High dietary copper induces oxidative stress and leads to decreased egg quality and reproductive performance of Chinese Yellow broiler breeder hens

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ABSTRACT The objective of this study was to investigate the effects of dietary copper (**Cu**) on production, egg quality, and hatchability of Chinese Yellow broiler breeder hens and growth performance of their offspring. A total of 576 30-week-old hens were randomly allotted into 6 groups, each with 6 replicates (8 cages for each replicate with 2 birds per cage). The basal diet contained 3.50 mg/kg Cu, and the other 5 treatment diets contained 8.5, 13.5, 23.5 43.5, and 83.5 mg/kg Cu, respectively, additionally supplemented with Cu on the basal diet. The trial lasted for 15 wk. Qualified egg rate of birds fed 23.5 or 83.5 mg/kg Cu was significantly decreased ($P < 0.05$) compared with those given 3.5, 8.5, or 13.5 mg/kg Cu. Plasma malondialdehyde concentration showed quadratic effect ($P = 0.002$) which that decreased first then increased with dietary Cu increased. Highest values of Cu content and hepatic activity of Cu-ATPase occurred in hens fed 83.5 mg/kg dietary Cu with linear ($P = 0.001$) and quadratic ($P = 0.001$) effects. Shell strength and proportion on 18th day of live embryos of hens fed 13.5 mg/kg Cu were the greatest compared with other groups respectively ($P < 0.05$); rate of qualified eggs for hatch and hatchability of fertilized eggs of hens fed 83.5 mg/kg Cu were the least ($P < 0.05$). In conclusion, both inadequate (3.5 mg/kg diet) and excess (83.5 mg/kg) of dietary Cu can induce oxidative stress in hens and lead to decreased egg quality. Hatchability and growth performance of offspring were decreased when breeder hens were fed excess Cu in spite of greater hatching weight. The appropriate dietary Cu level for Chinese Yellow broiler breeder hens during the egg-laying period is 15.7 to 21.2 mg/kg (1.81–2.44 mg Cu fed per day) when based on Cu level and Cu-ATPase activity in the liver. This dietary Cu requirement is approximately doubled (∼40 mg/kg, ∼4.60 mg Cu per bird per day) for maximal response of eggshell thickness.

Key words: copper, Chinese Yellow breeder hen, oxidative stress, egg quality, reproductive performance

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INTRODUCTION

Copper (**Cu**), an essential trace mineral nutrient for growth and development of animals, is involved in various physiological and biochemical processes. Feed supplementation of organic and inorganic Cu as a growth promoter for pigs or chickens has been documented in numerous studies (Gonzales-Eguia et al., 2009; Karimi et al., 2011; Dhama et al., 2014; Zhao et al., 2014). Copper plays an important role in development of the embryo, newborn, bone and connective tissues, and inflammatory processes (EDEC, 2003).

Biochemical processes are not properly completed when Cu is deficient (Kaya et al., 2010; Scheiber et al., 2014; Cao et al., 2016). Copper is an integral part of the important antioxidant enzyme Cu-Zn superoxide dismutase (**Cu-ZnSOD**). Copper-zinc superoxide dismutase is considered to be a good marker of Cu status (Gaetke and Chow, 2003). Restricting dietary Cu quickly impairs catalytic functioning of Cu-ZnSOD in numerous tissues (Harris, 1992). Copper-ATPase, known as ATPase Cu transporting protein consisting 2 isoforms: ATP7A and ATP7B (Minghetti et al., 2010), is an important ATPase that transports Cu across cellular membranes; it delivers Cu to cuproenzymes during their biosynthesis and

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exports Cu to maintain cellular homeostasis as a mechanism for protecting the cell from oxidative damage caused by excess Cu (Svetlana et al., 2007). Copper-ATPase exhibits Cu-dependent trafficking. In the presence of high levels of Cu, ATP7B relocates from the trans-Golgi network to the apical domain of hepatocytes, where it facilitates elimination of excess Cu into the bile (Braiterman et al., 2015). Antioxidant 1 Cu chaperone can transfer Cu to its destination molecules ATP7A/ATP7B (Dirksen et al., 2017).

Copper also plays an important role in the formation of the eggshell membrane, which then influences structure, texture, and shape of the eggshell (Baumgartner et al., 1978). The eggshell and its associated membranes contain high concentration of Cu (Baumgartner et al., 1978; Richards, 1997). Structure of the membrane, texture, shape, and pigments of eggshell, even egg weight can be influenced by a Cu-deficient diet (Baumgartner et al., 1978). It is noteworthy that the absolute amount of shell deposited by hens may not be affected by mineral deficiency, but egg size and thickness of shell may decrease (Roland et al., 1975; Favero et al., 2013).

Specific recommendations for Cu of broiler breeder hens were not included in the NRC (1994). Currently, the major information used by nutritionists to determine Cu concentrations in breeder diets was derived from primary broiler breeder manuals (Cobb-vantress, 2013; Aviagen, 2016), which specifically apply to fast-growing broiler breeder hens. Research on specific recommended amount of Cu in the diet of Chinese Yellow broiler breeder hens is lacking, despite China being the third largest producer of chickens, including approximately 4 billion per year Chinese Yellow, slower growing meat-type chickens.

High doses of Cu and zinc (**Zn**) were often used in diets of pigs because of their bactericidal effect (Dbski, 2016). Excessive Cu intake by adult birds caused accumulation of large amounts of Cu in the liver (Goldberg et al., 1956) where it results in production of reactive oxygen species (**ROS**) (Kadiiska et al., 1993). Trace elements such as Cu, iron, and Zn all have toxicity potential and ability to produce ROS, especially when they are at higher concentrations (Stohs and Bagchi, 1995; Valko et al., 2005). Their ability to cause metal-induced oxidative stress has been frequently studied in many organisms (Gaetke and Chow, 2003; Kim et al., 2009; Gou et al., 2018). Reduced glutathione (**GSH**), ratio of reduced to oxidized glutathione (GSH/**GSSG**), and superoxide dismutase serve as biomarkers reflecting oxidative stress in birds (Isaksson et al., 2005; Berglund et al., 2007; Koivula and Eeva, 2010).

