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Dietary levels of methionine plus cystine and chelated copper on the chemical composition of eggs and yolk cholesterol of 49-week-old brown laying hens

Níveis dietéticos de Metionina + Cistina e cobre quelatado sobre a composição química e colesterol dos ovos de poedeiras marrons com 49 semanas de idade

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Highlights:
Interaction of dietary levels of methionine + cystine and organic copper.
Chemical composition of the egg.
Met + Cys was determinant on yolk cholesterol concentration in laying hens.

Abstract

The present study aims to evaluate the possible interactions of dietary levels of methionine plus cystine (Met + Cys) and organic copper (Cu) on the chemical composition of eggs, using 320 Hy-Line Brown hens at 49 weeks of age. Composition and deposition variables were evaluated in a 4 × 5 factorial arrangement, with total analysed levels of 18, 44, 71, and 99 mg kg^{-1} of Cu and 0.613, 0.631, 0.816, 0.918, and 0.955% Met + Cys. We allocated four replicate cages (four hens/cage) to each treatment group. Two eggs per plot were sampled to determine the chemical composition of albumen and yolk, based on natural and dry matter and on daily rates of deposition. An interaction effect of Met + Cys and Cu levels was observed for deposition rates of ash and Cu in the yolk, and the chemical composition of Ethereal Extract (EE) and ash in eggs, EE in albumen and Nitrogen, EE, and ash in the yolk. An isolated effect was observed for Met + Cys on egg chemical composition and on shell and yolk fractions, as well as on deposition rates of albumen N, EE, and ash, and yolk EE. The yolk cholesterol content increased by 18.17% with increasing Met + Cys in diets. In conclusion, the chemical composition of eggs varied with dietary Met + Cys and organic Cu concentrations. Dietary levels of Met + Cys determined the yolk cholesterol concentration in laying hens.
Key words: Egg quality. Nutrients deposition. Organic minerals. Sulphur amino acids.

Resumo

O presente estudo teve como objetivo avaliar as possíveis interações dos níveis alimentares de metionina mais cistina (Met + Cys) e cobre orgânico (Cu) na composição química dos ovos, utilizando 320 galinhas Hy-Line Brown com 49 semanas de idade. As variáveis de composição e deposição foram avaliadas em arranjo fatorial 4 × 5, com níveis totais analisados de 18, 44, 71 e 99 mg kg-1 de Cu e 0,613, 0,631,

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0,816, 0,918 e 0,955% de Met + Cys. Alocaram-se quatro gaiolas replicadas (quatro galinhas / gaiola) para cada grupo de tratamento. Foram amostrados dois ovos por parcela para determinar a composição química do albúmen e da gema, com base na matéria natural e seca e nas taxas diárias de deposição. Foi observado um efeito de interação dos níveis de Met + Cys e Cu para as taxas de deposição de cinzas e Cu na gema e composição química do Extrato Etéreo (EE) e cinzas nos ovos, EE no albume e nitrogênio, EE e cinzas na gema. gema. Um efeito isolado foi observado para Met + Cys na composição química dos ovos e nas frações de casca e gema, bem como nas taxas de deposição de N no albúmen, EE e cinzas e gema EE. O teor de colesterol na gema aumentou 18,17% com o aumento de Met + Cys nas dietas. A composição do ovo variou com as concentrações de Met + Cys e Cu orgânico. Os níveis alimentares de Met + Cys determinaram a concentração de colesterol na gema em galinhas poedeiras.
Palavras-chave: Qualidade do ovo. Deposição de nutrientes. Minerais orgânicos. Aminoácidos sulfurados.

Introduction

In 2018, Brazil produced about 44.5 billion eggs, 11.45% more than in 2017. However, egg consumption is still considered low, representing only 212 per capita, as a consequence of contradictory information on the cholesterol content of eggs and possible harm that consumption causes to health (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2019). Good quality eggs are, unquestionably, a source of dietary nutrients for children and adults. Albumen and yolk contain several proteins with high biological value, with the most important being ovotransferrin and ovalbumin (Leeson & Summers, 2005).

Egg are composed of 9.5% eggshell, 24.0% yolk, and 67.5% albumen (Souza, 2017). On average, the cholesterol content of egg yolk varies according to weight: an egg with 60 g of yolk provides about 210 mg of cholesterol (Leeson & Summers, 2001; Spence, Jenkins, & Davignon, 2010).

