Methionine plus Cystine Levels for Light Laying Hens on Growth Phase

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ABSTRACT

The aim of this study was to evaluate the methionine plus cystine levels in the diets of laying hens during the rearing period (13-18 weeks old). The diets included a control diet formulated according to NRC and five other diets with different levels of this digestible methionine plus cystine (0.317, 0.356, 0.396, 0.436 and 0.475%) that have been based on the recommendations of the Brazilian poultry and swine tables. Performance, serological and histological variables were evaluated. There was a linear effect on final body weight, weight gain, methionine plus cystine intake and feed conversion, and quadratic effect on the serum albumin levels, serum protein levels and relative spleen weight. The estimated levels of digestible methionine plus cystine were 0.361%, 0.346% and 0.398%, for albumin activity, serum protein and relative weight of spleen, respectively. The different formulation bases significantly influenced the histology of the liver, small intestine and magnum. It is recommended the use of levels above 0.475% of digestible methionine plus cystine for light laying hens with 13-18 weeks old, which corresponded to a ratio of digestible methionine plus cystine: digestible lysine of more than 98%.

Keywords: Nutritional requirements. Productive performance. Sulfur amino acids.

RESUMO

O objetivo deste estudo foi avaliar os níveis de metionina + cistina nas dietas de galinhas poedeiras durante o período de crescimento (13 a 18 semanas). As dietas incluíram uma dieta controle formulada de acordo com o NRC e outras cinco dietas com diferentes níveis de metionina + cistina digestível (0,317, 0,356, 0,396, 0,436 e 0,475%), baseadas nas recomendações das tabelas brasileiras de aves e suínos. O desempenho produtivo, variáveis sorológicas e histológicas foram avaliadas. Houve efeito linear no peso vivo final, ganho de peso, consumo de metionina + cistina e conversão alimentar, e efeito quadrático nos níveis séricos de albumina, proteína sérica e peso relativo do baço. O nível estimado de metionina + cistina foi de 0,361%, 0,346% e 0,398%, para a atividade da albumina, proteína sérica e peso relativo do baço, respectivamente. As diferentes formulações influenciaram significativamente a histologia do fígado, intestino delgado e magno. Recomenda-se o uso de níveis acima de 0,475% de metionina + cistina digestível para galinhas poedeiras com 13 a 18 semanas de idade, o que corresponde a uma relação de metionina + cistina digestível: lisina digestível superior a 98%.

Palavras-chave: *Exigências nutricionais. Desempenho produtivo. Aminoácidos sulfurados.*

1 Introduction

Dietary protein, together with the energy content of the diet, is known to be one of the more costly nutrients in the feed of laying hens. As the first limiting amino acid in corn-soybean meal based diets for laying hens, methionine has an important role in the performance of animals. Providing the exact levels required by animals in the diet has a significant impact on economic results.

Amongst the main functions of sulfur amino acids, they are involved in the donation of methyl radicals to various metabolic reactions in the body; protein synthesis, especially the synthesis of proteins of the immune system in humans (GRIMBLE, 2006); regulation of gene expression (WU *et al.*, 2006); and they act as substrate for the synthesis of choline, phosphatidylcholine and acetylcholine, which are essential for nerve function and metabolism of leukocytes (KIM *et al.*, 2007).

During the growth phase of layers, there is an intense development of the reproductive organs and other tissues. Feeding diets deficient in methionine at this stage can affect the weight gain of the animals and sexual maturity. According to D'Agostini *et al.* (2012), the factors that interfere in birds a great sexual maturity are directly related to body weight and flock uniformity in the growing phase. Birds weighing less than optimal will begin to lay eggs later and egg production will be reduced. In contrast, over weight birds begin to lay earlier and produce small eggs, which is also not interesting for the producer.

This study determined the nutritional requirements of sulfur amino acid for replacement pullets in the growing phase (13 to 18 weeks old).