The trace minerals are traditionally supplemented in feeds of birds by using inorganic sources, such as oxides or sulfates (Vieira, 2008). Gene expression or activity of Cu-ATPase in the liver or other tissues of hens may be affected by dietary Cu. The one objective was to evaluate Cu requirements of Chinese Yellow broiler breeder hens during egg-laying period. Meanwhile, negative effects of excess Cu used in animal production were also concerned. To achieve these goals, laying performance, biochemical indices, organic indexes and gene expression of Chinese Yellow breeder hens, and egg quality, hatchability, and growth performance of offsprings were measured in hens fed 6 levels of dietary Cu supplemented with Cu sulfate. The appropriate dietary Cu level of Chinese Yellow boiler breeder hens during the egg-laying period would be obtained.

MATERIALS AND METHODS

Chickens and Husbandry

Breeder Hens A total of 576 27-week-old Chinese Yellow broiler breeder hens (Mahuang, an improved local breed, 2.09 ± 0.14 kg) were obtained from Guangdong Wiz Agricultural Science & Technology Co., Ltd. (Guangzhou, China). Birds were balanced for laying rate then randomly allotted into 6 groups, each consisting of 6 replicates (8 cages for each replicate with 2 birds per cage). All experimental methods conformed to guidelines established by the Guangdong Academy of Agricultural Sciences Institutional Animal Care and Use Committee. The breeder hens were under study for 15 wk until the trial was finished at 42 wk of age. All birds received 16 h of lighting daily from 06:00 am to 10:00 pm. Room temperature and humidity were recorded daily.

Offspring of Hens During the last 2 wk of the hen trial, all breeder hens were artificially inseminated every 3 d with 30 μL pooled semen. Qualified eggs from each replicate were collected in the second wk and incubated under standard conditions for hatching. A total of 1,003 chicks were hatched. Chicks from hens fed 3.5, 8.5, 13.5, 23.5, 43.5, and 83.5 mg/kg Cu were used for corresponding treatments with the numbers of 145, 166, 187, 156, 160 and 145 respectively due to different hatchability and healthy hatchlings. Hatchlings from the same replicate of hens were likewise allotted to the same replicate in floor pens (1 replicate per pen with length 2.5 m × width 0.8 m) with wood shavings as litter. All experimental methods conformed to guidelines established by the Guangdong Academy of Agricultural Sciences Institutional Animal Care and Use Committee. The environmental room temperature reduced by 1°C every 2 d from 35°C to 25°C, and continuous artificial light was provided until the trial finished at 28 d of age.

Diets

Hens The basal diet (Table 1) was formulated to meet or exceed the nutritional requirements of breeder hens (Ministry of Agriculture in China, 2004) with no added Cu (analyzed 3.50 mg/kg). Calcium and nonphytate phosphorus contents in the basal diet were 3.29 and 0.45%, respectively. Additional Cu as $CuSO_4 \bullet 5H_2O$ (Guangdong Newland Feed Science & Technology Co., Ltd., Guangzhou, China) was incorporated into the diets (0, 5, 10, 20, 40, and 80 mg Cu/kg of diet). All breeder hens received about 115 g mash diet (as needed, consistently increased or decreased by a few g based on egg production) every day and had ad libitum access to fresh water. Water was deionized to eliminate any possible

Table 1. Composition of the basal diet of breeder hens.

Ingredient, %	Value
Corn	67.8
Wheat bran	5.96
Soybean protein concentrate	15.8
DL-Methionine	0.28
Calcium carbonate (analytically pure)	6.84
Dicalcium phosphate (food-grade)	2.07
Salt (NaCl)	0.25
Premix[1]	1.00
Nutritional level[2]	
ME, Kcal/kg	2,748
CP, %	16.3
Lysine, %	0.80
Methionine + cysteine, %	0.80
Calcium, %	3.29
Nonphytate phosphorus, %	0.45
Cu, mg/kg[3]	3.71
Cu, mg/kg[4]	3.50

[1]Provided per kg of diet: vitamin A, 12,000 IU (transretinyl acetate); cholecalciferol, 2,500 IU; vitamin E, 30 IU (DL-α-tocopheryl acetate); vitamin K, 1.5 mg, thiamine, 2 mg; riboflavin, 10 mg; niacin, 30 mg; pantothenic acid, 10 mg; vitamin B_6, 4 mg; folic acid, 1.1 mg; cobalamin, 0.01 mg; biotin, 0.15 mg; 50% choline chloride, 900 mg; $FeSO_4 \bullet H_2O$, 240 mg; $ZnSO_4 \bullet H_2O$, 209 mg; $MnSO_4 \bullet H_2O$, 283 mg; $NaSeO_3$, 0.56 mg; $Ca(IO_3)_2 \bullet H_2O$, 1.46 mg; ethoxyquin, 150 mg; calcium propanoate, 1.00 g; $NaHCO_3$, 1.50 g; maize cob meal (carrier), 4.75 g.

[2]Values were calculated based on the data presented in the Chinese feed database (Chinese feed database. 2018).

[3]Calculated Cu content based on Cu analyses in corn, wheat bran, and soybean protein concentrate.

[4]Copper was analyzed by atomic absorption spectrophotometry.

source of Cu before drinking by breeder hens. The apparatus for removing Cu from water was bought from Guangzhou Chenxing Environmental Protection Technology Co., Ltd. (Guangzhou, China). Copper in water was absorbed by silica sand then further eliminated by ion exchange. All birds were fed the basal diet for 3 wk for partial depletion of Cu stored in the body then with the experimental diets for an additional 12 wk.

Table 2. Composition of the diet of offsprings.