Methionine is considered an essential amino acid and cystine is semi-essential, although this classification may vary among species. Methionine has an important role in metabolism, as it participates in the metabolic pathways of compounds such as adrenaline and creatine. It is a key component in protein synthesis, which is impaired if there is methionine deficiency, and it is present in most animal tissues (Dalibard et al., 2014). hens are fed inconsistent diets, with methionine content varying from high to deficient, laying performance is affected by an excess of fat in the ovary and liver (Bertechini, 2004). Methionine also participates in lipid biosynthesis, being involved in the transport of lipids in the blood (Patterson & Kung, 1988). DL-methionine is usually considered the first limiting amino acid for birds. When bird's diets are supplemented with amino acids, protein, nitrogen utilization and amino acid balance improve (Bunchasak & Silapasorn, 2005).

Both methionine and proteins influence egg size (Leeson & Summers, 2005) and methionine levels can be adjusted to control the egg size of late-cycle laying hens (Leeson & Summers, 2005). According to Summers, Atkinson and Spratt (1991), the use of methionine in a diet containing 10% crude protein (CP) produced a 10% increase in egg mass. However, Penz and Jensen (1990) found low egg and albumen weight for birds that received diets with only 13% CP. Increased egg production was observed by Solarte, Rostagno, Soares, Silva and Velasquez (2005) when laying hens received 0.684% methionine plus cystine (Met + Cys). According to Gambaro (2014), birds fed 0.77% Met + Cys had higher egg weights, compared to birds fed diets with only 0.66% Met + Cys. Barbosa et al. (1999) observed the same change in egg weight when Met + Cys were included in the diet of white and brown egg-laying hens. According to Brumano et al. (2010), increased Met + Cys consumption by light laying hens at 20 to 40 weeks resulted in increased

yolk percentage. Polese (2011) also found increasing Met + Cys levels increased the percentage of yolk, and the author argues that this was a consequence of choline synthesis, followed by the formation of yolk lipoproteins. Microminerals, which are also important for egg-laying, can be chelated or inorganic; chelates bind metal ions to other organic substances, making them more bioavailable than inorganic forms of minerals (Saldanha, 2008). For many years inorganic minerals have been used as low-cost dietary supplements, but recent studies (Nunes et al., 2013; Alves, Bastos-Leite, Goulart; Silva, & Medeiros 2015; Carvalho, Vilela, Fagundes, Souza, & Fernandes, 2016) suggest that use of organic minerals is more effective, because they are more bioavailable and are excreted less, reducing environmental pollution. Organic minerals (Cu and Zn) are involved in eggshell membrane formation (Baumgartner, Brown, Salevsky, Jr., & Leach, Jr., 1978), participate in enzymatic activities, and are important for animal health (Mabe, Rapp, Bain, & Nys, 2003; Richards, Zhao, Harrell, Atwell, & Dibner, 2010; Scottá et al., 2014; Rezende, 2016).

Mabe et al. (2003) reported that supplementing laying hen diets with organic Cu and Zn altered the mechanism of shell formation, probably by their interaction with calcium carbonate, modifying the texture of the shell. The addition of organic rather than inorganic minerals can increase egg weight and improve shell quality and bone strength of semi-heavy laying hens (Nunes et al., 2013). Carvalho et al. (2016) found it was possible to replace up to 70% of microminerals from inorganic sources with organic ones, improving shell quality, without affecting the birds' performance.

In 1883, Boutigny demonstrated the presence of copper in animal tissues. However, it was only in 1920 that studies in rats demonstrated that iron and copper were necessary for haemoglobin formation. Although not part of haemoglobin, copper is present in plasma proteins involved in the release of iron into plasma (Lesson & Summers, 2001). It is an important trace mineral for birds, being necessary in bone formation and as a component of extra and intracellular enzymes, such as cytochrome oxidase. Copper deficiency results in a difficulty in iron absorption and its mobilization from tissues (Lesson & Summers, 2001; Olgun, Yazgan, & Cufadar, 2013). Copper is absorbed by excess Ca, Fe, and Zn in the diet, and, in poultry, its deficiency produces anaemia, reproductive failure, and bone fragility (Scottá et al., 2014) and reduces lipoprotein lipase activity in extra-celular parts of tissues and liver cells (Valsala & Kurup, 1987).

According to Baumgartner et al. (1978), Cu deficiency in the diet affects eggshell quality. However, they found there was an increase in egg weight, which they contributed to changes in egg membranes and to the increase in albumen weight. The use of chelated Cu, Zn, and Mn microminerals associated with methionine resulted in improved bioavailability, compared to other sulphates added to the laying hen diet (Sun, Guo, Li, Zhang, & Wen 2012). Dobrzánski et al. (2008) found that including chelated copper in the laying diet resulted in an increase in Cu in the eggshell, blood and feathers, compared to birds that received inorganic copper.

