Effects of dietary supplementation with cupreous N-carbamylglutamate (NCG) chelate and copper sulfate on growth performance, serum biochemical profile and immune response, tissue mineral levels and fecal excretion of mineral in weaning piglets

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ABSTRACT
This experiment was conducted to measure the effects of dietary supplementation with copper sulfate and cupreous N-carbamylglutamate chelate (NCG-Cu) on the growth performance, serum biochemical profile and immune response, tissue mineral levels and fecal excretion of minerals of weaning piglets. Eighteen 28-d-old healthy weaning piglets (initial body weight = 6.34 ± 0.10 kg) were individually housed and randomly assigned to receive one of three diets containing no copper in either form (Control), 650 g/t copper sulfate (650 g/t Cu group) or 640 g/t NCG-Cu (640 g/t NCG-Cu group) in the final feed for 14 days. These data indicate that 640 g/t NCG-Cu was as effective as 650 g/t Cu for stimulating growth, immune response, and improving F/G in weaning piglets. Fecal Cu excretion decreased in piglets from the 640 g/t NCG-Cu group, which received 160 mg/kg Cu compared with the fecal Cu excretion observed in the piglets from the 650 g/t Cu group, which also received 160 mg/kg Cu. Therefore, 640 g/t NCG-Cu of dietary Cu, may provide an effective environmental alternative to 650 g/t Cu in weaning piglets.

Abbreviations: ADFI: average daily feed intake; ADG: average daily gain; ALB: albumin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate amino transferase; CK: creatine kinase; CREA: creatinine; D-BIL: direct-acting-bilirubin; F/G: feed/gain ratio; GLB: globulin; GLU: blood glucose; IgA: immunoglobulin A; IgG: immunoglobulin G; LDH: lactate dehydrogenase; NCG: N-
1. Introduction

Micromineral supplementation to meet requirements in swine diets is crucial for animal growth, reproduction and immune system development. Feeding high concentrations of copper is well recognized to have growth-promoting effects in weaned pigs and to reduce problems with post-weaning diarrhea, and the response appears to be additive to that obtained from feeding antibiotics (Beames & Lloyd, 1965; Case & Carlson, 2002; Hasman et al., 2006; Hedemann, Jensen, & Poulsen, 2006; Hill et al., 2000; Hill et al., 1983; Pluske, Pethick, Hopwood, & Hampson, 2002; Xing, Hao, Liu, Xu, & Kuang, 2014; Yuan et al., 2015). Minimally, piglets require 5–6 mg/kg, whereas growing pigs and slaughter pigs require 3–5 mg/kg copper in feed for normal growth according to the National Research Council’s recommended levels. However, supplemented levels often exceed the requirements, resulting in an enhanced excretion of minerals to the environment and raising concerns regarding environmental pollution (Hill et al., 2000; Jondreville, Revy, & Dourmad, 2003).

Interest in using organic minerals has increased because of the reported potential of their higher bioavailability compared with inorganic mineral sources (Apgar, Kornegay, Lindemann, & Notter, 1995). Studies have shown that organic copper binds to peptides and amino acids during digestion, which can aid in copper absorption and improve growth performance (Apgar et al., 1995; Beames & Lloyd, 1965; Bunch, McCall, Speer, & Hays, 1965; Coffey, Cromwell, & Monegue, 1994; Hill et al., 1983; Van Heugten & Coffey, 1992; Zhou, Kornegay, Van Laar, et al., 1994). Two reviews report a number of studies that have shown that organic copper is not more effective than inorganic copper in improving pig performance (Acda & Chae, 2002; Pluske et al., 2002). Discrepancies between effective and ineffective supplementation with organic and inorganic copper in improving pig performance may require further research.

Therefore, this study was designed to evaluate the effectiveness of dietary supplementation with copper sulfate (Cu) and cupreous N-carbamylglutamate chelate (NCG-Cu) on the growth performance, serum biochemical profiles and immune response, tissue mineral levels and fecal excretion of minerals of weaning piglets.

2. Materials and methods

2.1. Ethics statement

This study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving animal subjects were approved by the animal welfare committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (Changsha, Hunan Province, China).

2.2. Pigs management and sample collection

Eighteen 28-day-old healthy weaning pigs (Landrace × Large × Yorkshire) (Hunan New Wellful Co., Ltd., Hunan Province, China) with a mean body weight of 6.34 ± 0.10 kg...
were randomly assigned to three dietary treatments: (1) a diet without added copper (Control), (2) a diet with 650 g/t copper sulfate (Cu\(^{2+}\) elemental concentration of 160 mg/kg) (650 g/t Cu) and (3) a diet with 640 g/t NCG-Cu diet (Cu\(^{2+}\) elemental concentration of 160 mg/kg) (640 g/t NCG-Cu). Each group contained six pigs (half barrows and half gilts). All diets were formulated to meet the NRC’s (2012) recommended nutrient requirements for weaning pigs. The ingredient and nutrient composition of the diets are shown in Table 1 (Wu, Liao, He, Feng, et al., 2015). Pigs had free access to drinking water and their respective diets throughout the experimental period. After 14 days of dietary exposure to the different copper sources and immediately after electrical stunning, six pigs/group (three barrows and three gilts) were killed for analysis. Body weight and feed consumption as well as the presence of diarrhea were recorded. The diarrhea rate was calculated as follows: \[ \text{diarrhea rate} \times 100\% = \frac{\text{Diarrhea piglets}}{\text{Total experiment piglets} \times \text{Experiment time} \times d} \times 100\% \]

After 14 days of dietary exposure to the different copper sources, 5 mL of blood was collected aseptically in tubes from a jugular vein 2 h after feeding, centrifuged at 3000 \( \times g \) for 10 min at 4°C to obtain serum samples, and stored at \(-80^\circ C\) for further analysis. The liver, spleen, kidney and heart were removed and weighed. The weights were recorded both as the organ weight and as a percentage of the total body weight.