Ingredient, %	Value
Corn	65.3
Wheat bran	4.50
Soybean protein concentrate	22.8
DL-Methionine	0.15
Calcium carbonate (analytically pure)	1.00
Dicalcium phosphate (food-grade)	2.13
Salt (NaCl)	0.26
Maize cob meal	2.86
Premix[1]	1.00
Nutritional level[2]	
ME, Kcal/kg	2,883
CP, %	20.7
Lysine, %	1.11
Methionine + cysteine, %	0.77
Calcium, %	0.99
Nonphytate phosphorus, %	0.45
Cu, mg/kg[3]	3.56
Cu, mg/kg[4]	3.39

[1]Provided per kg of diet: vitamin A, 6,000 IU (transretinyl acetate); cholecalciferol, 500 IU; vitamin E, 20 IU (DL-α-tocopheryl acetate); vitamin K, 0.5 mg, thiamine, 3.8 mg; riboflavin, 4.0 mg; niacin, 42 mg; pantothenic acid, 10 mg; vitamin B_6, 3.5 mg; folic acid, 0.55 mg; cobalamin, 0.01 mg; biotin, 0.15 mg; 50% choline chloride, 1,200 mg; $FeSO_4 \bullet H_2O$, 267 mg; $ZnSO_4 \bullet H_2O$, 218 mg; $MnSO_4 \bullet H_2O$, 189 mg; $NaSeO_3$, 0.48 mg; $Ca(IO_3)_2 \bullet H_2O$, 0.57 mg; ethoxyquin, 225 mg; calcium propanoate, 2.10 g; $NaHCO_3$, 2.25 g; maize cob meal (carrier), 7.13 g.

[2]Values were calculated based on the data presented in the Chinese feed database (Chinese feed database. 2018).

[3]Calculated Cu content based on Cu analyses in corn, wheat bran, soybean protein concentrate and corn gluten meal.

[4]Copper was analyzed by atomic absorption spectrophotometry.

Chicks The diet (Table 2) was formulated based on corn and soybean protein concentrate with no Cu added in the premix to meet the nutritional requirements of Chinese Yellow broilers (Ministry of Agriculture in China, 2004). The Cu content in the diet was 3.39 mg/kg (analyzed value). All birds were fed the same diet (pellets), and feed and water were supplied ad libitum.

Measurements

Laying Performance Mortality was checked daily, and dead birds were recorded to adjust feed allowance, estimates of egg production and egg mass as appropriate. Number of total laid eggs, defective eggs (including those with double-yolk, soft-shell, cracked, very small, malformed, and so on), and total egg weight were recorded daily. At 42 wk of age (end of trial), egg laying rate, average egg weight, egg mass (egg weight of each breeder laid per day), and qualified egg rate (1 – total defective eggs/total eggs laid) were calculated. Qualified eggs met the criteria described by (Xu et al., 2010).

Sampling Two birds, representative of average egg production in each replicate, were individually weighed, and 5 mL blood was sampled from the brachial vein into evacuated tubes containing EDTA-K_2 (1 mg/mL blood). One milliliter of nonclotted blood was held to measure hemoglobin. The remainder (4 mL) was held on ice for < 1 h, then centrifuged at $860 \times g$ for 15 min at 4°C, and plasma aliquots were kept at −80°C until analysis. The birds were electrically stunned and exsanguinated to obtain tissues. Ovarian and oviductal weights were weighed and recorded. Ovarian index (%) = 100 × ovarian weight/live weight, and oviductal index (%) = 100 × oviductal weight/live weight. Oviductal length was measured with a ruler. The number of dominant follicles with diameter greater than 8 mm was recorded. The tibia was dissected from the right leg, and its breaking strength was determined in an Instron Universal Testing Machine with a 50-kg-load cell at 50-kg-load range with a crosshead speed of 50 mm/min (Park et al., 2003).

Biochemical Indices in Blood Hemoglobin in whole blood, malondialdehyde (**MDA**), GSH, GSSG, and Cu-ZnSOD in plasma was measured using MDA (thiobarbituric acid method), GSH (spectrophotometry), GSSG (spectrophotometry), and superoxide dismutase typed (hydroxylamine method) assay kits, respectively (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Biochemical Indices in the Liver The hepatic content of Cu and contents in the diets were analyzed by atomic absorption spectrophotometry (Shimadzu Corporation, Kyoto, Japan). Contents of MDA, GSH, and GSSG and activity of Cu-ZnSOD and Cu-ATPase in the liver were measured using MDA (thiobarbituric acid method), GSH (spectrophotometry), GSSG (spectrophotometry),

Table 3. qPCR primers.

Gene name	Sequence	GenBank no.
Atox1	f-5′- AGAAGACTGGAAAGAGCGCA-3′ r-5′- GCAGAGCAAGGTGGGAGATA-3′	NM_001277712.1
Atp7b	f-5′- CTGATAACGGGCGACAACAG-3′ r-5′- GTCACCAACCATTGCAACCT-3′	XM_015276078.2
Cox11	f-5′- CGAGTTTGCAGAGGACCCTA-3′ r-5′- GTTTCACAGGTGGCAGTCAG-3′	XM_001233972.5
Cox17	f-5′- GACGCCTGCATCATTGAGAA-3′ r-5′- ATCTTGCTCACCCCACTCAT-3′	NM_001302169.1
Commd1	f-5′-CACTCCTGCCCTCTACAACA-3′ r-5′- GCCATCACCTGCGTCAATAG-3′	XR_003074663.1
Cutc	f-5′- GAGTTTCACTGCTCTGCTCG-3′ r-5′- AATGGCATTCAGGGTCCTCA-3′	NM_001006503.1
β-actin	f-5′-GAGAAATTGTGCGTGACATCA-3′ r-5′-CCTGAACCTCTCATTGCCA-3′	L08165.1

Abbreviations: *Atox1*, Antioxidant 1 copper chaperone; *Atp7b*, ATPase copper transporting beta; *Commd1,* Copper metabolism domain containing 1; *Cox11*, Cytochrome c oxidase copper chaperone COX11; *Cox17*, COX17 cytochrome c oxidase copper chaperone; *Cutc*, cutC copper transporter; f, forward; r, reverse.