Egg quality can be improved by supplying the birds with microminerals (Sechinato, 2003), and the chelated forms may increase egg weight, as well as having good mineral bioavailability (Carvalho, 2012; Lim & Paik, 2006). In addition to being essential to lipid metabolism, copper can affect both shell formation and quality (Baumgartner et al., 1978). According to Pesti and Bakalli (1998) and Idowu, Kuye, Oladele-Ojo and Eruvbetine (2005), dietary supplementation of copper could decrease egg cholesterol. As an essential and limiting amino acid for laying hens, methionine has a significant effect on egg weight (Kakhki, Golian, & Zarghi, 2016; Leeson & Summers, 2005). Sulphur amino acids, such as methionine and cystine, can modify the composition of the egg (Barbosa et al., 1999; Brumano et al., 2010; Polese, 2011).

However, most studies using amino acids and chelated minerals have focused on isolated effects on birds and eggs, thus information on the combined effects of nutrients is still scarce. However, amino acid supplementation has been increasingly used in the poultry diets to reduce the pollution caused by the residues generated and the cost of feed production (Jenn-Chung, Chung-Yi, & Peter Wen-Shyg, 1998). Therefore, the present study evaluated the possible interaction of dietary levels of Met + Cys and chelated Cu on the chemical composition of eggs.

Material and Methods

The research was carried out in Pirassununga, São Paulo, Brazil (Latitude 21º 57 'S, Longitude 47º 27' W). All experimental procedures were approved by the Ethics Committee on Animal Use (1589/2009 CEUA). The laboratory procedures were performed at the Laboratory of Bromatology and Monogastric Nutrition.

Animals

A total of 320 laying hens (Hy-Line Brown) were used, at 48 weeks old (around 89% egg production), and the results of the treatments were assessed after 48 to 49 weeks. Laying hens and treatments were allocated, in a completely randomized way, to four replicate cages (4 hens/cage) with dimensions of 1.0 x 0.4 x 0.4 m with four divisions of 0.25 x 0.4 x 0.4 and two birds per division.

The birds were distributed in galvanized wire cages arranged in a line and equipped with a galvanized plate feeder and *nipple* drinking supply. Food and water were provided *ad libitum*. The artificial lighting was kept constant during the evaluation periods to ensure 17 hours of light every 24 hours.

Diets and analysis

Dietary treatments (Table 1) were assigned in a 4 x 5 factorial arrangement, using analysed levels of chelated Cu (18, 44, 71, and 99 mg kg^{-1}) and five levels of Met + Cys (0.613, 0.631, 0.816, 0.918, and 0.955%). The diets were based on corn and soybean meal and formulated to meet the minimum recommendations proposed by Rostagno et al. (2011), except for Met + Cys levels. The lowest level Met + Cys diet was divided into five equal batches. DL-methionine (RHODIMET®, DL-methionine 99%) was added to the diet to obtain increased levels of this amino acid.

We formulated diets with 0, 22, 43, and 88 mg kg^{-1} of supplied chelated copper. Differences between the formulated and analysed values resulted from mineral fractions in other dietary ingredients, such as corn and soybean meal. Therefore, Cu estimation was corrected for 18 mg kg^{-1}, corresponding to a concentration of 0 mg kg^{-1} (without supplementation). For estimated digestible Met + Cys, a level of 89.23% was used as an index, according to the digestible coefficient (National Research Council [NRC], 1994). This index accounted for the weighted average relationship between digestibility and the total Met + Cys from corn and soybean meal.

We sampled two eggs per plot to determine the chemical composition, including Crude protein (CP %), Ethereal Extract (EE %), Ash (%), Water (H_2O %), and cholesterol, of the eggs. The egg yolk and albumen were placed in separate containers, frozen at -40°C, freeze-dried and ground to powder for further analysis.

Crude protein, EE and MM were analysed according to the Association of Official Analytical Chemists (AOAC, 2005), copper levels were determined using inductively coupled plasma optical emission spectrometry (Model 710 ICP-OES, Agilent Technologies, Santa Clara, California, US).