2 Theoretical Reference

Body protein consists of approximately 20 amino acids and these are required for various functions in the body, such as organic protein synthesis, a constituent of body tissues, immunoglobulins, hormones, skin formation, feathers, muscles, organs, in addition to participating in the metabolism of other nutrients in the body. Conceptually, amino acids can be classified according to their ability to be synthesized by the poultry organism: the essential amino acids, which cannot be synthesized by the organism or their synthesis is insufficient to meet the metabolic demand; and non-essential amino acids, which can be synthesized in the bird's body from amino acids or other metabolites.

Thus, methionine, the first limiting amino acid in poultry diets, is the main donor of methyl groups (S-adenosylmethionine) for several metabolic reactions and participates directly in protein synthesis (LEESON; SUMMERS, 2001). Allied to this, it serves as an alternative source of cystine in a non-reversible process, and, for this reason, methionine and cystine requirements are usually considered together. These amino acids play a fundamental role in the structure of several proteins and in interconnecting polypeptide chains by means of disulfide bridges (LENNINGHER, 1996).

Methionine can be an alternative source of cystine, presenting important functions in the structures of various proteins, such as immunoglobulins and insulin, linking various polypeptide chains via disulfide bridges (NELSON; COX, 2005). Cystine still participates in the synthesis of glutathione, an important cellular antioxidant for the organism of animals (TESSERAUD *et al.*, 2008).

Another aspect that deserves mention in the methionine metabolism is that this amino acid acts as a lipotropic agent, due to its role as the donor of methyl groups or also due to its involvement in the metabolism of choline, betaine, folic acid and vitamin B12 (CHEN *et al.*, 1993). According to Brumano (2008), methionine deficiency can cause severe damage to the kidneys and liver, since methylation reactions are essential in the metabolism of fat in the liver, preventing fatty liver syndrome.

Recently, Figueiredo Júnior *et al.* (2020) observed a positive effect of methionine on zootechnical performance, liver activity, and better development of intestinal villi in light laying hens during the rearing phase.

Thus, the formulation of diets with levels of sulfur amino acids in quantities required by laying hens, allows better use of the genetic potential of hens and enables the production of high-quality food, with reduced costs with protein ingredients and improved productivity performance of the animals.

3 Research method

The study was conducted at the Poultry Unit, Department of Animal Science, from the Federal University of Paraíba, Campus II, Areia-PB. Four hundred and eighty Dekalb White birds with an initial age of 13 weeks were used. The birds were housed in production cages and were given food and water ad libitum. The project had ethical approval from

the Animal Use and Care Committee of the Federal University of Paraiba, Brazil, under protocol number 149/2015.

The animals were distributed in a completely randomized design with six treatments and ten replicates, with eight birds per experimental unit. The experimental diets (Table 1) included a control treatment formulated to meet the nutritional requirements according to NRC (1994); and five other treatments with increasing levels of methionine plus cystine (0.317; 0.356; 0.396; 0.436 and 0.475%) that were established as 80, 90, 100, 110 and 120% of the recommendations of the Brazilian Poultry and Swine Tables (ROSTAGNO et al., 2005). These diets were supplemented with DL-Methionine-99% (0; 0.039; 0.078; 0.117; 0.156%), replacing corn gluten meal, to achieve the dietary levels.

Animals and diets were weighed at the beginning and at the end of the trial period to assess final body weight (g/bird), weight gain (g/bird), feed intake (g/bird), feed conversion (g/g) and methionine plus cystine and lysine intakes (mg/bird/day). It was also assessed the relative weight (%) of the liver, abdominal fat pad, and spleen, besides serum levels of alanine aminotransferase (U/L), aspartate amino transferase (U/L), gamma-glutamyl transferase (U/L) creatinine (mg/dL) albumin (g/dL) and total protein (g/dL). Histopathological analyses of the liver, small intestine and magnum were performed, and the growth curve from 13-18 weeks old was assessed.

For serological and histopathological analyses and organ weight assessment, five birds per treatment were slaughtered at the end of the experimental period. Serological analyses were performed using the VetTest Blood Chemistry Analyzer (Idexx Laboratories,

Table 1 – Ingredients and composition of experimental diets.