superoxide dismutase typed (hydroxylamine method), and Cu-ATPase (colorimetry) assay kits, respectively (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Quantitative RT-PCR Total RNA from stroma of the ovary was prepared using TRIzol (Invitrogen, Carlsbad, CA) and adjusted to 500 ng/mL for all samples before synthesizing first-strand cDNA (Promega, Beijing, China). Specific transcripts (mRNA) were quantified by quantitative PCR with an ABI 7500 Real-time Detection System (Applied Biosystems, Foster, CA) using a SYBR Premix Ex Taq II kit (Takara, Dalian, China). The primers for antioxidant 1 Cu chaperone (*Atox1*), ATPase Cu transporting beta (*Atp7b*), cytochrome c oxidase Cu chaperone COX11 (*Cox11*), COX17 cytochrome c oxidase Cu chaperone (*Cox17*), Cu metabolism domain containing 1 (*Commd1*), cutC Cu transporter (*Cutc*), and the house-keeping gene β-actin (Table 3) were based on chicken sequences and were synthesized by Sangon Biological Engineering Co., Ltd. (Shanghai, China).

Egg Quality At completion of feeding breeder hens the experimental diets (42 wk of age), 2 eggs per replicate, representative of the mean egg weight, were used to measure related indices of egg quality. Egg shape index, the ratio of vertical length to diameter at the midlength, was calculated from measurements made with a vernier caliper. Shell strength was determined with an Egg Force Reader (EFR-01; Orka, Ramat HaSharon, Israel). Eggshell was separated from the albumen and yolk, washed to remove residual albumen, and dried at 65°C for 4 h, then weighed. Eggshell proportion (%) = 100 × eggshell weight/egg weight. Shell thickness, yolk color, and Haugh unit were measured using an egg multitester EMT-5200 (Robotmation Co. Ltd., Tokyo, Japan). Shell thickness was calculated as the average thickness at the blunt end, sharp end, and middle of eggs. The yolk color was determined as per the La Roche scale (scores 1–15) (Zita et al., 2013).

Hatching Performance During the last 1 wk, qualified eggs (51–60 g) for hatch from each replicate were collected and recorded. Rate of qualified eggs for hatch (total qualified eggs for hatch/total eggs laid) was calculated. Forty qualified eggs for hatch per replicate consisting of equal numbers from each day were selected, weighed individually, and incubated under standard conditions for hatching. On the fifth day after the start of incubation, unfertilized eggs were recorded and eliminated. On the 18th day, eggs with dead embryos also were recorded and eliminated. The number of hatched chicks on day 21 and 22 was recorded and hatchling weight per replicate was recorded. Hatchability of fertilized eggs (number of hatched chicks/[total qualified eggs for hatch-unfertilized eggs]) and hatchability of live embryos (number of hatched chicks/live embryos on the 18th day) were calculated.

Growth Performance of Offsprings Mortality was checked daily, and dead birds were recorded and

Table 4. Effects of dietary copper on laying performance of Chinese Yellow broiler breeders.

	Dietary Cu content, mg/kg							*P*-value[2]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Egg production, %	64.3	63.4	64.6	63.2	62.3	62.9	1.07	0.682		
Egg weight, g	52.4	53.1	53.0	53.0	53.2	53.7	0.350	0.299		
Egg mass, g/d	33.7	33.7	34.2	33.2	33.2	33.7	0.567	0.807		
Qualified egg rate, %	97.5^{a}	97.2^{a}	97.4^{a}	95.4^{c}	97.1^{a,b}	96.1^{b,c}	0.348	0.001	0.050	0.076

[1]SEM from ANOVA (n = 6).
[2]Linear and quadratic contrasts examined only when Cu level was significant.
$^{a-c}$Means with no common superscript differ ($P < 0.05$).

Table 5. Biochemical indices in plasma of Chinese Yellow broiler breeders fed diets with different copper contents.

	Dietary Cu content, mg/kg							*P*-value[2]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Hemoglobin, g/L	111	124	113	101	84	125	3.44	0.055		
MDA, nmol/mL	5.72^{a}	5.10^{a,b}	3.04^{b}	2.91^{b}	4.01^{b}	7.08^{a}	0.777	0.004	0.246	0.002
GSH/GSSG, mol/mol	14.8^{b}	12.8^{b}	33.3^{a}	27.4^{a,b}	28.4^{a}	17.7^{b}	4.10	0.004	0.902	0.052
Cu-ZnSOD, U/mL	94.5	87.4	89.2	89.8	92.6	89.2	5.54	0.938		

Abbreviations: Cu-ZnSOD, copper-zinc superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde.
[1]SEM from ANOVA (n = 6).
[2]Linear and quadratic contrasts examined only when Cu level was significant.
[a,b]Means within row with no common superscript differ ($P < 0.05$).

weighed to adjust estimates of gain, intake, and feed conversion ratio, as appropriate. At 28 d of age, birds were deprived of feed overnight and weighed. During the trial of chicks, only initial and final weights were measured. Accumulated feed intake and just the rest of feed at the age of 28 d when trial was finished were recorded. BW gain and feed intake were determined, and ADFI, ADG, and feed/gain ratios were calculated.

Statistical Analysis

The effects of dietary Cu treatment were assessed by 1-way GLM ANOVA procedures of SAS (version 8.1) with replicates as the experimental unit for each variable. When needed for normality and homogeneity of variance, data were transformed. When the major effect was significant ($P < 0.05$), linear and quadratic effects of Cu content were determined. For variables of hens with significant effects, optimal dietary Cu level was determined using quadratic polynomial (**QP**) and broken-line (**BL**) (2-slope BL or BL with plateau) regression models (Gou et al., 2019). The QP model ($Y = \alpha + \beta \times Cu + \gamma \times Cu^2$) had Y as the dependent variable; α was the intercept; β was the linear coefficient; γ was the quadratic coefficient. The optimal response for Cu was defined as $Cu = -\beta/(2 \times \gamma)$. The 2-slope BL model ($Y = \alpha + \beta \times Cu$, $Cu \leq \gamma$; $Y = \delta + \varepsilon \times Cu$, $Cu > \gamma$) had Y as the dependent variable; α and δ were both intercepts; β and ε were slopes of the 2 lines. The Cu level at the break point (γ) was considered as that providing optimal response. The BL with plateau model ($Y = \alpha + \beta \times \gamma$, $Cu \leq \gamma$; $Y = \alpha + \beta \times Cu$, $Cu > \gamma$) had Y as the dependent variable; α was the intercept; β was the slope of line; the value ($\alpha + \beta \times \gamma$) was the plateau. The Cu level at the break point (γ) was considered to be that providing the optimal response.