### 2.3. Analysis of serum biochemical, amino acid profile and immunoglobulin

Serum biochemical parameters, including albumin (ALB), blood glucose (GLU), creatinine (CREA), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate amino transferase (AST), globulin (GLB), total cholesterol (TC), total protein (TP), Urea (urea), direct-acting-bilirubin (D-BIL), total bilirubin (T-BIL), urate (UA), lactate dehydrogenase (LDH) and creatine kinase (CK), were measured using spectrophotometric kits in accordance with the manufacturer’s instructions (Nanjing Jiangcheng Biotechnology Institute, Jiangsu Province, China) and identified using an Automatic Biochemistry Radiometer (Au640, Olympus) as described previously (Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015).

| Ingredients                  | Contents (%) | Nutrient composition | Contents   |
|------------------------------|--------------|----------------------|------------|
| Corn (43%CP)                 | 63.70        | Digestive energy, MJ/kg | 14.60      |
| Soybean meal                 | 19.80        | Crude protein, %     | 20.27      |
| Whey powder                  | 4.30         | Lysine-HCl, %        | 1.48       |
| Fish meal (64%CP)            | 9.00         | Methionine, %        | 0.42       |
| Soybean oil                  | 0.80         | Threonine, %         | 0.90       |
| Lysine hydrochloride         | 0.38         | Calcium, %           | 0.80       |
| Hydroxy methionine           | 0.10         | Available Phosphorus, % | 0.45      |
| L-threonine                  | 0.09         |                       |            |
| L-tryptophan                 | 0.01         |                       |            |
| CaHPO\(_3\)                  | 0.00         |                       |            |
| Rock-powder                  | 0.52         |                       |            |
| Salt                         | 0.30         |                       |            |
| 1% Premix\(^a\)             | 1.00         |                       |            |
| Total                        | 100.00       |                       |            |

\(^a\)Premix provided the following per kilogram of the diet: Vitamin A 2000 IU; Vitamin D\(_3\) 200 IU; Vitamin E 12 IU; Vitamin K 0.5 mg; Vitamin B\(_1\) 0.016 mg; Vitamin B\(_2\) 3 mg; Vitamin B\(_3\) 12.5 mg; folic acid 0.3 mg; Vitamin B\(_5\) 10 mg; Choline chloride 0.5 mg; Vitamin B\(_1\) 1 mg; Vitamin B\(_6\) 1.6 mg; Vitamin B\(_7\) 0.05 mg; Fe 80 mg; Mn 3 mg; Zn 46.8 mg; I 0.1 mg; Se 0.3 mg.
Thirty-seven amino acids were identified in serum by LC–MS/MS (HPLC Ultimate 3000 and 3200 QTRAP LC–MS/MS) as described previously (Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015). The concentrations of immunoglobulin (Ig) G and IgA were measured using ELISA kits in accordance with the manufacturer’s instructions (Cusabio Biotech Co., Ltd., Hubei, China).

2.4. Analysis of mineral levels

The mineral values in the serum, fecal and liver, longissimus dorsi, spleen and kidney were analyzed according to previously described methods, with minor adjustments (Subramanian, 1996; Xing et al., 2014). All samples were obtained, placed in plastic bags, and immediately preserved on ice for an acceptable length of time prior to being analyzed for heavy metals. All samples were weighed before and after being cut into small pieces and ground thoroughly to achieve homogeneity. Then, 5 g of each sample was placed in a 125 mL Erlenmeyer flask, 10 mL of concentrated nitric acid was added, and the sample was warmed on a hot plate until solubilized. The temperature of the hot plate approached the boiling point until the solution turned brown. Then, the sample was allowed to cool, and an additional 5 mL of concentrated nitric acid was added for repeated heating and cooling. Another 2 mL of nitric acid was added before the flask was heated again on the hot plate until the volume of the sample was reduced to 10 mL. Once cooled, 2 mL of 30% hydrogen peroxide was added. Once again, the sample was heated until the volume of the sample was reduced to 5–10 mL. After being allowed to cool, another 2 mL of hydrogen peroxide was added. This step was repeated until a total of 10 mL of hydrogen peroxide had been added. Then, the sample was allowed to cool, and 2 mL of concentrated hydrochloric acid was added. The sample was then returned to the hot plate until the volume of the sample was reduced to 5–10 mL. The sample was allowed to cool and transferred into a 100 mL volumetric flask. The sample was then topped-up with deionized water to the mark for ICP-OES analysis.

2.5. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the general linear model (GLM) procedure in the SPSS19.0 software program (Chicago, IL, USA) (Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015). Duncan’s multiple range test was applied to compare the differences among the treatments. Differences were considered significant at $P < .05$.

3. Results

3.1. Growth performance and rate of diarrhea

The growth performance results of weaning piglets are shown in Table 2. A significant difference in average daily gain (ADG) $(P < .05)$ was observed between the 640 g/t NCG-Cu group and the 650 g/t Cu group, but this value was not significantly different
between the control group and the 640 g/t NCG-Cu group (P > .05). The average daily feed intake (ADFI) was significantly different between the 640 g/t NCG-Cu group and the 650 g/t Cu group (P < .05), but in the control and 640 g/t NCG-Cu groups, this value was significantly higher than in the 650 g/t Cu (P > .05). The control group showed the lowest feed/gain ratio (F/G).