Ingredients	NRC, 1994*	Rostagno et al. (2005)				
		80%	90%	100%	110%	120%
Corn	77.653	79.847	79.847	79.847	79.847	79.847
Soybean meal, 45%	18.518	15.212	15.212	15.212	15.212	15.212
Calcium phosphate	2.994	1.180	1.180	1.180	1.180	1.180
Sodium chloride	0.332	0.329	0.329	0.329	0.329	0.329
Choline chloride	0.070	0.070	0.070	0.070	0.070	0.070
Mineral premix[1]	0.050	0.050	0.050	0.050	0.050	0.050
Vitamin premix[2]	0.050	0.050	0.050	0.050	0.050	0.050
Antioxidant[3]	0.010	0.010	0.010	0.010	0.010	0.010
Potassium carbonate	0.00	0.005	0.005	0.005	0.005	0.005
Limestone	0.323	1.123	1.123	1.123	1.123	1.123
Corn gluten meal, 60%	0.00	2.124	2.085	2.046	2.007	1.968
DL-Methionine	0.00	0.00	0.039	0.078	0.117	0.156
Total	100	100	100	100	100	100
Calculated chemistry composition						
Crude Protein, %	15	14	14	14	14	14
ME, kcal/kg	2900	2900	2900	2900	2900	2900
Calcium, %	0.800	0.780	0.780	0.780	0.780	0.780
Available phosphorus, %	0.646	0.310	0.310	0.310	0.310	0.310
Digestible Arginine, %	0.670	0.531	0.531	0.531	0.531	0.531
Digestible Isoleucine, %	0.400	0.372	0.372	0.372	0.372	0.372
Digestible Lysine, %	0.450	0.483	0.483	0.483	0.483	0.483
Digestible Met + Cys, %	0.420	0.317	0.356	0.396	0.436	0.475
Digestible Threonine, %	0.370	0.333	0.333	0.333	0.333	0.333
Digestible Tryptophan, %	0.110	0.106	0.106	0.106	0.106	0.106
Sodium, %	0.150	0.150	0.150	0.150	0.150	0.150
Chlorine, %	0.243	0.244	0.244	0.244	0.244	0.244
Potassium, %	0.544	0.500	0.500	0.500	0.500	0.500
Electrolytic Balance, mEq/kg	135.87	124.36	124.36	124.36	124.36	124.36

* Feed formulated based on total amino acid basis. [1] Inorganic mineral premix per kg of product: Mn, 20 g; Fe, 10 g; Zn, 13,7 g; Cu, 2,5 g; Se, 0,063 g; I, 0,19 g; and vehicle q.s.p., 500 g. [2] Vitaminic premix per kg of feed: Vit. A - 15.000.000 UI, Vit. D_3 - 1.500.000 UI, Vit. E - 15.000 UI, Vit.B_1 - 2,0 g, Vit.B_2-4,0 g, Vit B6 - 3,0 g, Vit.B_{12} - 0,015 g, Nicotinic acid - 25 g, Pantotenic acid - 10 g, Vit.K_3 - 3,0 g, Folic acid- 1,0 g, Bacitracin zinc - 10 g, Selenium - 250 mg.[3] Antioxidant BHT - 10 g, and vehicle. q.s.p. - 1.000 g.

Maine, USA). For histopathological analyses, fragments of the small intestine, liver and magnum were immersed in fixative metacarn (60% methanol, 30% chloroform and 10% acetic acid) for 12 hours and then transferred to 70% alcohol. The inclusion of the fragments was performed in Paraplast, followed by 5μm-serial sectioning. Diverse sections were stained with hematoxylin and eosin, periodic acid Schiff (PAS) and Masson-trichrome. The photomicrographs were obtained using a microscope coupled to a camera (BX-51, Olympus, Japan) and images were processed with a Zeis software (KS 400.3, Zeiss, Germany).

Samples of the duodenum of 5 animals per treatment were routinely processed for scanning electron microscopy (GUERRA et al., 2010). Briefly, the samples were fixed at 4° C in 2.5% glutaraldehyde in phosphate buffer (0.1M, pH 7, 4) and post-fixed in osmium tetroxide for 2 hours.