RESULTS

Laying Performance of Chinese Broiler Breeder Hens

Egg laying rate, egg weight, and egg mass of the breeder hens were not influenced ($P > 0.05$) by the different levels of Cu fed (Table 4); highest mean values for egg laying rate and egg mass occurred with 13.5 mg/kg Cu. Qualified egg rate was affected ($P = 0.001$) by dietary Cu, with both linear ($P = 0.050$) and quadratic ($P = 0.076$) effects. Qualified egg rates of hens fed 3.5, 8.5, or 13.5 mg/kg Cu all were greater ($P < 0.05$) than that of hens fed 23.5 or 83.5 mg/kg Cu; besides, qualified egg rate of hens fed 43.5 mg/kg Cu was increased by 1.7 percent point ($P < 0.05$) compared with that of hens fed 23.5 mg/kg Cu.

Biochemical Variables

Blood hemoglobin was a tendency to be affected ($P = 0.055$) by dietary Cu (Table 5). Plasma MDA concentration was affected ($P = 0.004$) by dietary Cu with a quadratic ($P = 0.002$) effect. Plasma MDA concentration decreased first then increased with dietary Cu increased. Plasma MDA of hens fed 13.5, 23.5, or 43.5 mg/kg Cu all were less ($P < 0.05$) than that of hens fed 3.5 or 83.5 mg/kg Cu. The ratio of GSH/GSSG (mol/mol) was the highest in breeder hens fed

Table 6. Biochemical indices in the liver of Chinese Yellow broiler breeders fed diets with different copper contents.

	Dietary Cu content, mg/kg							*P*-value[2]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Copper, mg/kg DM	14.7^{b}	14.8^{b}	15.7^{b}	16.7^{b}	17.5^{b}	26.6^{a}	1.62	0.001	0.001	0.001
MDA, nmol/mg protein	0.884	0.916	0.728	0.770	1.090	0.956	0.113	0.272		
GSH/GSSG, mol/mol	0.865	0.931	0.969	0.880	0.894	1.273	0.114	0.345		
Cu-ZnSOD, U/mg protein	152	171	157	150	152	154	6.25	0.432		
Cu-ATPase, μmolPi/mg protein/h	5.36^{c}	5.97^{b,c}	5.72^{c}	5.91^{b,c}	6.53^{b}	7.80^{a}	0.272	0.001	0.001	0.001

Abbreviations: Cu-ZnSOD, copper-zinc superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde.
[1]SEM from ANOVA (n = 6).
[2]Linear and quadratic contrasts examined only when Cu level was significant.
[a-c]Means within row with no common superscript differ ($P < 0.05$).

Table 7. Organ and tissue indices of Chinese Yellow broiler breeders fed diets with different copper content.

	Dietary Cu content, mg/kg							*P*-value[2]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Weight of liver, g	38.6^{b}	34.1^{c}	35.7^{c}	33.1^{c}	34.1^{c}	41.8^{a}	0.892	0.001	0.007	0.001
Hepatic index, %	1.69^{a}	1.37^{b}	1.48^{b}	1.44^{b}	1.50^{b}	1.74^{a}	0.051	0.001	0.014	0.002
Weight of comb, g	7.33^{b}	6.37^{b}	7.77^{b}	7.63^{b}	9.66^{a}	6.92^{b}	0.574	0.009	0.630	0.007
Comb index, %	0.297^{b,c}	0.255^{c}	0.355^{a,b}	0.323^{a,b}	0.411^{a}	0.290^{b,c}	0.023	0.002	0.631	0.004
Femoral breaking strength, kgf	26.7	23.1	27.1	25.7	20.4	23.8	2.17	0.309		
Femoral index, %	0.484	0.497	0.473	0.511	0.479	0.454	0.014	0.082		

[1]SEM(n = 6).
[2]Linear and quadratic contrasts examined only when Cu level was significant.
[a-c]Means within row with no common superscript differ ($P < 0.05$).

13.5 mg/kg Cu with a significant ($P = 0.004$) effect being demonstrated.

Of the variables measured in the liver (Table 6), only Cu content and activity of Cu-ATPase were affected ($P = 0.001$) by different levels of dietary Cu; highest values occurred with 83.5 mg/kg Cu with both linear ($P = 0.001$) and quadratic ($P = 0.001$) effects.

Organ and Tissue Indices

There was a significant effect ($P < 0.05$) of dietary Cu level on liver weight and index of breeder hens, with both linear and quadratic ($P < 0.05$) effects (Table 7). Both weight of comb and comb index were affected ($P < 0.05$) by dietary Cu in a quadratic manner ($P < 0.05$). The highest value of femoral breaking strength occurred with 13.5 mg/kg Cu, and the lowest femoral index occurred with 83.5 mg/kg Cu, although no significant ($P > 0.05$) effects were demonstrated.

The relevant ovarian and oviductal variables, ovarian weight and index, oviductal weight and index, oviductal length, and number of dominant follicle all were not affected ($P > 0.05$) by dietary Cu content (data not shown).

Expression of Cu-Related Genes in Stroma of the Ovary There were significant effects of dietary Cu level on relative abundance of *Atp7b* ($P = 0.004$) and *Commd1* ($P = 0.001$) in ovarian stroma of breeder hens (Table 8). Breeder hens fed 13.5 mg/kg Cu had more *Atp7b* transcripts ($P < 0.05$) compared with others except the hens fed 23.5 mg/kg Cu. Breeder hens fed 13.5 mg/kg Cu had higher expression of *Commd1* ($P < 0.05$) than all others except for hens fed 8.5 mg/kg Cu.