The rate of diarrhea of the weaning piglets is shown in Table 3. The diarrhea rate varied significantly (P < .05) between the 640 g/t NCG-Cu group and the 650 g/t Cu group, but this value was not significantly different (P > .05) between the control group and the 640 g/t NCG-Cu group.

### 3.2. Relative organ weights

Table 4 shows the effects of the two diets with different copper sources on relative organ weight. No significant differences were observed among the three groups with respect to heart, liver or spleen weight (P > .05). However, the relative kidney weights in the piglets of the 650 g/t Cu group and 640 g/t NCG-Cu groups were lower than those in the control group (P < .05). No differences were observed among the groups with respect to contamination resulting from the different copper sources.

### 3.3. Serum biochemical parameters and free amino acid concentrations

Table 5 shows the effects of the two doses of copper supplementation in the diet on the serum biochemical parameters of the weaning piglets. No difference was observed in

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**Table 2.** Effect of dietary supplementation with cupreous N-carbamylglutamate (NCG) chelate and copper sulfate on growth performance in weanling pigs (n = 6).

| Items       | Dietary supplementation | SEM ±      | P value |
|-------------|-------------------------|------------|---------|
|            | Control1                 | 650 g/t Cu2 | 640 g/t NCG-Cu^3 |         |
| Initial BW, kg | 6.36                    | 6.35                   | 6.31                | 0.106              | 0.243              |
| Final BW, kg  | 9.81^1                   | 8.56^b                  | 9.56^b              | 0.232              | .047               |
| ADFI, g/d    | 195.83^b                 | 134.40^b                | 198.81^b            | 8.793              | .011               |
| F/G         | 1.71                     | 1.86                   | 1.62                | 0.657              | .065               |

Notes: Means within the same row with different superscript differ significantly (P < .05). The experiment lasted 14 days.

1 Control = basal diet (Cu^2+ elemental concentration of 16.58 mg/kg).

2 650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^2+ elemental concentration of 160 mg/kg).

3 640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu^2+ elemental concentration of 160 mg/kg).

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**Table 3.** The effects of dietary supplementation with cupreous N-carbamylglutamate (NCG) chelate and copper sulfate on diarrhea rate in weanling pigs (n = 6).

| Items         | Dietary supplementation | SEM ± | P value |
|---------------|-------------------------|-------|---------|
| Diarrhea rate (%) | Control1                  | 650 g/t Cu2 | 640 g/t NCG-Cu^3 |         |
|               | 5.30^a                   | 7.50^b                  | 5.20^a              | 0.247              | .038               |

Notes: Means within the same row with different superscript differ significantly (P < .05). The experiment lasted 14 days.

1 Control = basal diet (Cu^2+ elemental concentration of 16.58 mg/kg).

2 650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^2+ elemental concentration of 160 mg/kg).

3 640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu^2+ elemental concentration of 160 mg/kg). The diarrhea rate was calculated as follows: diarrhea rate (%) = (Diarrhea piglets [n]/(Total experiment piglets [n] × Experiment time [d])) × 100%.

The diarrhea rate of the weaning piglets is shown in Table 3. The diarrhea rate varied significantly (P < .05) between the 640 g/t NCG-Cu group and the 650 g/t Cu group, but this value was not significantly different (P > .05) between the control group and the 640 g/t NCG-Cu group.

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Table 4. The dietary supplementation with cupreous N-carbamylglutamate (NCG) chelate and copper sulfate on relative organ weights (g/kg BW) in weanling pigs (n = 6).

| Items | Dietary supplementation |
|-------|------------------------|
|       | Control¹ | 650 g/t Cu² | 640 g/t NCG-Cu³ | SEM ± | P value |
| Heart | 4.77     | 4.85       | 5.15           | 0.129 | .476    |
| Liver | 25.78a   | 22.97b     | 25.03b         | 0.561 | .038    |
| Spleen| 2.21     | 2.27       | 2.42           | 0.137 | .925    |
| Kidney| 6.50     | 6.21       | 6.46           | 0.199 | .051    |

Notes: Means within the same row with different superscript differ significantly (P < .05). The experiment lasted 14 days.
¹Control = basal diet (Cu²⁺ elemental concentration of 16.58 mg/kg).
²650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu²⁺ elemental concentration of 160 mg/kg).
³640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu²⁺ elemental concentration of 160 mg/kg).

Table 5. Serum biochemical chemical parameters of weanling pigs fed with diets containing cupreous N-carbamylglutamate (NCG) chelate and copper sulfate (n = 6).

| Items | Dietary supplementation |
|-------|------------------------|
|       | Control¹ | 650 g/t Cu² | 640 g/t NCG-Cu³ | SEM ± | P value |
| ALB (g/L) | 41.85a | 39.45a | 38.43a | 0.930 | .324 |
| GLU (mmol/L) | 5.34a | 6.27a | 6.85a | 0.285 | .044 |
| CREA (mmol/L) | 130.47a | 150.42b | 133.20a | 3.193 | .048 |
| ALP (U/L) | 345.77a | 559.42b | 425.45b | 32.668 | .027 |
| ALT (U/L) | 60.45a | 80.42b | 64.93a | 6.262 | .002 |
| AST (U/L) | 122.38a | 181.57b | 126.96a | 32.960 | .038 |
| GLB (g/L) | 11.35a | 17.67b | 11.55a | 0.750 | .034 |
| TC (mmol/L) | 2.23a | 3.67b | 2.29a | 0.079 | .052 |
| TP (g/L) | 53.20a | 78.32b | 51.46a | 1.279 | .023 |
| UREA (mmol/L) | 3.88a | 4.27b | 3.78a | 0.212 | .042 |
| D-BIL (μmol/L) | 5.56a | 7.25a | 5.01a | 0.518 | .054 |
| T-BIL (μmol/L) | 5.38a | 6.94b | 4.62a | 0.608 | .027 |
| UA (μmol/L) | 0.45a | 1.05a | 0.65a | 0.163 | .045 |
| LDH (U/L) | 872.45a | 896.78b | 841.13b | 96.786 | .800 |
| CK (U/L) | 565.23a | 2444.97b | 1400.87ab | 329.755 | .056 |