The fragments were then dehydrated in increasing concentrations of ethanol (70-100%) and then critical point dried with CO_2, mounted on stubs, and surfaces were coated with gold. Finally, the samples were observed under a scanning electron microscope and the height of the duodenal villi was assessed qualitatively by the same person.

The growth curve was built using the weights collected of all birds weekly from 13-18 weeks old. The data collected were subjected to equation models proposed by Von Bertalanffy, Brody, Gompertz, Logistic and Richards, using the software GOSA (Bio-Log, Ramonville, France) to obtain the best fit of the mathematical equations. Akaike's information criterion (AIC) was used to select the mathematical model with the best fit for the data of the weight and age of the birds.

Statistical analyses were performed using SAS (2011). The control treatment (NRC, 1994) was compared with the other treatments (varying levels of methionine plus cystine, according to Rostagno et al., 2005) by Dunnett's test at 5% probability. The application of methionine plus cystine was estimated by regression analysis, in order to consider the value of R2 and the biological response of birds.

4 Research Results

All variables were affected by treatment (P <0.05), except for feed intake (Table 2).

Final weight and weight gain were lower in the birds fed diets with levels recommended by the NRC (1994) compared with animals fed diets with 100, 110 and 120% of methionine plus cystine as recommended by Rostagno et al. (2005). Birds fed the level of 120% of the requirements of this amino acid as recommended by the Brazilian tables showed improved feed conversion compared with the control treatment.

These results can be explained by better amino acid balance of diets based on digestible amino acids compared with total amino acids. NRC tables recommended levels of essential amino acids below the requirements of the birds, as well as sulfur amino acid levels, when the values of digestible amino acids are considered. It is thus possible that birds fed the control diet showed poor performance due to lower availability of amino acids in the diet that are considered essential to the normal physiology of birds. On the other hand, diet formulation based on digestible amino acid results in greater accuracy in amino acid supplementation, since it considers the ability of the bird to utilize dietary amino acids and overcome any

Table 2 – Live weight (LW, g/bird), weight gain (WG, g/bird), feed intake (FI, g/bird), feed conversion (FC, g/g) and methionine + cystine intake (MCI, mg/bird/day) of light laying hens of 13 to 18 weeks old

Treatment		LW[2]	WG[3]	FI	FC[4]	MCI[5]
NRC		1207.36	350.30	2207.80	6.32	220.78
	80%	1209.17	351.16	2131.47	6.08	160.24¥
	90%	1215.09	361.28	2121.64	5.88	179.83¥
Rostagno et al. (2005)	100%	1235.62¥	378.18¥	2174.54	5.75	205.03¥
	110%	1240.53¥	382.73¥	2132.83	5.58	221.41
	120%	1253.60¥	395.87¥	2129.85	5.38¥	240.88¥
Regression[1]		L**	L**	ns	L**	L**
C.V.(%)		1.53	4.33	3.17	4.89	3.13

[1]Based on Rostagno et al. (2005) levels. C.V.(%): Coefficient of variation; ¥: Different means by Dunnett test at 5% probability; L**: linear effect at 1% probability; ns: not significant; [2]$\hat{Y} = 1.143x + 1116.5$, $R^2 = 0.966$; [3]$\hat{Y} = 1.108x + 262.97$, $R^2 = 0.979$; [4]$\hat{Y} = -0.017x + 7.434$, $R^2 = 0.995$; [5]$\hat{Y} = 2.028x - 1.382$, $R^2 = 0.996$.

differences in digestibility of ingredients (ROMBOLA *et al.*, 2008).

Methionine plus cystine and lysine intakes were higher in the birds fed the control diet compared with those given diets with 80, 90 and 100% of the requirements of these amino acids considering Brazilian tables (Table 2). Birds fed the diet with 120% methionine plus cysteine showed the highest intake of sulfur amino acids, when compared with the control diet. Although no significant effect (P<0.05) was observed between treatments for feed intake, higher consumption of sulfur in birds fed the control diet amino acids can be explained by the higher feed intake of animals when compared to treatments with 80, 90 and 100% of dietary methionine plus cystine recommended by Rostagno *et al.* (2005).

