AA were analyzed in accordance with the method 994.12 of the Association of Official Analytical Chemists (1995), with minor laboratory adaptations as described by Siegert et al. (2015a). In brief, samples were oxidized in an ice bath for 16 h, and were hydrolyzed at 110 °C for 23 h by using a mixture of hydrochloric acid and phenol. Norleucine was used as an internal standard. The AA were separated and detected with the Biochrom 30 system (Biochrom Ltd., Cambridge, United Kingdom) using various buffer solutions with different pH values and postcolumn ninhydrin derivatization. All AA were determined at a wavelength of 570 nm, except proline, which was determined at 440 nm. The amide residues in Asn and Gln are lost during acid hydrolysis, forming Asp and Glu, respectively (Fontaine, 2003). Hence, Asn and Gln were determined together with Asp and Glu. The concentration of Trp was analyzed after barium hydroxide hydrolysis by using reverse-phase HPLC by using the Shimadzu type 20 A HPLC system (Shimadzu Corp., Kyoto, Japan). Concentrations were detected by fluorescence detection at an excitation wavelength of 283 nm and an emission wavelength of 355 nm.

Excreta samples were treated and analyzed for DM, N, NH₃, and UA as described by Hofmann et al. (2019). In brief, excreta samples obtained from all collections of each metabolism unit were pooled before being weighed and homogenized. Excreta N concentration was determined by Kjeldahl digestion using the Vapodest 50S analyzing system (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Excreta NH₃ concentration was measured by using steam distillation and titration by using the Vapodest 50 analyzing system (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). The UA analysis was carried out according to Marquardt et al. (1983) and Shingfield and Offer (1999) with minor laboratory adaptations as described by Hofmann et al. (2019). In brief, glycine buffer was added to freeze-dried and pulverized samples. After stirring this solution, the mixture was centrifuged at 20,000 g and room temperature for 15 min, and was then diluted with a solution of sodium dihydrogen phosphate, phosphoric acid, and acetonitrile. The mixture

Table 2. Analyzed nutrient composition of the experimental diets (g/kg on a 88% DM basis unless otherwise stated).

Treatment ¹	CP180	CP160	CP160+Ala	CP160+Pro	CP160+Asp	CP160+Asp+Asn	CP160+Glu	CP160+Glu+Gln	CP160+NH ₄ Cl
DM (g/kg)	912	913	911	909	912	917	913	915	919
Crude fat ²	77.9	59.1	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Crude fiber ²	29.1	26.0	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Crude ash ²	51.7	46.2	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Starch ²	450	519	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
AME _N (MJ/kg on a 88% DM basis) ^{3,4}	13.4	13.4	n. c.	n. c.	n. c.	n. c.	n. c.	n. c.	n. c.
CP	178	156	159	156	158	162	161	165	180
Lys	11.3	11.5	11.2	10.9	11.0	11.2	11.4	11.3	11.3
Met	5.3	5.4	5.4	5.2	5.1	5.1	5.2	5.3	4.9
Cys	2.7	2.7	2.6	2.6	2.6	2.6	2.6	2.6	2.7
Met+Cys	8.0	8.1	8.0	7.8	7.7	7.7	7.8	7.9	7.6
Thr	7.6	7.6	7.4	7.3	7.3	7.4	7.5	7.5	7.5
Trp	1.9	1.7	1.7	1.7	1.7	1.8	1.7	1.7	1.8
Arg	12.1	12.3	11.9	11.7	11.7	12.0	12.0	12.1	12.1
Ile	7.9	8.0	7.8	7.7	7.7	7.9	7.9	8.0	7.9
Leu	14.0	12.6	12.7	12.5	12.6	12.7	12.6	12.7	12.5
Val	10.9	11.1	10.8	10.7	10.6	10.7	10.9	10.9	10.9
His	4.1	3.8	3.7	3.7	3.7	3.8	3.8	3.8	3.8
Phe	7.8	7.4	7.4	7.2	7.4	7.5	7.5	7.6	7.4
Pro	9.7	7.4	7.6	9.3	7.7	7.8	7.8	7.7	7.4
Ala	8.4	6.6	8.4	6.7	6.7	6.8	6.7	6.8	6.7
Asx ⁵	15.7	10.2	10.5	10.1	15.5	15.9	10.3	10.4	10.3
Glx ⁶	28.0	20.0	20.4	20.0	20.2	20.5	28.5	28.8	20.2
Gly	10.1	12.1	11.5	11.3	11.2	11.3	11.5	11.7	11.6
Ser	7.7	5.4	5.5	5.4	5.4	5.5	5.4	5.4	5.5
Gly _{equi} ⁷	15.6	16.0	15.4	15.1	15.1	15.2	15.4	15.6	15.5
Met/(Met+Cys) ratio	0.66	0.67	0.67	0.67	0.66	0.66	0.67	0.67	0.65