Notes: Means within the same row with different superscript differ significantly (P < .05). The experiment lasted 14 days.
¹Control = basal diet (Cu²⁺ elemental concentration of 16.58 mg/kg).
²650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu²⁺ elemental concentration of 160 mg/kg).
³640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu²⁺ elemental concentration of 160 mg/kg).

the concentrations of the ALB, GLU, UA and LDH between the control and the two copper-treatment groups (P > .05). The CREA, ALT, AST, GLB, TC, TP, Urea, D-BIL, T-BIL, ALP, UA and CK values were not different between the control group and the 640 g/t NCG-Cu group, but these values were significantly different between the 650 g/t Cu group and the 640 g/t NCG-Cu group (P < .05). The ALP activity in the control group was significantly lower than that in the 650 g/t Cu group and that in the 640 g/t NCG-Cu group, but the value was significantly different between the 650 g/t Cu group and the 640 g/T NCG-Cu group (P > .05).

Table 6 shows the effects of the diets with 2 different sources of copper supplementation on the serum for 37 free amino acid concentrations in weaning piglets. The concentrations
of DL-α-amino-n-butyric acid, L-alanine, L-cystathionine, L-lysine, L-ornithine, L-phenylalanine and L-tyrosine in the 650 g/t Cu group and the 640 g/t NCG-Cu group varied significantly from those of the control group ($P < .01$), but these values did not differ significantly between the 650 g/t Cu group and the 640 g/t NCG-Cu group ($P > .05$).

The L-arginine and L-leucin concentrations in the control group were lower than those in the 650 g/t Cu group ($P < .05$), but these values were not differ significantly between the 650 g/t Cu group and 640 g/t NCG-Cu group ($P > .05$). The concentrations of L-cystine, L-threonine and L-valine varied significantly among the three groups ($P < .01$).

The levels of IgG and IgA in serum are also shown in Table 7. The concentration of IgA did not vary significantly among the treatments. The level of IgG in the 640 g/t NCG-Cu group was significantly ($P < .05$) higher than that in the control group, but did not vary significantly ($P > .05$) between the 650 g/t Cu and 640 g/t NCG-Cu groups.

### Table 6. Serum free amino acid parameters of weanling pigs fed with diets containing cupreous N-carbamylglutamate (NCG) chelate and copper sulfate ($n = 6$).

| Items                      | Control $^1$ | 650 g/t Cu$^2$ | 640 g/t NCG-Cu$^3$ | SEM ± | $P$ value |
|----------------------------|--------------|----------------|--------------------|-------|-----------|
| L-1-methylhistidine        | 0.00         | 0.00           | 0.00               | 0.000 | .000      |
| L-3-methylhistidine        | 1.10$^a$     | 1.25$^a$       | 0.93$^b$           | 0.044 | None      |
| L-alpha-aminoadipic acid   | 4.80$^a$     | 5.36$^b$       | 3.81$^a$           | 0.328 | .003      |
| DL-α-amino-n-butyric acid  | 2.30$^a$     | 1.46$^b$       | 1.49$^a$           | 0.138 | .152      |
| L-alanine                  | 68.80$^a$    | 46.95$^b$      | 43.53$^b$          | 4.363 | .013      |
| L-anserine                 | 0.25$^a$     | 0.23$^a$       | 0.27$^a$           | 0.137 | .026      |
| L-arginine                 | 16.37$^a$    | 24.93$^b$      | 20.28$^{a,b}$      | 1.384 | .995      |
| L-aspartic acid            | 3.41$^a$     | 3.34$^a$       | 2.64$^a$           | 0.202 | .029      |
| DL-β-aminoisobutyric acid  | 0.00         | 0.00           | 0.00               | 0.000 | .237      |
| β-Alanine                  | 3.31$^b$     | 3.17$^a$       | 3.67$^a$           | 0.225 | None      |
| L-carnosine                | 4.34$^{a,b}$ | 5.40$^b$       | 3.49$^a$           | 0.362 | .674      |
| L-citrulline               | 14.80$^a$    | 23.71$^b$      | 18.56$^{a,b}$      | 1.544 | .028      |
| L-cystathionine            | 3.28$^a$     | 1.72$^b$       | 1.69$^b$           | 0.239 | .050      |
| L-cystine                  | 0.76$^a$     | 2.16$^b$       | 3.28$^a$           | 0.318 | .002      |
| Ethanolamine               | 0.48$^a$     | 0.41$^a$       | 0.67$^a$           | 0.727 | .001      |
| γ-Aminobutyric acid        | 0.18$^a$     | 0.22$^a$       | 0.39$^a$           | 0.068 | .335      |
| L-glutamic acid            | 46.70$^a$    | 33.26$^b$      | 32.21$^a$          | 3.201 | .434      |
| Glycine                    | 100.04$^a$   | 74.36$^a$      | 75.95$^a$          | 5.647 | .116      |
| L-histidine                | 4.25$^a$     | 5.64$^a$       | 4.77$^a$           | 0.352 | .110      |
| DL- plus allo-δ-hydroxylysine | 0.54$^a$    | 0.77$^a$       | 2.05$^a$           | 0.343 | .277      |
| Hydroxy-L-proline          | 16.07$^a$    | 17.74$^b$      | 16.61$^a$          | 0.864 | .155      |
| L-isoleucine               | 15.55$^a$    | 14.34$^a$      | 12.19$^a$          | 0.688 | .749      |
| L-leucin                   | 12.16$^a$    | 18.03$^b$      | 15.51$^{a,b}$      | 0.978 | .128      |
| L-lysine                   | 20.60$^a$    | 41.33$^b$      | 38.64$^b$          | 3.054 | .037      |
| L-methionine               | 9.46 ± 1.52$^a$ | 9.58 ± 1.07$^a$ | 10.11$^a$        | 0.624 | .003      |
| L-ornithine                | 12.02$^a$    | 17.26$^b$      | 17.79$^b$          | 1.003 | .913      |
| O-phosphoethanolamine      | 0.00         | 0.00           | 0.00               | 0.000 | .237      |
| L-phenylalanine            | 8.53$^a$     | 12.67$^b$      | 12.08$^b$          | 0.587 | None      |
| L-proline                  | 30.97$^a$    | 27.31$^a$      | 25.52$^a$          | 1.201 | .002      |
| O-phospho-L-serine         | 2.73$^a$     | 3.21$^a$       | 3.87$^a$           | 0.261 | .171      |
| Sarcosine                  | 3.03$^a$     | 3.01$^a$       | 2.82$^a$           | 0.149 | .205      |
| L-serine                   | 18.74$^a$    | 18.50$^a$      | 20.13$^a$          | 0.744 | .840      |
| Taurine                    | 13.38$^a$    | 12.34$^a$      | 11.74$^a$          | 0.954 | .654      |
| L-threonine                | 11.49$^a$    | 23.21$^b$      | 21.79$^c$          | 2.442 | .799      |
| L-tyrosine                 | 10.11$^a$    | 20.10$^b$      | 19.98$^b$          | 1.509 | <.0001    |
| Urea                       | 114.73$^a$   | 92.13$^a$      | 112.35$^a$         | 7.678 | .008      |
| L-valine                   | 10.07$^a$    | 23.16$^b$      | 20.78$^a$          | 1.719 | .444      |