Regression analysis evidenced a linear effect (P<0.01) on final body weight, weight gain, feed conversion and methionine plus cystine intake (Table 2).Supplementation with sulfur amino acids improved the growth performance of birds during the rearing period; however, it was not possible to estimate the nutritional requirement for this period. Thus, the requirement of methionine plus cystine for replacement pullets between 13 and 18 weeks is higher than the level of 0.475% in the diet.

D'Agostini *et al.* (2012) evaluated the requirement of methionine plus cystine for replacement pullets in the growing phase (13 to 18 weeks of age) and observed that the requirement of this amino acid for weight gain was 0.502% based on digestible amino acids, which is higher than suggested in the present study (0.475%). Furthermore, the Dekalb White Management Guide suggests a methionine plus cystine level of 0.640% for birds aged 11-15 weeks, which is also higher than the level suggested herein (0.475%).

Feed conversion was also improved in birds fed 120% of the recommendation of sulfur amino acid of the Brazilian tables during the experimental period. These results are in agreement with the data found by D'Agostini *et al.* (2012), who observed a linear decreasing effect of methionine plus cystine on feed conversion of Lohmann LSL laying hens from 13 to 18 weeks old.

According to Silva *et al.* (2009), meeting the requirements of amino acids for replacement birds during the growth phase is a critical step in optimizing the performance in the production phase. Lower levels of methionine plus cystine in the diets of laying hens during growth impaired performance in this study, possibly because of the importance of

sulfur amino acids as donors of methyl groups to various metabolic reactions, as well as the direct participation in protein synthesis. Methionine in the form of S-adenosylmethionine is required for the biosynthesis of substances that are essential during the growth period of the birds, such as cysteine, carnitine, polyamines, epinephrine, choline and melatonin (BAKER *et al.*, 1996) Thus, the observed deficiency of this amino acid in treatments with lower levels of supplementation may explain the low performance of the animals.

There was no effect of treatments on feed intake of birds during the trial period (Table 2). Although methionine plus cystine are considered one of the most toxic amino acids to birds when consumed in excess, the highest levels of them diets were not able to impair feed intake in the period.

Brody model provided the best fit of the growth curve of laying hens aged between 13 and 18 weeks, showing the lowest AIC (1610.565; Table 3). The average growth curve of the birds of 13-18 weeks old is shown in Figure 1.

Figure 1 – Growth curves (adjusted to Brody model) of laying hens from 13 to 18 weeks old fed different levels of methionine plus cystine.
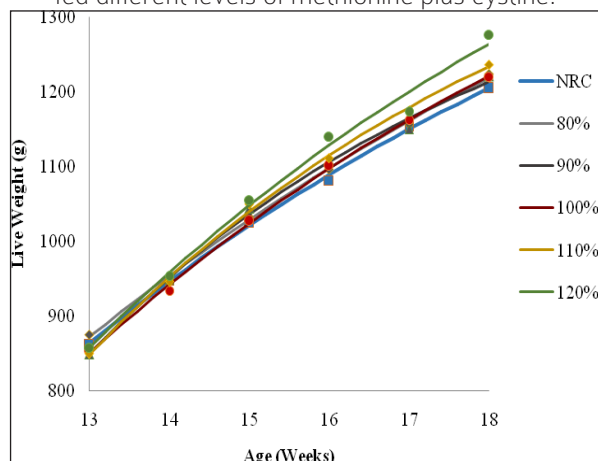
Source: Autor himself

It is observed that birds fed with 120% of the Brazilian tables© recommendations for methionine plus cystine showed higher growth in the period of 13-18 weeks old, followed by treatments of 110 and 100%. Impaired growth was seen when birds were fed the control diet (NRC, 1994) and lower levels of supplementation of methionine plus cystine (80 and 90% of Rostagno *et al.*, 2005). It is also observed that the positive effects of dietary supplementation of sulfur amino acid on the growth of laying hens were more evident after 15 weeks of age, mainly for the treatment

with 120% of methionine plus cystine of Brazilian tables. This result confirms regression analysis data, in which the highest levels of supplementation of methionine plus cystine in the diet resulted in better performance. It is worth noting that birds with the worst performance in the growth curve, will take longer to achieve the ideal weight at laying onset.