¹Nutrient composition (on a 88% DM basis): CP160 = 160 g CP/kg; CP180 = 180 g CP/kg.

²n. a. = not analyzed.

³Apparent metabolizable energy (N-corrected), calculated according to the estimation equation of the WPSA (1984). Analyzed nutrient concentrations and table values for sugar in corn and soybean meal (Centraal Veevoederbureau, 2016) were used.

⁴n. c. = not calculated.

⁵Asp and Asn together.

⁶Glu and Gln together.

⁷Gly + 0.7143 × Ser.

was analyzed by HPLC (Agilent 1100; Agilent Technologies Inc., Santa Clara, CA) at 286 nm. Urea was analyzed in a two-step process by quantifying ammonia before and after hydrolyzing urea by adding urease with an enzymatic assay kit (Catalog No., 10542946035, R-Biopharm AG, Darmstadt, Germany). Glutamate dehydrogenase was then added to catalyze ammonia, 2-oxoglutarate, and NADH to L-Glu, NAD^+ , and H_2O . The amount of oxidized NADH is equivalent to the ammonia concentration and was measured in a photometer (Evolution 201, Thermo Scientific Inc., Waltham, MA) at 340 nm. To ensure maximum ammonia concentrations were not exceeded in the samples, 10- and 5-fold dilutions were prepared for CP160+ NH_4Cl and the other treatments, respectively. This dilution defined the detection limit of ~ 1460 mg/kg DM for CP160+ NH_4Cl and ~ 460 mg/kg DM for the other treatments, with minor deviations due to varying DM contents of the excreta samples. However, urea concentrations were below the limit of detection in all measured samples. Therefore, measurements were discontinued after all replicates of CP180 and CP160, and 3 replicates of the other treatments were obtained.

Calculations and Statistical Analysis

Accretion of N and N-utilization efficiency (NUE) from day 18 to 21 of the experiment were calculated as follows:

$$\text{N accretion (g/d)} = \text{N intake (g/d)} - \text{N excretion (g/d)} \quad (1)$$

$$\text{NUE (\%)} = \text{N accretion (g/d)} / \text{N intake (g/d)} \times 100 \quad (2)$$

The uric acid–nitrogen/(uric acid–nitrogen+ammonia–nitrogen) [UA-N/(UA-N+ NH_3 -N)] ratio was calculated because it was shown to be useful for interpreting physiological processes of N excretion in broiler chickens (Siegert et al., 2016; Hofmann et al., 2019).

Statistical analyses were carried out using the MIXED procedure of the software package SAS (version 9.4, SAS Institute Inc., Cary, NC). All data were evaluated by one-way ANOVA after testing for normal distribution and homogeneity of variance. Estimation of the variance components was carried out by the restricted maximum likelihood method. Kenward–Roger method was used for computing degrees of freedom and SEs. The metabolism unit was considered as the experimental unit. The applied statistical model was

$$y_{ij} = \mu + trt_i + block_j + e_{ij} \quad (3)$$

where y_{ij} is the dependent trait, μ is the overall mean, trt_i is the fixed effect of treatment i , $block_j$ is the random effect of block j , and e_{ij} is the residual error. Statistical significance was set at $P < 0.050$. Comparisons between treatments were made with 2-paired t tests.

RESULTS

Total bird weight among metabolism units ranged between 1,365 and 1,460 g (SD = 20 g) on d 7 and did not differ significantly between treatments ($P = 0.701$). The survival rate during the experimental period was 99% and independent of treatment (9 of 729 birds died in 6 different treatments).