Table 1.
Ingredients and nutritional composition of experimental diets

	Met + Cys Total(%) [1]				
	0.613	0.631	0.816	0.912	0.955
	Met + Cys Digestible [2]				
Ingredients (%)	0.491	0.606	0.708	0.781	0.846
Corn grain	61.028	61.094	61.231	61.105	60.767
Soybean meal	23.299	23.063	22.813	22.827	23.024
Soybean oil	1.893	1.895	1.839	1.800	1.800
L-Lysine HCl	0.109	0.117	0.125	0.125	0.119
DL Methionine	0.060	0.214	0.368	0.519	0.669
L-Threonine	0.030	0.034	0.037	0.037	0.035
L-Tryptophan	0.003	0.004	0.005	0.005	0.004
Salt	0.221	0.221	0.221	0.221	0.221
Limestone	11.314	11.314	11.314	11.314	11.314
Dicalcium phosphate	1.471	1.474	1.476	1.476	1.475
Choline chloride	0.030	0.030	0.030	0.030	0.030
Baking soda	0.279	0.279	0.279	0.279	0.279
Kaolin or Carboquelate Cu -16% [5]	0.063	0.063	0.063	0.063	0.063
Premix Vit [3]	0.100	0.100	0.100	0.100	0.100
Premix Min [3]	0.100	0.100	0.100	0.100	0.100
Total	100.00	100.00	100.00	100.00	100.00
Analyzed values - % [4]					
Dry Matter	89.30	89.88	89.47	89.80	89.22
Crude protein	16.52	16.19	16.54	16.47	15.63
Met + Cys	0.613	0.631	0.816	0.918	0.955
Methionine[3]	0.313	0.373	0.536	0.635	0.702
Cystine	0.299	0.258	0.280	0.282	0.253
Lysine	0.914	0.840	0.887	0.917	0.870
Threonine	0.722	0.626	0.680	0.737	0.622
Arginine	0.964	0.788	0.835	0.878	0.861
Isoleucine	0.682	0.608	0.652	0.692	0.589
Leucine	1.545	1.411	1.479	1.561	1.337
Valine	0.762	0.681	0.760	0.798	0.699
Alanine	0.916	0.809	0.861	0.922	0.812
Histidine	0.437	0.377	0.384	0.417	0.408
Phenylalanine	0.876	0.802	0.833	0.910	0.780
Áspartic acid	1.621	1.425	1.524	1.639	1.387
Glutamic acid	3.082	2.778	2.925	3.099	2.706
Glicine	0.634	0.615	0.626	0.669	0.573
Serine	0.851	0.751	0.798	0.864	0.739
Tyrosine	0.650	0.606	0.619	0.652	0.559

continue

continuation

Calculated values - % [1]					
Metabolizable Energy - kcal/kg	2763	2768	2770	2770	2.770
Calcium	4.7	4.7	4.7	4.70	4.7
Phosphor avaliable	0.36	0.36	0.36	0.36	0.36
Sodium	0.18	0.18	0.18	0.18	0.18
Chlorine	0.18	0.18	0.18	0.18	0.18
Linoleic acid	2.368	2.368	2.368	2.317	2.312
Digestible tryptophan	0.168	0.168	0.168	0.168	0.168
Digestible Methionine + Cystine	0.500	0.650	0.800	0.950	1.100
Digestible Lysine	0.800	0.800	0.800	0.800	0.800
Digestible Threonine	0.560	0.560	0.560	0.560	0.560

[1] (Natural matter) Reviewed by Ajinomoto Biolatin Industry & Com. Ltda. [2]Estimated based on laboratory analysis and the weighted average ratio of 89.23% of digestibility, used in relation to the total Methionine + Cystine of the main ingredients, according to NRC (1994). [3] Provided per kg of diet: folate, 145 mg; pantothenate, 5.930 mg; niacin, 12 g; Vit.A 5,000,000 IU; B12 6,500 mcg; B2 2,000 mg; B6 300 mg; D3 1,850,000 IU; Vit.E. 4,500 IU; Vit.K 918 mg. If (selenate) 500 mg; Fe 55g, I 1,550mg; Mn 70 g; Zn (min.) 50 g. [4] Provided per kg premix: Manganese, 88 g; iron, 55g; Zinc, 88g; iodine, 1.7g; Selenium, 3g. [5] Analyzed by CBO Brazil - 100% chelated copper: N, 3.07%; Ala, 0.06%; Arg, 0.04%; Aspartic acid, 0.02%; Gly, 0.10%; Ile, 0.02%; Leu, 0.05%; Glutamic acid, 0.12%; Lys, 0.00; Cys, 0.03%; Met, 0.01%; Phe, 0.03%; Tyr, 0.09%; Thr, 0.06%; Pro, 0.44%; Val, 0.05%; His, 0.00%; Ser, 0.04%; sum amino acids, 1.1.