Notes: Means within the same row with different superscript differ significantly ($P < .05$). The experiment lasted 14 days.

1Control = basal diet (Cu$^{2+}$ elemental concentration of 16.58 mg/kg).

2650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu$^{2+}$ elemental concentration of 160 mg/kg) and

3640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu$^{2+}$ elemental concentration of 160 mg/kg).
3.4. Feed, serum, tissue and fecal mineral values

The mineral values of the three feeds used for the weaning piglets are shown in Table 8. Ten types of minerals, namely P, Mg, Ca, Cd, Cu, Fe, Mn, Ni, Pb and Zn, were detected in the feeds. Among these minerals, the concentrations of Cu were 16.58, 654.82 and 443.78 mg/kg in the control group, 650 g/t Cu group and 640 g/t NCG-Cu group, respectively.

The serum mineral values obtained for the weaning pigs are shown in Table 9. Five types of minerals, namely Ca, P, Cu, Zn and Fe, were detected in the serum. The concentration of Cu in the control was significantly lower than the Cu concentration in the 650 g/t Cu and 640 g/t NCG-Cu groups (\( P < .05 \)), but the Cu concentrations in the 650 g/t Cu and 640 g/t NCG-Cu groups were not significantly different (\( P > .05 \)).

The fecal mineral values of the weaning piglets are shown in Table 9. Three types of minerals, namely Cu, Zn and Fe, were detected in the feces. The concentration of Cu in the control was significantly lower than that in the 650 g/t Cu group and the 640 g/t NCG-Cu group (\( P < .05 \)), but the Cu concentrations in the 650 g/t Cu and 640 g/t NCG-Cu groups were not significantly different (\( P > .05 \)).

### Table 7. Serum immune parameters of weanling pigs fed with diets containing cupreous N-carbamylglutamate (NCG) chelate and copper sulfate (\( n = 6 \)).

| Items     | Dietary supplementation | SEM ±  | \( P \) value |
|-----------|-------------------------|--------|--------------|
|           | Control\(^1\)           | 650 g/t Cu\(^2\) | 640 g/t NCG-Cu\(^3\) |
| IgG (mg/mL) | 7.03\(^a\)              | 8.26\(^b\)      | 8.63\(^b\)     | 0.307 | .044 |
| IgA (mg/mL) | 7.14                    | 7.22              | 7.31             | 0.218 | .057 |

Notes: Means within the same row with different superscript differ significantly (\( P < .05 \)). The experiment lasted 14 days.

\(^1\)Control = basal diet (Cu\(^{2+}\) elemental concentration of 16.58 mg/kg).
\(^2\)650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu\(^{2+}\) elemental concentration of 160 mg/kg).
\(^3\)640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu\(^{2+}\) elemental concentration of 160 mg/kg). IgG: immunoglobulin G, IgA: immunoglobulin A.