Egg Quality

Relevant indices of egg quality, viz. egg shape index, shell strength, shell thickness, eggshell proportion, yolk color score, albumen height, and Haugh unit, are presented in Table 9. There were significant ($P < 0.05$) effects of dietary Cu on shell strength, yolk color score, albumen height, and Haugh unit. Shell thickness was affected ($P = 0.001$) by dietary Cu with a quadratic ($P = 0.001$) response. The highest value of egg shape index and eggshell proportion occurred with 13.5 mg/kg Cu, although no significant ($P > 0.05$) effects were demonstrated.

Hatching Performance

Some relevant data on hatching performance, fertility rate, hatchability, production of healthy hatched chicks, and hatchling weight are presented in Table 10. Of the variables examined, production of qualified eggs for hatch, proportion of live embryos on 18th day, hatchability of fertilized eggs and live embryos that hatched, and hatching weight of chicks were affected ($P < 0.05$) by dietary Cu; there was a linear effect ($P = 0.016$) on proportion of live embryos on the 18th day with both linear ($P = 0.001$) and quadratic ($P = 0.004$) effects on hatchling weight. Maximal production of qualified eggs for hatch and proportion of live embryos on the 18th day, occurred with diets of breeder hens containing

Table 8. Gene expression in stroma of ovary of Chinese Yellow broiler breeders fed diets with different copper content.

	Dietary Cu content, mg/kg							*P*-value[2]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Atox1	2.07	2.14	2.22	2.11	2.07	2.03	0.086	0.685		
Atp7b	0.261^{b,c}	0.319^{b,c}	0.594^{a}	0.479^{a,b}	0.154^{c}	0.362^{b}	0.076	0.004	0.618	0.875
Cox11	1.02	1.04	1.09	1.03	1.09	0.99	0.054	0.740		
Cox17	1.92	1.96	2.05	1.85	2.05	1.81	0.063	0.054		
Commd1	0.384^{b}	0.860^{a}	0.848^{a}	0.447^{b}	0.363^{b}	0.565^{b}	0.090	0.001	0.339	0.325
Cutc	1.33	1.41	1.37	1.22	1.26	1.22	0.055	0.097		

Abbreviations: *Atox1*, Antioxidant 1 copper chaperone; *Atp7b*, ATPase copper transporting beta; *Commd1*, Copper metabolism domain containing 1; *Cox11*, Cytochrome c oxidase copper chaperone COX11; *Cox17*, COX17 cytochrome c oxidase copper chaperone; *Cutc*, cutC copper transporter.
[1]SEM (n = 6).
[2]Linear and quadratic contrasts examined only when Cu level was significant.
[a-c]Means within row with no common superscript differ ($P < 0.05$).

Table 9. Egg quality of Chinese Yellow broiler breeders fed diets with different copper contents.

	Dietary Cu content, mg/kg							*P*-value[3]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Egg shape index	1.31	1.28	1.32	1.29	1.32	1.30	0.012	0.331		
Shell strength, kgf[2]	3.82^{c}	$4.44^{a,b}$	4.58^{a}	$4.42^{a,b}$	4.18^{b}	$4.14^{b,c}$	0.107	0.001	0.509	0.293
Shell thickness, mm	0.345^{b}	$0.353^{a,b}$	0.358^{a}	0.355^{a}	0.363^{a}	0.339^{c}	0.004	0.001	0.066	0.001
Eggshell proportion, %	9.05	9.09	9.35	9.17	9.17	9.09	0.108	0.433		
Yolk color score	4.02^{c}	5.07^{b}	6.70^{a}	5.23^{b}	4.93^{b}	5.13^{b}	0.296	0.001	0.972	0.673
Albumen height, mm	4.61^{b}	4.70^{b}	6.53^{a}	4.42^{b}	4.88^{b}	4.96^{b}	0.392	0.010	0.788	0.958
Haugh unit	66.1^{b}	66.7^{b}	79.5^{a}	63.0^{b}	67.6^{b}	69.8^{b}	2.75	0.006	0.984	0.966

[1]SEM (n = 6).
[2]kg-force.
[3]Linear and quadratic contrasts examined only when Cu level was significant.
[a-c]Means within row with no common superscript differ ($P < 0.05$).

13.5 mg/kg Cu, and minimal values of those variables and hatchability of fertilized eggs occurred with diets containing 83.5 mg/kg.

Growth Performance of Offsprings

Growth performance–related variables BW, ADFI, ADG, and F/G are presented in Table 11. There were significant effects ($P = 0.005$) of dietary Cu fed to breeder hens on both BW and ADG of their offsprings, with both linear ($P < 0.01$) and quadratic ($P < 0.05$) effects of dietary content.

Regression Analyses

The data of MDA in plasma, Cu in the liver, Cu-ATPase in the liver, and thickness of eggshell, all presenting significant quadratic effects, were selected for further analysis by QP and BL regressions related to the dietary Cu level. In some cases, QP models were not listed when the derived optimal values of dietary Cu were negative and inconsistent with reality (Table 12). As per the optimal dietary Cu response from regression models multiplied by the ADFI allowance of 115 g, the optimal daily Cu fed allowance of Chinese Yellow broiler breeder hens during the laying period was calculated (Table 12). When the 2 regression models were compared, for plasma MDA content, the BL model was better than QP with smaller *P*-value and greater R^2. For least values of plasma and hepatic MDA and hepatic activity of Cu-ATPase, the appropriate dietary Cu was between 15.7 and 21.2 mg/kg diet with 1.81 to 2.44 mg Cu daily allowance for these breeders weighing ~2.23 kg BW. A maximum response for thickness of eggshell was obtained at 38.6 mg/kg (4.44 mg/day/hen) and 41.1 mg/kg dietary Cu (4.73 mg/day/hen) for the QP and 2-slope BL models, respectively.