Cholesterol determination was performed using the direct saponification technique, according to the methodology described by Mazalli, Saldanha and Bragagnolo (2003). We weighed 0.25 g of yolk sample in 70 m L test tubes with screw cap, then 10 m L of 2% potassium hydroxide solution (KOH) in ethyl alcohol PA (absolute alcohol) was added. The tubes containing the samples and solution were placed in a 50°C water bath with stirring for 2 hours. Subsequently, 5 m L of distilled water was added and we allowed the tubes to cool. Each tube received 10 m L of hexane, was then vortexed (tube shaker) for 1 minute to extract the hexanic phase (unsaponifiable matter), then this phase was transferred to other test tubes with screw caps. The hexanic phase extraction process was repeated twice for each tube. We determined cholesterol levels using a high-performance liquid chromatography (HPLC) method adapted from Mazalli et al. (2003). A solution (mobile phase) of acetonitrile and isopropanol in the ratio of 85:15 was prepared. Subsequently, in properly identified 1.5 mL vials (Vials, Agilent Technologies), 0.5 mL of the hexane phase was added and inserted into the lyophilizer for drying for 20 minutes. All the liquid in each vial was transferred to 5 m L syringes (with pore membrane), then transferred to other vials and subsequently analysed by HPLC.

Statistical analysis

The data were analysed using the SISVAR procedure 5.6. Significance was $P < 0.05$. The variables were submitted to regression analysis by orthogonal polynomials.

Results and Discussions

We analysed the interactions of Met + Cys and Cu with following variables: yolk ash deposition and yolk Cu (Table 2); egg EE and ash on natural matter; and yolk N, EE, ash, and Cu concentrations (Table 3).

We did not find relationships between the levels of Met + Cys and chelated-Cu and the egg weight and egg content, as shown by the statistical

results. However, an increase in Met + Cys levels increased yolk weight. The response obtained for the variable 'egg weight' in the present study differs from Barbosa et al. (1999), who found an increase in Met + Cys levels (0.434 to 0.734%) resulted in an increased egg weight for light laying hens and semi-heavy laying hens. In contrast, Bendezu et al. (2015) observed that the increase in Met + Cys content coincided with a lower egg weight for laying hens in the first production cycle. Although different studies provide contrasting results, the level of methionine in laying diets is related to egg weight (Bunchasak & Silapasorn, 2005; Brumano, 2009; Gambaro, 2014).

The effects of dietary Met + Cys and Cu on egg characteristics and contents and on deposition rates are presented in Table 2. Dietary increase in amino acids produced a linear increase in yolk weight ($P = 0.006$). The same tendency ($P < 0.10$) was observed for albumen and shell weights, according to equations presented in Table 2. Effects of Met + Cys also were observed on albumen N, EE, and Ash deposition, resulting in a positive quadratic response ($P < 0.01$). The mean estimated by equations of total Met + Cys was 0.769 ± 0.002% and digestible Met + Cys was 0.666 ± 0.002, based on the index from the digestible coefficients (NRC, 1994). In yolk, there was higher EE deposition at an estimated level of 0.839% of total Met + Cys or 0.749% digestible Met + Cys.

Table 2.
Effect of dietary levels Met + Cys and Cu on the weight of egg fractions and daily deposition rates

	Methionine + Cystine%						Copper(mg kg-1)						*P* - value		
Variables	**0.613**	**0.631**	**0.816**	**0.918**	**0.955**	**SEM**	**18**	**44**	**71**	**99**	**SEM**	**Mean**	**Met+Cys[1]**	**Cu[2]**	**Met*Cu[2]**
Egg weight (g)	64.23	67.45	66.65	67.08	66.58	0.631	66.38	66.74	65.66	66.80	0.564	66.40	0.152	0.469	0.221
Eggshell (g)	6.13	6.38	6.33	6.40	6.40	0.077	6.34	6.38	6.22	6.36	0.069	6.33	0.061	0.303	0.747
Yolk weight (g)	15.45	16.20	16.40	16.18	16.58	0.223	16.04	15.86	15.90	16.34	0.199	16.10	0.005	0.285	0.339
Alb weight (g)	43.38	44.53	44.00	44.50	43.50	0.534	44.04	44.28	43.48	44.12	0.478	43.98	0.083	0.546	0.347
N in the Alb (g/day)[3]	0.70	0.85	0.85	0.94	0.71	0.041	0.88	0.80	0.80	0.77	0.036	0.81	0.004	0.680	0.168
EE in the Alb (g/day)[3]	0.47	0.63	0.62	0.61	0.47	0.027	0.59	0.55	0.59	0.51	0.024	0.56	0.001	0.103	0.154
Alb in Ash (g/day)[3]	0.18	0.20	0.21	0.20	0.17	0.009	0.20	0.19	0.19	0.18	0.008	0.19	0.012	0.483	0.365
N in yolk (g/day)[3]	0.37	0.40	0.40	0.41	0.41	0.007	0.43	0.38	0.37	0.40	0.006	0.40	0.091	0.865	0.621
EE in yolk (g/day)[3]	4.80	5.08	5.28	5.18	5.23	0.078	5.22	5.10	4.96	5.16	0.069	5.11	0.001	0.110	0.144
Yolk Ash (g/day)[3]	0.37	0.40	0.29	0.34	0.43	0.013	0.38	0.31	0.38	0.39	0.012	0.36	0.001	0.001	0.001
Cu in the yolk (mg/day)[3]	0.05	0.07	0.08	0.08	0.09	2.948	0.07	0.07	0.08	0.08	2.637	0.07	0.001	0.037	0.001
Totals Ash (g/day)[4]	0.54	0.60	0.50	0.55	0.60	0.017	0.58	0.51	0.57	0.57	0.015	0.56	0.001	0.005	0.001