### Table 8. Analysis of the innate micromineral concentration of the basal diets, cupreous N-carbamylglutamate (NCG) chelate and copper sulfate.

| Items     | Dietary supplementation | SEM ±  | \( P \) value |
|-----------|-------------------------|--------|--------------|
|           | Control\(^1\)           | 650 g/t Cu\(^2\) | 640 g/t NCG-Cu\(^3\) |
| P (mg/kg) | 617.60                  | 2049.90 | 1225.60      |
| Mg (mg/kg) | 926.55                  | 888.15  | 933.23       |
| Ca (mg/kg) | 8873                    | 9536.40 | 9660.60      |
| Cd (mg/kg) | 0.40                    | 0.38    | 0.41         |
| Cu (mg/kg) | 16.58                   | 654.82  | 443.78       |
| Fe (mg/kg) | 438.88                  | 536.77  | 520.94       |
| Mn (mg/kg) | 78.64                   | 85.99   | 71.24        |
| Ni (mg/kg) | 2.07                    | 2.14    | 2.12         |
| Pb (mg/kg) | 0.08                    | 0.15    | 0.98         |
| Zn (mg/kg) | 1654.18                 | 2002.83 | 1980.06      |

Note: The experiment lasted 14 days.

\(^1\)Control = basal diet (Cu\(^{2+}\) elemental concentration of 16.58 mg/kg).
\(^2\)650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu\(^{2+}\) elemental concentration of 160 mg/kg).
\(^3\)640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu\(^{2+}\) elemental concentration of 160 mg/kg). Each diet was analyzed in duplicate.
Table 9. Analyzed micromineral concentration of serum, feces and different organs in weanling pigs fed with diets containing cupreous N-carbamylglutamate (NCG) chelate and copper sulfate (n = 6).

| Dietary supplementation             | Control1 | 650 g/t Cu2 | 640 g/t NCG-Cu3 | SEM ± P value |
|-------------------------------------|----------|-------------|-----------------|---------------|
| **Serum**                           |          |             |                 |               |
| Ca (mmol/L)                         | 2.94a    | 2.89b       | 2.69a           | 0.100 .042    |
| P (mmol/L)                          | 2.54a    | 1.65b       | 2.97a           | 0.220 .000    |
| Cu (μmol/L)                         | 17.28b   | 25.75b      | 27.56b          | 3.050 .030    |
| Zn (μmol/L)                         | 106.28a  | 94.47a      | 98.54a          | 27.990 .323   |
| Fe (μmol/L)                         | 61.16a   | 78.96b      | 87.27b          | 0.100 .035    |
| **Fecal**                           |          |             |                 |               |
| Cu (mg/kg)                          | 1074.65a | 2069.52b    | 1220.77c        | 0.100 .042    |
| Zn (mg/kg)                          | 2958.28a | 3047.17a    | 2434.54a        | 0.100 .035    |
| Fe (mg/kg)                          | 1961.61a | 2078.96b    | 1987.37b        | 0.100 .035    |
| **Liver**                           |          |             |                 |               |
| Cr (mg/kg)                          | 5.22a    | 8.04a       | 8.53a           | 0.100 .565    |
| Mg (mg/kg)                          | 98.04a   | 107.31a     | 105.22a         | 3.810 .121    |
| Ca (mg/kg)                          | 53.76a   | 57.47a      | 58.26a          | 9.600 .512    |
| Cd (mg/kg)                          | 4.01a    | 5.22a       | 5.82a           | 1.220 .034    |
| Cu (mg/kg)                          | 18.80a   | 123.55b     | 115.03b         | 0.220 .081    |
| Fe (mg/kg)                          | 257.34a  | 318.02a     | 301.76a         | 0.220 .235    |
| **Longissimus dorsi**               |          |             |                 |               |
| Cr (mg/kg)                          | 13.23a   | 19.40a      | 10.34a          | 1.560 .318    |
| Mg (mg/kg)                          | 1160.12a | 1147.89a    | 1249.48b        | 55.020 .062   |
| Ca (mg/kg)                          | 2478.61a | 1748.97b    | 2400.96a        | 223.110 .028  |
| Cd (mg/kg)                          | 0.56a    | 0.11a       | 0.15a           | 0.140 .786    |
| Cu (mg/kg)                          | 37.20a   | 50.88b      | 37.83a          | 4.160 .040    |
| Fe (mg/kg)                          | 158.80a  | 192.81b     | 158.70a         | 0.100 .061    |
| **Spleen**                          |          |             |                 |               |
| Cr (mg/kg)                          | 7.21a    | 7.55a       | 7.78a           | 0.890 .413    |
| Mg (mg/kg)                          | 150.13a  | 152.04a     | 153.01a         | 31.430 .521   |
| Ca (mg/kg)                          | 2078.31a | 2309.65a    | 2159.51a        | 67.450 .741   |
| Cd (mg/kg)                          | 0.15a    | 0.17a       | 0.18a           | 0.080 .579    |
| Cu (mg/kg)                          | 26.81a   | 33.14a      | 28.54a          | 2.050 .617    |
| Fe (mg/kg)                          | 178.04a  | 196.05a     | 185.21b         | 45.550 .034   |
| **Kidney**                          |          |             |                 |               |
| Cr (mg/kg)                          | 10.11a   | 9.94a       | 10.27a          | 0.691 .217    |
| Mg (mg/kg)                          | 1078.02a | 1053.64a    | 1011.01b        | 24.180 .203   |
| Ca (mg/kg)                          | 2968.15a | 3514.95b    | 3014.05b        | 145.250 .037  |
| Cd (mg/kg)                          | 0.51a    | 0.48a       | 0.49a           | 0.012 .963    |
| Cu (mg/kg)                          | 85.11a   | 154.62b     | 135.57b         | 3.173 .063    |
| Fe (mg/kg)                          | 305.13a  | 452.65a     | 402.65a         | 57.021 .214   |
| Notes: Means within the same row with different superscript differ significantly (P < .05). The experiment lasted 14 days.  
1Control = basal diet (Cu2+ elemental concentration of 16.58 mg/kg).  
2650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu2+ elemental concentration of 160 mg/kg).  
3640 g/t NCG-Cu = basal diet + 640 g/kg cupreous N-carbamylglutamate chelate diet (Cu2+ elemental concentration of 160 mg/kg). Each diet was analyzed three times.
The liver mineral values obtained for the weaning pigs are shown in Table 9. Ten types of minerals, namely P, Mg, Ca, Cd, Cu, Fe, Mn, Ni, Pb and Zn, were detected in the liver. The concentrations of Cu and Zn in the control were significantly lower than those in the 650 g/t Cu group and the 640 g/t NCG-Cu group (P < .05), but these values did not vary significantly between the 650 g/t Cu group and the 640 g/t NCG-Cu group (P > .05). The concentration of Fe in the control group was slightly lower than that in the 650 g/t Cu group and that in the 640 g/t NCG-Cu group (P > .05).