DISCUSSION

In the present study with Mahuang breeder hens, dietary Cu levels of 3.5, 8.5, 13.5, 23.5, 43.5, or 83.5 mg/kg had no significant effect on egg production, egg weight, and egg mass. Similar results were obtained in breeder hens fed diets with Cu content ranging from 10 to 120 mg/kg (Attia et al., 2011) and in laying ducks fed diets with 4 to 24 mg/kg Cu (Fouad et al., 2016). Even in laying hens, production performance was not affected by dietary Cu level between 100 and 300 mg/kg (Kim et al., 2016). In most cases, production performance of hens was affected with positive or negative effects by high level of dietary Cu (higher than 125 mg/kg) (Jackson and Stevenson, 1981; Al Ankari et al., 1998; Pesti and Bakalli, 1998; Pekel and Alp, 2011).

Copper is a redox-active metal, playing a major role along with iron in the production of ROS (Koivula and Eeva, 2010). Copper can cause oxidative stress and intracellular oxidative damage by increasing ROS formation (Stohs and Bagchi, 1995; Gaetke and Chow, 2003; Valko et al., 2005). In the present study, susceptibility to lipid oxidation reflected by MDA production

Table 10. Hatching performance of Chinese Yellow broiler breeders fed diets with different copper content.

	Dietary Cu content, mg/kg							*P*-value[2]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Rate of qualified eggs for hatch, %	73.6^{b}	$77.2^{a,b}$	85.6^{a}	71.2^{b}	72.4^{b}	69.5^{b}	3.44	0.050	0.075	0.208
Hatching egg weight, g	55.9	56.5	56.1	56.2	56.5	56.3	0.175	0.111		
Fertility rate, %	96.3	98.3	97.1	97.9	100.0	97.1	1.34	0.457		
Proportion of live embryos on 18th day, %	$95.9^{a,b}$	$91.2^{b,c}$	97.9^{a}	$94.4^{a,b,c}$	$91.7^{b,c}$	88.9^{c}	1.96	0.043	0.016	0.057
Hatchability of fertilized eggs, %	86.7^{a}	72.9^{c}	$83.3^{a,b}$	$75.7^{b,c}$	$82.5^{a,b}$	72.7^{c}	2.77	0.007	0.088	0.219
Hatchability of live embryos, %	87.2^{a}	76.1^{c}	$84.3^{a,b}$	$77.7^{b,c}$	88.2^{a}	$77.3^{b,c}$	2.86	0.023	0.408	0.487
Proportion of healthy hatchlings, %	92.7	94.7	98.0	96.0	98.5	93.9	1.68	0.109		
Hatchling weight, g	35.9^{b}	36.5^{b}	$36.7^{a,b}$	36.2^{b}	$36.7^{a,b}$	37.3^{a}	0.219	0.009	0.001	0.004

[1]SEM(n = 6).
[2]Linear and quadratic contrasts examined only when Cu level was significant.
[a-c]Means with no common superscript differ ($P < 0.05$).

Table 11. Growth performance of offspring of Chinese Yellow broiler breeders fed diets with different copper content.

	Dietary Cu content, mg/kg							*P*-value[2]		
Variable	3.5	8.5	13.5	23.5	43.5	83.5	SEM[1]	Cu level	Linear	Quadratic
Final BW, g	559[a]	574[a]	564[a]	516[b,c]	553[a,b]	512[c]	12.2	0.005	0.006	0.022
ADFI, g	34.6	35.8	33.9	33.9	34.6	32.0	1.14	0.341		
ADG, g	18.7[a]	19.2[a]	18.8[a]	17.1[b,c]	18.4[a,b]	16.9[c]	0.433	0.005	0.005	0.017
F/G	1.85	1.87	1.80	1.98	1.88	1.89	0.043	0.190		

Abbreviation: F/G, feed/gain.
[1]SEM (n = 6).
[2]Linear and quadratic contrasts examined only when Cu level was significant.
[a-c]Means within row with no common superscript differ ($P < 0.05$).

and oxidative stress occurred in hens fed the highest dietary Cu (83.5 mg/kg); in contrast, antioxidative and oxidative status (GSH/GSSG) of hens were both improved by moderate dietary supplementation (13.5–43.5 mg/kg Cu). Similar results were observed in pigs (Lauridsen et al., 1999; Rey and López-Bote, 2010).

The content of Cu in the liver is influenced by dietary Cu supply, and generally, when dietary Cu level is low, hepatic Cu is diminished and vice versa (Cousins, 1985). In the present study, hepatic content of Cu increased slightly without significant difference when hens were fed dietary Cu from 3.5 to 43.5 mg/kg but was significantly increased when the diet contained 83.5 mg/kg Cu. Skřivan et al. (2006) similarly found that when Cu level in the diet changed from 9.2 or 34.0 to 72.5 mg/kg or higher, hepatic content of Cu was significantly increased. In earlier studies, the amount of Cu accumulated in the liver also can be markedly influenced by doses of Cu (Bremner and Davies, 1976; Theil and Calvert, 1978; Weiner and Cousins, 1980). Powell (2000) indicated that once Cu accumulates at a site, it might cause repetitive radical formation through redox cycling. In the present study, oxidative stress was not observed in the liver but was evident in blood, presumably because increased activity of Cu-ATPase in the liver of hens fed 83.5 mg/kg Cu exported excess Cu into bile to protect against hepatic oxidative damage (Braiterman et al., 2015). The increased weight of the liver observed here with deficiency or excess of Cu needs to be confirmed and explored further in additional studies.

Inconsistent with changes in the hepatic activity of Cu-ATPase with increase of dietary Cu fed to hens, transcripts of *Atp7b* (one of the Cu-ATPases) in ovarian stroma were highest in number in the hens fed 13.5 mg/kg Cu. Highest expression of *Cox17* and higher expression of *Commd1* and *Cutc* were also observed in ovarian stroma of hens fed 13.5 mg/kg Cu. These probably indicate that Cu-related metabolism in ovarian stroma was optimal when hens were fed a normal level of dietary Cu. Copper metabolism domain containing 1 has a role in Cu homeostasis (Alina et al., 2014), and expression of *COMMD1* measured here was consistent with that of the other transcripts.