[1]Methionine + Cystine, [2]Met + Cys * Cu (Interaction methionine + cystine and copper), [3] Daily deposition rates, [4]Daily deposition (egg without shell)
Response equations for the level of Methionine + Cystine in the composition and chemical deposition of eggs. Eggshell weight (g): $Y= 5.912+ 0.000522X$, Yolk weigth (g): $Y= 14.579 + 0.002026X$; N in the Alb (g/day): $Y= -2.038+ 8.032X - 5.231X^2$; EE in the Alb (g/day): $Y= - 2.477+ 8.136X - 5.266X^2$; Ash in Alb (g/day): $Y=- 0.567+ 2.038X- 1.3233X^2$; EE in the yolk (g/day): $Y= 0.575+ 11.196X-6.671X^2$; N in the Alb (%) : $Y= -3.7690+ 16.054X -10.526X^2$;EE in the Alb (%)4 $Y= -5.133+17.209X-11.1653X^2$;Ash in the Alb (%): $Y= 1.106+ 4.159X- 2.712X^2$; Cholesterol (mg):$Y= -173,281+932,457X-536,581X^2$.

Table 3.
Effect of dietary levels of Met + Cys and Cu on the chemical composition of egg, albumen, yolk and cholesterol content of eggs

	Methionine + Cystine (%)					SEM	Copper (mg kg-1)				SEM	*P* - value			
Variables	**0.613**	**0.631**	**0.816**	**0.918**	**0.955**		**18**	**44**	**71**	**99**		**Mean**	**Met[1]**	**Cu[2]**	**Met*Cu[3]**
EE in the egg (%) [4]	9.10	9.48	9.78	9.55	9.48	0.104	9.70	9.38	9.40	9.42	0.093	9.48	0.001	0.055	0.039
Ash in the Egg (%) [4]	0.93	0.98	0.83	0.90	0.99	0.026	0.96	0.84	0.95	0.95	0.023	0.93	0.001	0.001	0.001
N in the Alb (%)[5]	2.03	2.28	2.23	2.23	1.88	0.082	2.18	2.14	2.14	2.14	0.073	2.14	0.009	0.480	0.128
EE in the Alb (%)[5]	1.11	1.42	1.41	1.37	1.07	0.058	1.35	1.25	1.35	1.16	0.051	1.27	0.001	0.031	0.070
Ash in the Alb (%)[5]	0.41	0.46	0.47	0.45	0.38	0.021	0.46	0.44	0.43	0.41	0.019	0.43	0.024	0.350	0.388
N in the yolk (%) [5]	2.88	2.83	2.88	2.83	2.80	0.016	2.82	2.86	2.86	2.82	0.015	2.84	0.195	0.047	0.051
EE in the yolk (%) [5]	31.15	31.43	32.33	32.00	31.33	0.169	31.80	31.84	31.28	31.66	0.151	31.65	0.001	0.064	0.001
Ash in the yolk (%)[5]	2.37	2.42	1.79	2.13	2.57	0.079	2.29	1.95	2.39	2.39	0.071	2.26	0.001	0.001	0.001
Cholesterol (mg) [6]	189.53	209.78	228.23	231.63	227.90	3.530	220.32	214.92	218.46	215.94	3.157	217.41	0.001	0.618	0.154
Cu in the yolk (mg) [7]	6.50	7.93	9.95	9.53	10.98	0.348	8.50	8.42	9.16	9.82	0.312	8.98	0.001	0.007	0.003

[1]Methionine +Cystine , [2]Copper, [3]Met+ cys*Cu (Interaction Methionine + Cystine and Copper), [4] Chemical composition of egg, [5] Composition in the Natural Matter,[6] Total choelesterol in yolk, [7]Concentration the Copper in the yolk.