The values for minerals found in the longissimus dorsi of the weaning pigs are shown in Table 9. Ten types of minerals, namely P, Mg, Ca, Cd, Cu, Fe, Mn, Ni, Pb and Zn, were detected in the longissimus dorsi. The concentrations of Cu, Ni and Zn in the control group did not vary significantly from those of the two copper-treatment groups (P > .05), but these values were significantly different between the 650 g/t Cu group and the 640 g/t NCG-Cu group (P < .05). The concentration of Fe in the control group was slightly lower than that in the 650 g/t Cu and 640 g/t NCG-Cu groups (P > .05).

The mineral values obtained from the spleens of the weaning pigs are shown in Table 9. Ten types of minerals, namely P, Mg, Ca, Cd, Cu, Fe, Mn, Ni, Pb and Zn, were detected in the spleen. The concentration of Ni in the control was significantly different from the concentrations observed in the two copper-treatment groups (P < .05), but this value did not vary significantly between the 650 g/t Cu group and the 40 g/t NCG-Cu group (P > .05). No differences were observed in the concentration of Cu among the three groups (P > .05).

The mineral values measured in the kidneys of the weaning piglets are shown in Table 9. Ten types of minerals, namely P, Mg, Ca, Cd, Cu, Fe, Mn, Ni, Pb and Zn, were detected in the kidney. The concentrations of Ca and Cu in the control differed significantly from those measured in the two copper-treatment groups (P < .05), but these values were not significantly different between the 650 g/t CuSO4 group and the 640 g/t NCG-Cu group (P > .05).

4. Discussion
Copper plays an important role in the normal metabolism of piglets. Several studies have evaluated the performance of weaning piglets fed diets supplemented with Cu either as copper sulfate or as organic Cu. Compounds such as Cu-carbonate, Cu-lysine, tribasic Cu-chloride and organic Cu chelates have been studied, and these compounds, as observed for copper sulfate, appear to stimulate growth in piglets and reduce diarrhea (Apgar et al., 1995; Bunch et al., 1965; Coffey et al., 1994; Stansbury, Tribble, & Orr, 1990; Zoubek, Peo, Moser, Stahly, & Cunningham, 1975). In our study, the 640 g/t NCG-Cu group showed a significant advantage in measures of ADG, ADFI, F/G and diarrhea compared with the 650 g/t Cu group (Tables 2 and 3). These results are consistent with previous studies in which the growth of piglets was stimulated and the diarrhea rate was reduced.

In the present study, the relative kidney weights of pigs in the 650 g/t Cu and 640 g/t NCG-Cu groups were lower than those of pigs in the control group (P < .05), but no significant differences in the weight of other organs were observed (Table 4). This result is not consistent with previous studies in which heart weight increased significantly in both male and female rats exposed to copper-deficient diets, with the heart of the males being more severely enlarged, and in which no changes were observed in the weights of the liver, kidney or spleen (Allen, Hassel, & Lei, 1982; Koller, Mulhern, Frankel, Steven, & Williams,
A possible explanation for the discrepancies between these studies and our present study could be that the effects of relative organ weights depend on animal species, sexuality and copper dose.

The measured serum biochemical parameters reflected the metabolism and visceral organ status of the pigs (Table 5). The CREA, ALT, AST, GLB, TC, TP, Urea, D-BIL, T-BIL, UA and CK values in the 650 g/t Cu group and the 640 g/t NCG-Cu group were significantly different ($P < .05$). This finding is consistent with previous results showing that dietary supplementation with copper for quail and hen has a significant effect on ALT; however, it is not consistent with previous results indicating increased AST levels in pigs fed a copper-contaminated diet (Almansour, 2006; Berrin Kocaoğlu Güçlü et al., 2008). Serum AST and ALT levels have been reported to be sensitive indicators of liver injury because an increase in these values reflects leakage from injured hepatocytes (Nyblom, Berggren, Balldin, & Olsson, 2004). Changes in blood chemistry variables occur before the formation of physiological and morphological lesions. Although no changes were observed in the ratio of liver weight to body weight, the alterations observed in the serum enzyme activities, particularly in the animals fed diets supplemented with 160 mg/kg Cu, may suggest that when the animals are exposed to this concentration of copper or higher for prolonged periods, potentially hazardous effects may arise.