Growth Performance and Nitrogen Accretion

Overall the highest levels of G:F, ADG (Figure 1), and N accretion (Table 3) were observed for CP180, and they were lower at CP160 ($P < 0.001$). The treatments CP160+Asp, CP160+Asp+Asn, CP160+Glu, and CP160+Glu+Gln had higher G:F compared with CP160 ($P \leq 0.033$). The G:F was higher for CP160+Asp+Asn and CP160+Glu+Gln, than for CP160+Asp ($P \leq 0.027$). No difference in G:F was found between CP160+Ala, CP160+Pro, and CP160+Asp ($P \geq 0.237$). Supplementing Asp+Asn, Glu, and Glu+Gln to CP160 increased ADG ($P \leq 0.011$) and N accretion ($P < 0.001$). The ADG was higher for CP160+Asp+Asn than for CP160+Asp ($P = 0.025$), and was not different among CP160+Asp, CP160+Glu, and CP160+Glu+Gln ($P \geq 0.159$). The ADFI for CP180 was higher than for CP160, CP160+Ala, CP160+Pro, CP160+Asp, CP160+Glu, and CP160+Glu+Gln ($P \leq 0.038$) (Figure 1). No difference in ADFI was found for CP180 and CP160+Asp+Asn ($P = 0.206$).

Nitrogen Utilization Efficiency

The NUE at CP180 was lower than at CP160 and at CP160 supplemented with neAA ($P < 0.001$) (Figure 1). The NUE for CP160, CP160+Ala, CP160+Pro, and CP160+Glu was higher than for any other treatment ($P \leq 0.033$). The NUE decreased on addition of Asp, Asp+Asn, and Glu+Gln to CP160 ($P < 0.001$) and was lower at CP160+Asp compared with CP160+Asp+Asn ($P = 0.024$).

Excretion of Uric Acid–Nitrogen and NH_3 -Nitrogen

Excretion of NH_3 -N was lowest, whereas that of UA-N was highest at CP180 (Table 3). Excretion of UA-N was lower at CP160 than at CP180 ($P < 0.001$), and was increased on supplementation of Ala, Asp, Asp+Asn, Glu, and Glu+Gln ($P \leq 0.039$). The NH_3 -N excretion at CP160 was higher than that at CP180 ($P < 0.001$). Compared with CP160, NH_3 -N excretion decreased with CP160+Pro ($P < 0.039$) and increased with CP160+Glu+Gln ($P < 0.030$). Supplementation of individual neAA to CP160 increased the UA-N/(UA-N+ NH_3 -N) ratio compared with CP160 ($P \leq 0.003$) (Table 3). The UA-N/(UA-N+ NH_3 -N) ratio was highest at CP180 and lowest at CP160 ($P < 0.001$). No