Equations of the interaction response Met + Cys * Cu in the composition and chemical deposition in the egg: EE in the egg (%): Met within Cu 18: $Y= - 5.169+ 39.357X-25.2077X^2$, Met within Cu 44: $Y= -9.573+ 49.701X- 31.530X^2$, Cu within Met 0.816: $Y= 10.476-0.012X$; Ash in the egg (without peel)(%): Met within Cu 99: $Y= 6.837- 15.538X+9.919X^2$, Cu within Met 0.631:$Y=0.610+ 0.017X- 0.00015X^2$; Ash in the Yolk (g/day): Met within Cu 99: $Y= 4.372 -10.5840X+ 6.8007X^2$, Cu within Met 0.613: $Y= 0.459-0.0072X+0.000076X^2$, Cu within de Met 0.955 :$Y= 0.898-0.0197X+0.00016X^2$;Cu in the yolk (g/day): Met within Cu 18: $Y= - 461.707+ 1313.523X-781.201X^2$, Met within Cu 44: $Y=-3.625+93.518X$, Met within Cu 71: $Y= -14.107+114.652X$, Cu within Met 0.631: $Y= 35.326+0.540X$, Cu within Met 0.816: $Y=95.64+0.199X$; N in the yolk (%) : Met within Cu 99: $Y= 3.054-0.318X$, Cu within Met 0.816: $Y=2.678+0.007X-0.000060X^2$, Cu within Met 0.955: $Y=2.747+0.0054X-0.000054X^2$; EE in the yolk (%): Met within Cu 18: $Y= - 5.326+98.688X- 63.434X^2$, Cu within Met 0.613: $Y= 32.586 - 0.0245X$;Cu within Met 0.918:$Y= 34.174-0.103X+0.000896X^2$; Ash in the Yolk (%): Cu within Met 0.631: $Y= 1.113+ 0.057X- 0.000464$.

Regarding the chemical deposition rates, the average increase with Met + Cys was 0.759 ± 0.013%, which showed a positive effect on the deposition rates of N, EE, and ash in albumin, similar to the results observed for EE deposition in the yolk. The deposition rates observed in the EE yolk and N albumen variables increased by 10 to 26% in response to the increase in total Met + Cys levels from 0.613 to 0.918%.

We found that amounts of amino acids and trace mineral effected yolk ash and copper deposition ($P < 0.001$). The increase in Met + Cys levels in the 99 mg kg^{-1} Cu diet estimated 0.778% of total Met + Cys. In hens fed the diet with 18 mg kg^{-1} Cu, increasing of total Met + Cys concentrations up to 0.841% increased Cu deposition. In contrast, the variation of Cu in the diets with 0.631 and 0.816% of Met + Cys coincided with a linear increase ($P < 0.05$) in the deposition of Cu in the yolk. Whereas the varying Cu levels in the diets with 0.613 and 0.955% of Met + Cys resulted in an estimated ($P < 0.001$) minimum and maximum deposition of 50 and 65 mg kg^{-1} Cu, respectively, in the yolk.

The percentages of chemicals in the egg, albumen, yolk and cholesterol, according to the levels of Met + Cys and Cu are presented in (Table 3). Overall, the increase in amino acids within Cu concentrations was estimated 0.784 ± 0.004% of total Met + Cys. However, the Cu concentration changes within the Met + Cys diets were found for 60.24 ± 10.51 mg kg^{-1} or 42.24 ± 10.51 mg kg^{-1} of supplied chelated inorganic copper.

We found that an increase in Met + Cys concentrations in diets with higher Cu levels produced a linear decrease ($P = 0.003$) in N concentrations in the yolk. In the diet with 0.816% of Met + Cys, Cu increased by 60 mg kg^{1}, as estimated by the quadratic equation. The EE composition of yolk negatively correlated with varying Met + Cys levels in the diet containing 18 mg kg^{-1} of Cu, and the EE was estimated to be optimum for 0.778% of the total amino acid. In the diet with 0.918% Met + Cys, 58 mg kg^{-1} of Cu led to the lowest EE content in the yolk.

With regards to the chemical composition of the egg and its fractions, we observed that increasing Met + Cys, in diets containing 18 and 44 mg kg^{-1} Cu, to a mean level of 0.785% increased the EE as estimated by quadratic equations. The same interaction showed the opposite effect when we increased Cu in the diet containing of 0.816% of total Met + Cys, as there was a linear reduction in the EE content of eggs. As regards the egg (without shell), we observed that increasing Met + Cys levels in the diet with 99 mg kg^{-1} Cu resulted in a reduction of egg ash content. As reported by Medici et al. (2013), Cu may regulate the metabolism of methionine, which is an essential amino acid linked to polyunsaturated fatty acids (PUFA) metabolism (Maroufyan et al., 2013). Therefore, Cu may also affect the lipid composition of eggs.