Table 3 – Properties of non-linear growth curve models for laying hens from 13 to 18 weeks old

Model	Equation	AIC
Von Bertalanffy	$Y = A(1 - Be^{-Kt}) \, 3 + \varepsilon$	1610.650
Brody	$Y= A(1 - Be^{-Kt}) + \varepsilon$	1610.565
Gompertz	$Y = Ae^{-Be^{-Kt}} + \varepsilon$	1610.747
Logistic	$Y= A(1 + Be^{-Kt})^{-1} + \varepsilon$	1611.064
Richards	$Y= A(1 - Be^{-Kt})^M + \varepsilon$	1612.620

According to Kwakkel (1999), the donor of genetic material companies emphasize the importance of the birds reach the so-called "target weight" that would make them suitable for the production. However, the physiological reactions between target weight and production are not so clear.

All serological variables were different (P <0.05) between the control treatment (NRC, 1994) and the other treatments (ROSTAGNO *et al.*, 2005) according to Dunnett's test (Table 4).

Serum albumin was lower (P <0.05) in birds of the control treatment when compared with animals receiving the levels of sulfur amino acids according to the Brazilian tables, except for the treatment 120% of methionine plus cystine. The normal albumin range is from 0.8 to 2.0 g/dL (SCHMIDT *et al.*, 2007); similar values were reported herein. Total serum protein levels showed similar results than those of albumin. Besides, there was a quadratic effect (P <0.05) of the levels of methionine plus cystine based on Rostagno *et al.* (2005) on both albumin and total protein, as shown in Table 3. The estimated levels of methionine plus cystine for albumin and serum proteins are 91.25 and 87.40%, which corresponded to 0.324 and 0.311% methionine plus cystine, respectively.

The highest levels of albumin and serum protein in pullets fed methionine plus cysteine based on Brazilian tables© recommendations can be explained by the onset of egg production. Pullets may present hyperproteinemia before laying onset what is induced by estrogens (CAMPBELL, 2004), which may explain the high levels of albumin and serum proteins of birds. According to Schmidt *et al.* (2007), egg production can affect serum levels of total proteins, and also albumin levels.

Birds fed the diets containing 80 and 100% of methionine plus cystine showed higher levels of aspartate amino transferase compared with the control treatment. As for alanine aminotransferase, only the treatment with 100% of the nutritional requirements of methionine plus cystine was different from the control treatment.

Table 4 – Serum albumin level (ALB, g/dL), total protein (PTN, g/dL), aspartate aminotransferase (AST, U/L), alanine aminotransferase (ALT, U/L), gamma-glutamiltransferase (GGT, U/L) and creatinine (CRE, mg/dL) of laying hens with 18 weeks old

Treatment		ALB[2]	PROT[3]	AST	ALT	GGT	CRE
NRC (1994)		1.562	3.48	123.4	3.4	28.4	0.508
	80%	2.334¥	4.74¥	175.2¥	3.8	37.6¥	0.654¥
	90%	2.384¥	4.92¥	157.8	3.2	37.2	0.626¥
Rostagno et al. (2005)	100%	2.414¥	4.56¥	176.4¥	5.4¥	32.2	0.676¥
	110%	2.258¥	4.44¥	156	5.2	33.6	0.744¥
	120%	1.852	3.74	124.2	4.4	28.6	0.604¥
Regression[1]		Q*	Q*	ns	ns	ns	ns
C.V. (%)		16.273	13.319	15.48	21.84	12.17	12.412
REQ[4]		0.324	0.311	–	–	–	–

[1] Based on Rostagno et al. (2005) levels. C.V.(%): Coefficient of variation; ¥: Different means by Dunnett test at 5% probability; Q*: quadratic effect at 5% probability; ns: not significant. [2]$\hat{Y}$ = -0.0008x^2 + 0.146x − 4.3476, R^2 = 0.974; [3]$\hat{Y}$ =-0.001x^2 + 0.1923x − 3.68 R^2 = 0.955. [4]Requirement of methionine plus cysteine (%) according to derivation of polynomial equation.