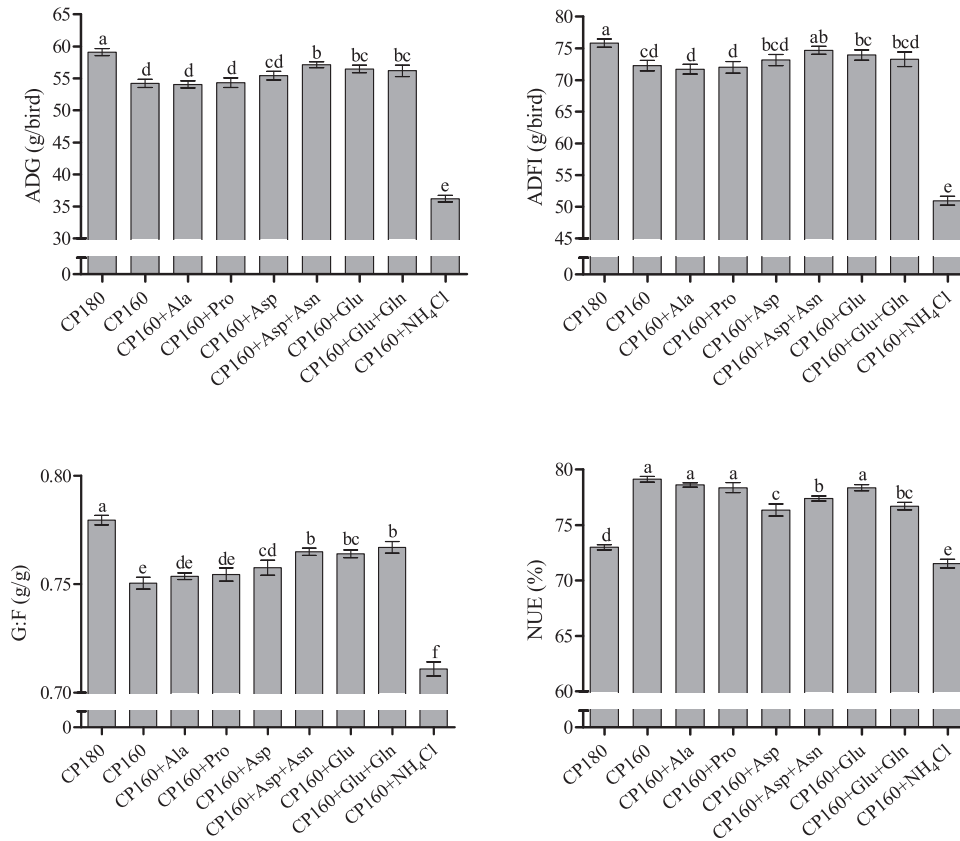


Figure 1. Effect of treatments on ADG, ADFI, and gain:feed ratio (G:F) from day 7 to 21 and nitrogen utilization efficiency (NUE) from day 18 to 21. Columns without a common letter differ significantly ($P < 0.050$). Error bars represent the SEM.

difference in the $UA-N/(UA-N+NH_3-N)$ ratio was found between CP160+Asp+Asn, CP160+Glu, and CP160+Glu+Gln ($P \geq 0.510$). The $UA-N/(UA-N+NH_3-N)$ ratio was lower for CP160+Asp compared with CP160+Asp+Asn ($P \leq 0.031$).

Effects of Ammonium Chloride Supplementation

Among all treatments, CP160+NH₄Cl resulted in the lowest ADG, ADFI, G:F, N accretion, NUE, and $UA-N/(UA-N+NH_3-N)$ ratio ($P < 0.050$), and the highest NH₃ concentration in excreta ($P < 0.001$) (Table 3, Figure 1).

DISCUSSION

Effects of Supplemented Amino Acids

The primary objective of the present study was to investigate whether single neAA can prevent or diminish the growth-reducing effect of CP when essential AA and Gly_{equi} concentrations are sufficient. The addition of Asp, Asp+Asn, Glu, and Glu+Gln increased growth compared with CP160. However, ADG and G:F of the CP180 diet were not achieved. Therefore, these data suggest that growth was limited by reasons other than the supply of respective neAA.

Growth performance at CP180 was higher than the performance objectives of the breeder (Aviagen, 2019). This suggests that the nutrient requirements of the

broiler chickens were met. The reduced growth at CP160 was consistent with the findings of a previous study (Hofmann et al., 2019) in which similar effects on growth were observed with a similar supply of essential AA and neAA. Hofmann et al. (2019) concluded that Gly_{equi} concentrations higher than 15 g/kg had no effect on growth. Therefore, the Gly_{equi} concentration of 15 g/kg used in the present study was likely adequate, and limited concentrations of neAA other than Gly_{equi} or other nitrogenous compounds in the feed most likely account for the reduced growth at CP160.

Growth Limitation Due to Deficiency of More than One Specific Nonessential Amino Acid

It is possible that individual neAA did not increase growth to the level of a diet adequate in neAA because more than one specific neAA limited growth at CP160. Addition of Asp+Asn, Glu, and Glu+Gln to CP160 caused similar growth responses, indicating that these neAA limited growth. Supplementing these neAA to CP160 might be related to increased UA formation. In birds, excess metabolic N is mainly excreted in the form of UA; however, ammonia and urea are also relevant nitrogenous compounds in avian urine. One molecule of Gly and Asp, and 2 molecules of Gln are needed to synthesize the purine ring of UA (Seegmiller, 1975). Different proportions of dietary AA can alter the urine composition (Namroud et al., 2008; Siegert et al., 2016; Hofmann et al., 2019), which consists of 72–91% of

Table 3. Effect of dietary treatments on nitrogen intake, nitrogen accretion, and excreta characteristics from day 18 to 21.