The composition of N in the yolk was affected by the interaction between amino acids and mineral, suggesting a probable antagonistic effect. An increase in Cu concentrations in the diets containing 0.816 and 0.955% of Met + Cys indicated a reduction in Cu requirement. Therefore, when Met + Cys levels increased in the diet, the demand for Cu was reduced. Comparing the results of the present study with the recommendations of Rostagno et al. (2017) for productive laying hens, we found a lower average level for Met + Cys and a similar inorganic Cu level. The results suggest, therefore, that there was a dependant relationship in the increase in the mineral in the diets with Met + Cys, coinciding with lower need for both, according to the changes in the chemical compositions of the eggs.

The chemical composition of the eggs, expressed in Natural Matter, also presented a positive quadratic variation in response to sulphur amino acids.

The concentrations of N, E, and ash in the Albumen increased when the concentration of Met + Cys were increased in the diet, to the mean level

of 0.767% total Met + Cys. Dietary levels of sulphur amino acids above the estimated would have a negative effect on these variables, as observed from the experimental treatment containing 0.816% of total Met + Cys.

Sulphur amino acids were observed (P = 0.001) to affect the cholesterol content, as an increase of 0.613 to 0.918% Met + Cys resulted in an increase in the cholesterol content, as presented in Table 3. The extent to which the levels of Met + Cys increased the quadratic response was observed (P < 0.001)

The effect of Met + Cys on the increase of yolk cholesterol was about 18%, suggesting that the sulphur amino acids were favourable for lipid synthesis. The increasing levels of digestible Met + Cys resulted in a higher percentage of yolk, because choline, which is synthesised from methionine, is used to make yolk lipoproteins and phospholipids (Polese, 2011). The effects of Met + Cys on the yolk cholesterol found in the present study differ from those found by Kakhki et al. (2016), who did not report an effect of these amino acids on the yolk.

The highest level of organic copper used in this study (99 mg kg^{-1}) did not produce significant effects, so the effects of the interaction were characterized as a regression deviation. This differs from the results found by Idowu et al. (2005) and Lien, Chen, Wu and Lu (2004), which suggested Cu reduced yolk cholesterol when the mineral concentration was raised to 250 mg kg^{-1}. Balevi and Coskun (2004), when analysing the effect of copper, including 150 mg kg^{-1} of Cu, on production and cholesterol, observed a reduction in yolk cholesterol without affecting the performance of the birds. However, this effect was not observed by Lim and Paik (2006), as they concluded that Cu had no effect on egg yolk cholesterol.

We observed a linear increase in the concentration of Cu in the yolk (P < 0.01) with increasing Met + Cys content in the diets with 18, 44, and 71 mg kg^{-1} copper. A similar linear increase (P < 0.05) of Cu in yolk was observed with increasing Cu supplementation in the diets with 0.631 and 0.816% Met + Cys.

The amount of Cu in the yolk was dependant on the levels of Met + Cys in the diet. The same effect was observed when the concentration of copper was changed in the diets with 0.631 and 0.816% Met + Cys. The results of the present study agree with those reported by Pesti and Bakalli (1998): they observed that increasing Cu in the diet increased its concentration in the yolk. These observations suggest that a variation of Cu and Met + Cys intake may affect the metabolism of hens. Using a rat model, Medici et al. (2013) demonstrated the interactions between Cu and methionine through the accumulation of Cu, reflecting metabolism. As reported by Medici et al. (2013), Cu may regulate the metabolism of methionine, an essential amino acid linked to polyunsaturated fatty acids (PUFA) metabolism (Maroufyan et al., 2013).

As reviewed by Trindade Neto et al. (not published), methionine and Cu are related to metabolism through their known inhibitory effect on S-adenosylhomocysteine hydrolase, which inhibits transmethylation reactions. In this same review, the authors reported that Cu can also coordinate a range of ligands, including cysteine thiolate and methionine thioether groups, and engage in cation interactions. In addition, a number of copper-dependent proteins and their functions have yet to be identified. Thus, many questions in the field of copper metabolism have yet to be answered.

The effects of copper on human nutrition are correlated to the immune system and the actions of antioxidants, as well as to the prevention of degenerative diseases. According to Lien et al. (2004), high levels of copper in the diet of laying hens increased the retention of Cu in the egg, but consuming eggs with high concentrations of Cu would cause health problems. In a review, Sahoo, Kataria and Mehta (2016) observed that the maximum daily intake of Cu should not be above 10

mg/day for humans because, although Cu helps in reducing LDL and cellular oxidation, high amounts could affect the use of Zn by the body. In the present study, the average Cu in the yolk was 8.98 mg kg^{-1}, within the limit suggested by Sahoo, Kataria and Mehta (2016).

Conclusion

The chemical composition of the eggs varied with the dietary concentrations of Met + Cys and organic Cu. The Met + Cys content of the laying hen diet interfered with the amount of yolk cholesterol.

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