Amino acids play important roles as metabolic intermediates in nutrition, immune response and growth performance (Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015). In the present study, the concentrations of DL-$\alpha$-amino-$n$-butyric acid, L-alanine, L-cystathionine, L-lysine, L-ornithine, L-phenylalanine and L-tyrosine in the 650 g/t Cu group and the 640 g/t NCG-Cu group were significantly different from those of the control group ($P < .01$) (Table 6). This finding is not consistent with previous results showing increased citrulline and arginine levels in pigs fed a single NCG-contaminated diet (Wu et al., 2010). One possible reason is that the degradation of dietary alanine, cystathionine, lysine, ornithine, phenylalanine and tyrosine by the small intestine is increased by the two doses of copper-supplemented feed, resulting in deficiencies of these amino acids in the animals.

Some reports have also indicated that a diet supplemented with Cu could stimulate immune capacity (Dorton, Engle, Hamar, Siciliano, & Yemm, 2003; Gonzales-Eguia, Fu, Lu, & Lien, 2009). In the present study, the level of serum IgA did not vary significantly among the treatments. The level of IgG in the 640 g/t NCG-Cu group was significantly ($P < .05$) higher than that in the control group (Table 7). Cu metabolism affects T and B cells, neutrophils and macrophages (Cao et al., 2015; Herich, 2017; Punyokun, Hongprayoon, Srisapoome, & Sirinarumitr, 2013; Qiao et al., 2017). An impaired humoral immune response was observed in mice with hypocuprosis (Prohaska, 1983). Previous studies have shown that NCG supplementation could increase intestinal mucosal immunity function in *Escherichia coli* challenged neonatal piglets (Zhang et al., 2013); however, some reports have also indicated that diet supplemented with Cu could stimulate immune capacity (Dorton et al., 2003; Gonzales-Eguia et al., 2009). The immune-stimulatory properties of NCG-Cu may be superior to those of Cu measured in the present study. These findings suggest that dietary supplementation with NCG-Cu at 640 g/t may induce an immune response in weaning piglets by modulating immunoglobulin levels.

In the present study, serum Cu concentrations were not affected by the dietary supplementation provided to the 650 g/t Cu and 640 g/t NCG-Cu groups, but the
concentration of copper in the control group (16.58 mg/kg) was significantly lower than that in the 650 g/t Cu group and that in the 640 g/t NCG-Cu group (Table 9). These data are consistent with previous results that demonstrate an increase in plasma Cu when 225 mg/kg Cu in the form of copper sulfate was supplemented in the diets of nursery piglets (Armstrong, Williams, Spears, & Schiffman, 2000). Additionally, plasma Cu increased with the supplementation of dietary Cu at concentrations of 100, 150 and 200 mg/kg (Apgar et al., 1995). However, a previous study showed an increase in plasma Cu concentrations only at supplemental dietary Cu concentrations of 375 and 500 mg/kg but not at or below 250 mg/kg (Roof & Mahan, 1982). A possible explanation for the discrepancies between that study and our present study could be that the different Cu serum concentrations depend on animal species and life stage, sex, different copper sources and doses, and experiment duration.

The liver and kidney are natural storage sites for copper (Luo & Dove, 1996). The Cu concentrations in these tissues might indicate the Cu status of pigs. In the present study, the concentration of Cu in the liver and kidney increased in the three groups, but no significant differences were observed in the spleen and longissimus dorsi (Table 9). Most previous studies have shown a large increase in liver Cu concentration and an increase in kidney concentration of Cu in piglets fed high-concentration Cu diets, which is consistent with the results of our present study (Cromwell, Stahly, & Monegue, 1989; Kline, Hays, & Cromwell, 1972; Luo & Dove, 1996; Zhou, Kornegay, Lindemann, et al., 1994). All data reported in these previous studies and our results indicated that when high-concentration Cu is supplemented in the diet, the distribution of Cu varies greatly in the different tissues, with more Cu being distributed to the liver and kidney within the physiological range according to the changes in the level of supplementation of Cu.

Previous research has indicated that pigs normally excrete 70–95% of the Cu consumed in diets (Bunch et al., 1965). This poor retention of copper by pigs fed high concentrations of copper presents an environmental concern because excess copper in swine feces results in copper pollution in the soil and water. In the present study, weaning piglets fed 160 mg/kg Cu in the 640 g/t NCG-Cu group showed decreased fecal excretion of Cu compared with weaning piglets fed 160 mg/kg Cu in the 650 g/t Cu group, which is consistent with the results of a previous study (Armstrong, Cook, Ward, Williams, & Spears, 2004) (Table 9). However, these data likely reflect the concentration of the supplemented dietary Cu rather than the source. Fecal Cu concentration did not vary significantly between the control group (Cu$^{2+}$ elemental concentration of 16.58 mg/kg) and the 640 g/t NCG-Cu group (Cu$^{2+}$ elemental concentration of 160 mg/kg), and the values for the ADG and ADFI did not differ significantly between the control group and the 640 g/t NCG-Cu group (Tables 2 and 9) ($P > .05$).

5. Conclusions

Data show that the growth- or immune-stimulatory properties of 160 mg/kg Cu from 640 g/t NCG-Cu are superior to those of 160 mg/kg Cu from 650 g/t Cu. Results suggest that, as a result of the reduction in fecal copper concentrations, 160 mg/kg NCG-Cu dietary copper may provide an effective environmental alternative to 160 mg/kg Cu for weaning piglets. Data also show that copper has limited effects on tissue mineral deposition, except for deposition in the spleen and liver. Thus, it appears that the tradition of
adding 250 mg/kg Cu in the form of copper sulfate to diets to stimulate growth or immune
should be reconsidered. From an environmental perspective, the implications of feces
excretion on copper pollution in the soil and water worldwide should be a concern.

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