Treatment ¹	CP180	CP160	CP160 + Ala	CP160 + Pro	CP160 + Asp	CP160 + Asp + Asn	CP160 + Glu	CP160 + Glu + Gln	CP160 + NH ₄ Cl	Pooled SEM	P-value
Daily N intake (g/bird)	3.29 ^a	2.72 ^d	2.79 ^{c,d}	2.76 ^d	2.86 ^c	2.97 ^b	2.94 ^b	2.99 ^b	2.14 ^e	0.042	<0.001
Daily N excretion (g/bird)	0.89 ^a	0.57 ^e	0.60 ^d	0.60 ^d	0.68 ^b	0.67 ^b	0.64 ^c	0.70 ^b	0.61 ^{c,d}	0.013	<0.001
Daily N accretion (g/bird)	2.40 ^a	2.15 ^c	2.20 ^c	2.16 ^c	2.18 ^c	2.30 ^b	2.31 ^b	2.29 ^b	1.53 ^d	0.035	<0.001
Daily excreta NH ₃ -N (mg/bird)	34.6 ^f	47.9 ^{c,d}	47.8 ^{c,d}	43.6 ^e	51.6 ^{b,c}	48.7 ^{b,c,d}	45.6 ^{d,e}	52.4 ^b	178.8 ^a	1.69	<0.001
Daily excreta uric acid-N (mg/bird)	324 ^a	167 ^{fg}	185 ^e	184 ^{e,f}	231 ^{c,d}	239 ^{b,c}	218 ^d	256 ^b	164 ^g	7.3	<0.001
Uric acid-N/(uric acid-N + NH ₃ -N) ratio	0.903 ^a	0.776 ^f	0.794 ^e	0.808 ^d	0.817 ^{c,d}	0.830 ^b	0.827 ^{b,c}	0.829 ^{b,c}	0.478 ^g	0.0049	<0.001

^{a-g}Means in a row without a common letter differ significantly ($P < 0.050$).

¹Nutrient composition (on a 88% DM basis): CP160 = 160 g CP/kg; CP180 = 180 g CP/kg.

UA-N plus NH₃-N and 3–10% of urea-N (Goldstein and Skadhauge, 2000). Urea was not detected in excreta in the present study. Given the limit of quantification, the proportion of urea-N in urinary N must have been lower than 1–2%, assuming that urinary N only consists of UA-N, NH₃-N, and urea-N. Therefore, not considering urea-N when deducing urinary N from nitrogenous compounds in excreta did not lead to substantial error in the present study.

Reducing the neAA concentration at CP160 decreased the UA-N/(UA-N+NH₃-N) ratio, indicating that low dietary neAA concentrations might diminish UA formation. The UA-N/(UA-N+NH₃-N) ratio was increased on supplementation of Asp+Asn, Glu, and Glu+Gln but not to the level of CP180. Therefore, UA synthesis at CP160+Asp+Asn, CP160+Glu, and CP160+Glu+Gln was probably limited by components other than the supplemented neAA, which may indicate that growth was limited by more than one neAA. Hence, it is possible that combinations of Asp, Asn, Glu, and Gln would overcome the growth-reducing effect of CP160.

Concentrations of Ala, Pro, and Asp seem to have been sufficient at CP160. Supplementation of Ala and Pro to CP160 had no effect on ADG and G:F. Addition of Asp to CP160 had no effect on ADG, but increased G:F. The increased G:F at CP160+Asp might indicate that water or fat accretion increased because N accretion was not affected by Asp supplementation. It is possible that Asp was catabolized to fumarate or oxaloacetate, or both (D’Mello, 2003), which was then used to generate energy via the citric acid cycle. Hence, increased G:F at CP160+Asp may not indicate that Asp was growth-limiting. Nevertheless, Ala, Pro, and Asp may have induced limited growth at CP160, but this was not detectable because other neAA included in this study were limiting. Thus, individual or combined supplementation of Ala, Pro, and Asp to CP160+Glu and CP160+Glu+Gln, and Ala and Pro to CP160+Asp+Asn would overcome the growth-reducing effect of CP160.