Copper deprivation can impair eggshell quality, as observed by scanning electronic microscopy (Berwanger et al., 2018). Efficiency of Cu deposition in eggshell is much higher than that of other mineral elements such as Zn and Mn (Skřivan et al., 2006; Dobrzanski et al., 2007; Abbas Ali et al., 2011). Copper may affect eggshell quality by its catalytic properties in enzymes involved in processes of membrane and eggshell formation and their interaction with calcite crystals in the forming eggshell (Abbas Ali et al., 2011). Therefore,

Table 12. Dose response regressions for Chinese Yellow broiler breeder hens fed diets with different copper content.

Variable	Model	Regression equation[1]	Optimal dietary Cu level, mg/kg	Optimal daily Cu fed allowance, mg	*P*-value	R^2
MDA[2] in plasma, nmol/mL	QP[2]	$Y = 5.65 - 0.128 \times X - 1.77 \times 10^{-3} \times X^2$	36.2	4.16	0.002	0.438
	Two-slope BL[3]	$Y = 6.77 - 0.261 \times X$ ($X \leq 17.2$) $Y = 1.06 + 0.071 \times X$ ($X > 17.2$)	17.2	1.98	<0.001	0.561
Copper in liver, mg/kg DM	BL with plateau[4]	$Y = 15.0$ ($X \leq 21.2$) $Y = 11.2 + 0.180 \times X$ ($X > 21.2$)	21.2	2.44	<0.001	0.636
Cu-ATPase in liver, μmolPi/mg protein/hour	BL with plateau[4]	$Y = 5.66$ ($X \leq 15.7$) $Y = 5.16 + 0.032 \times X$ ($X > 15.7$)	15.7	1.81	<0.001	0.706
Shell thickness, mm	QP[2]	$Y = 0.344 + 9.04 \times 10^{-4} \times X - 1.17 \times 10^{-5} \times X^2$	38.6	4.44	0.001	0.458
	Two-slope BL[3]	$Y = 0.348 + 4.20 \times 10^{-4} \times X$ ($X \leq 41.1$) $Y = 0.390 - 6.20 \times 10^{-4} \times X$ ($X > 41.1$)	41.1	4.73	<0.001	0.453

Abbreviation: MDA, malondialdehyde.
[1]Regression equations obtained using the analyzed Cu in the trial diets (3.5, 8.5, 13.5, 23.5, 43.5 and 83.5 mg/kg).
[2]QP: Quadratic polynomial; QP model: $Y = \alpha + \beta \times X + \gamma \times X^2$, where Y is the response variable, X is the dietary Cu, α is the intercept; β and γ are the linear and quadratic coefficients respectively. The optimal response was obtained by $-\beta/(2 \times \gamma)$.
[3]BL: Broken line; 2-slope BL model: $Y = \alpha + \beta \times Cu$, $Cu \leq \gamma$; $Y = \delta + \varepsilon \times Cu$, $Cu > \gamma$, where Y is the response variable, X is the dietary Cu, both α and δ are intercepts, both β and ε are slopes of lines. The Cu level at the break point (γ) was considered as the one providing the optimal response.
[4]BL with plateau model: $Y = \alpha + \beta \times \gamma$, $Cu \leq \gamma$; $Y = \alpha + \beta \times Cu$, $Cu > \gamma$, where Y is the response variable, X is the dietary Cu, α is the intercept, β is the slope of line, the value ($\alpha + \beta \times \gamma$) is the plateau. The Cu level at the break point (γ) was considered to be that providing the optimal response.

Cu plays an important role in eggshell quality. In the present study, both the lowest shell strength and reduced shell thickness in hens fed 3.50 mg/kg Cu are indicative of Cu deficiency. Lower qualified egg production, shell strength, and lowest shell thickness occurred in hens fed 83.5 mg/kg Cu here are also indicative of Cu excess, exceeding the level permitted earlier by the European Union (35 mg/kg) (Skřivan et al., 2006) and now reduced to 25 mg/kg (EFSA, 2016).

Deficiencies or excesses of Cu can affect egg quality and subsequent performance of the progeny (Berwanger et al., 2018). Hatching of embryos can be inhibited by Cu via inducing ROS and downregulating Wnt signaling (Zhang et al., 2018). In the present study, hatching of embryos was indeed reduced when hens were fed excess dietary Cu (83.5 mg/kg) resulting in more dead embryos. The reason may be that more ROS was produced in hens fed the highest level of Cu and oxidative stress occurred, as described before. Roychoudhury et al. (2016) indicated that Cu hampers embryo development in a dose-dependent manner. Copper is an essential trace element that plays vital roles in the physiology of animals including during fetal growth and development (El-Hussein et al., 2018).

In conclusion, Cu can accumulate in the liver and induce oxidative stress when hens are fed the highest tested level of dietary Cu. Qualified egg rate and plasma GSH/GSSG were of hens, and growth and development of the progeny were all decreased when hens were fed 83.5 mg/kg Cu. Lowest values for hatching performance occurred with this highest dietary Cu level. Breeder hens fed the lowest dietary Cu (3.5 mg/kg) also suffered some degree of oxidative stress, as did that of hens fed 83.5 mg/kg Cu. It can be concluded that 3.5 mg/kg dietary Cu for breeder hens in the laying period was insufficient. The negative impacts of Cu deficiency were relatively mild, relative to those of excess dietary Cu. The appropriate dietary Cu level for Chinese Yellow broiler breeder hens weighing about 2.36 kg during the egg-laying period is between 15.7 and 21.2 mg/kg with a daily allowance of 1.81 to 2.44 mg Cu per day when based on Cu level and Cu-ATPase activity in the liver.

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DISCLOSURES

There is no conflict of interest.

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