Growth Limitation due to Nonspecific Nonessential Amino Acid–Nitrogen

Alternatively, the birds may need nonspecific neAA-N rather than specific neAA. Nonessential AA can be converted into other neAA via various pathways. A precursor for the synthesis of most other neAA is Glu (Bequette, 2003), which can be reversibly metabolized to Ala, Pro, Asp, and Gln. In addition, Asn and Glu can be reversibly converted to each other via the intermediate Gln or Asp (Berg et al., 2007). Therefore, individual dietary neAA fed to birds above their specific requirement can be used to synthesize other neAA that are not sufficiently supplied with the diet. This could partly overcome the growth-reducing effect of diets with insufficient neAA concentrations, provided that conversion is sufficient. This would be in line with the

results of [Bezerra et al. \(2016\)](#), who observed that reduced growth with a diet deficient in neAA could be increased to the level of a diet adequate in neAA when Glu concentrations were higher than in the positive control. It is possible that Glu, as a source of nonspecific neAA-N, can be used to meet the requirement of neAA. The increased UA-N/(UA-N+NH₃-N) ratio at CP160+Ala and CP160+Pro showed that a higher proportion of urinary N was excreted as UA, and that part of Ala and Pro was used to synthesize UA; however, these AA are not involved in synthesizing the purine ring of UA ([Seegmiller, 1975](#)). This might indicate that Ala and Pro were converted to Glu, which, in turn, formed Gln or Asp to synthesize UA. If so, the amount of Glu formed from supplemented Ala and Pro may have been too low to increase growth. Furthermore, the UA-N/(UA-N+NH₃-N) ratio was not increased to the level of CP180 after supplementation of individual neAA. This may indicate that neAA necessary to form UA were metabolized to other neAA and consequently, were not available for the synthesis of UA.

Interconversion of Asp and Asn, and Glu and Gln

It is likely that the requirement for Asn was not met when only Asp was supplemented to CP160 because ADG and G:F were higher at CP160+Asp+Asn. By contrast, ADG and G:F did not differ between CP160+Glu and CP160+Glu+Gln. This indicates that dietary Gln, or the rate of Glu conversion to Gln was sufficient to meet the requirement of Gln. Reversible interconversions are described for Asp and Asn, as well as for Glu and Gln ([Prusiner and Milner, 1970](#)). Therefore, these results suggest that the conversion of Asp to Asn was limited, whereas the interconversion of Glu and Gln was not. The lower NUE at CP160+Asp compared with CP160+Asp+Asn indicated that part of additional Asp that could not be used by the birds to form Asn had to be excreted. The conversion of Asp to Asn may have been limited by Gln because Gln is necessary when Asp is metabolized to Asn by asparagine synthetase (EC 6.3.5.4) ([Arfin, 1967](#)). Limited conversion of Asp to Asn due to insufficient dietary Gln supply is indicated by the lower UA-N/(UA-N+NH₃-N) ratio at CP160+Asp compared with CP160+Asp+Asn. This is a consequence of reduced urinary UA excretion. An insufficient supply of dietary Gln might have limited UA formation at CP160+Asp, whereas at CP160+Asp+Asn, more Gln was available for UA formation because less Gln was necessary for the conversion of Asp to Asn.

Influence on Nitrogen Utilization Efficiency

A low need for the catabolism of digested AA probably contributed to the very high NUE of 73–79% observed in the present study. The low amount of excreted urea suggests that arginine degradation was low. Urea excretion is an indicator of arginine catabolism in poultry ([Austic](#)

and [Nesheim, 1970](#); [Fernandes and Murakami, 2010](#)) because urea is synthesized when arginine is degraded by arginase ([Tamir and Ratner, 1963](#)). This indicates that the supply of digestible arginine was close to the amount that could be used for metabolic processes, which contributed to the high NUE.

Reductions in neAA at CP160 resulted in increased NUE. This is consistent with the results of [Hofmann et al. \(2019\)](#), who suggested that NUE increased because urinary N was reabsorbed in the hindgut and used for neAA synthesis ([Karasawa, 1989](#); [Karasawa and Maeda, 1994](#)) and less muscle protein was degraded ([Urdaneta-Rincon and Leeson, 2004](#)). In addition, the increased NUE at CP160 may have resulted from utilization of ammonia, produced by the breakdown of urinary N compounds in the ceca, by microorganisms to synthesize AA. Such AA were shown to be absorbed by chicken ([Karasawa and Maeda, 1995](#); [Karasawa, 1999](#)), possibly after being transferred to the small intestine by reverse peristalsis.

With increased growth, NUE decreased at CP160+Asp+Asn and CP160+Glu+Gln, but was not affected at CP160+Glu. This indicates that most of the additional Glu was not excreted, and was almost completely used by the animals. By contrast, part of supplemented Asp+Asn and Glu+Gln was excreted, indicating that Asp+Asn and Glu+Gln were provided above the levels required by the animals, or were used to form other neAA. Therefore, supplementation of Glu is more efficient than adding Asp+Asn and Glu+Gln when the aim is to increase NUE, although growth did not differ between these treatments.

Effects of Supplemented Ammonia

The second objective of the present study was to investigate the effect of ammonia supplementation to provide NPN to broiler chickens when feeding diets deficient in neAA. In previous studies, triammonium citrate was used to investigate the effect of NPN in broiler chickens ([Lee and Blair, 1972](#); [Bregendahl et al., 2002](#)). However, it was not possible to pellet the feed supplemented with triammonium citrate because the pelleting machine became blocked. This was not foreseen because the other studies feeding triammonium citrate did not mention whether feed was pelleted. We then used NH₄Cl used as a readily available alternative source of ammonia in the present study. Supplementation of NH₄Cl decreased growth and was therefore unsuitable to overcome the growth-reducing effect of diets deficient in neAA.

Decreased growth on NH₄Cl supplementation may have been a consequence of metabolic acidosis because excess Cl⁻ as a source of mineral anions represents an acid load ([Patience, 1990](#)). Addition of NH₄Cl has been shown to induce metabolic acidosis in poultry ([Craan et al., 1982](#); [Coon and Balling, 1984](#); [Toyomizu et al., 1999](#)), and metabolic acidosis decreases growth ([Coon and Balling, 1984](#); [Borges et al., 2003a,b](#)). The lowest UA-N/(UA-N+NH₃-N) ratio was observed at

CP160+NH₄Cl. This indicates that NH₄Cl induced metabolic acidosis because ammonia excretion increases when acid is excreted (Patience, 1990). This occurs because NH₄⁺ is produced when NH₃ binds a proton (Hamm and Simon, 1987). The decreased UA-N/(UA-N+NH₃-N) ratio on NH₄Cl supplementation may also be explained by the incomplete absorption of NH₄Cl, increasing the amount of ammonia in excreta.

These results do not show that NPN, and particularly ammonia, provided with the diet per se cannot be used to increase growth when dietary neAA concentrations are deficient. The addition of urea, triammonium phosphate, and diammonium and triammonium citrate did increase growth in chickens (Featherston et al., 1962; Lee and Blair, 1972). Furthermore, supplementation of a diammonium citrate and diammonium phosphate mix increased the growth of pigs (Mansilla et al., 2017). Ammonia from NPN can be used by chickens and pigs to synthesize neAA (Karasawa, 1989; Karasawa and Maeda, 1994; Mansilla et al., 2015), or by microorganisms in the ceca of chickens to produce AA (Karasawa and Maeda, 1995; Karasawa, 1999), which may then be absorbed in the small intestine after being transported by reverse peristalsis. Therefore, feeding a source of NPN other than NH₄Cl might be suitable to increase growth with diets deficient in neAA.

In conclusion, these results show that individual supplementation of Asp+Asn, Glu, and Glu+Gln partly compensates for the growth-reducing effects of very low CP diets adequate in Gly_{equi}. Addition of these neAA increased growth to a similar extent, but not to the level of CP180. Addition of NH₄Cl as source of NPN to diets deficient in neAA was unable to increase growth.

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