The significant increase in aspartate aminotransferase suggests severe and diffuse liver damage (KANEKO *et al.*, 1997), or even muscle disorders, since aspartate amino transferase is not hepato-specific (SOUZA *et al.*, 2013). However, significant liver injury is indicated by levels greater than 275 U/L (SCHMIDT, 2007), which was not seen in the present study; therefore, it does not characterize a lesion in the liver of birds that received supplementation of methionine plus cystine.

Birds fed the treatment with 80% of methionine plus cystine had higher levels of the enzyme gamma-glutamyl transferase (GGT), compared with the control treatment. The elevated activity of this enzyme is caused by higher production and release by the hepatobiliary tissue, denoting cholestasis (ROSTAGNO *et al.*, 2005). Probably the deficiency of sulfur amino acids may have caused the elevation of the gamma-glutamyl transferase activity in birds fed the diet with 80% of methionine plus cystine. The high GGT levels found in this study may be explained by the age of the birds, since young animals exhibit high levels of this enzyme (KANEKO *et al.*, 1997). Although some high levels for GGT, AST and ALT were seen, increased levels of methionine plus cystine in the diet did not cause significant liver damage (Figure 2).

Animals fed diets with varying levels of methionine plus cystine (80-120% methionine plus cystine) had higher serum creatinine compared with control animals treatment. However, the increase was not enough to lead to any impairment in birds renal function (Data not shown).

According to the Figure 2 (C and D), it is observed that birds fed 100 and 110% of methionine plus cystine of the Brazilian tables had lower liver glycogen accumulation, with lower positivity for periodic acid Schiff staining. Liver glycogen is a major source of carbohydrate in the body, serving as an energy source and supplier of glucose to maintain glucose homeostasis. The lower accumulation of glycogen in birds fed 100 and 110% of methionine plus cystine of Brazilian tables can also be attributed to the energy allocation for organ development instead of stocking as liver glycogen.

These findings are corroborated by the histological results shown below. Furthermore, hepatic steatosis was not observed in any of the treatments. This fact may be attributed to the protective effect of methionine on liver cells, since this amino acid is a major donor of methyl radicals in the body which are essential to the metabolism of the liver.

Figure 2 – Photomicrographs of the liver of laying hens at 18 weeks old supplemented with methionine plus cystine levels. A) Image representing the control treatment (NRC, 1994); B) Image representing the treatments with 80 and 90% of methionine plus cystine (ROSTAGNO *et al.*, 2005) ; C) Image representing the treatment with 100% of methionine plus cysteine (ROSTAGNO *et al.*, 2005); D) Image representing treatment with 110% of methionine plus cystine (ROSTAGNO *et al.*, 2005); E) Image representing treatment with 120% of methionine plus cystine (ROSTAGNO *et al.*, 2005). Periodic acid Schiff staining. 100x increase.
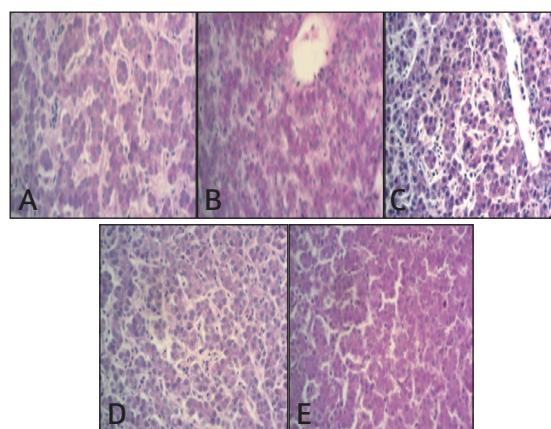
Source: Autor Himself

Scanning electron microscopy of the small intestine of laying hens at 18 weeks old (Figure 3) showed an increase in the height of the intestinal villi with methionine plus cystine supplementation in the diets.

Figure 3 – Images of scanning electron microscopy of the small intestine of laying hens at 18 weeks old supplemented with methionine plus cystine. A) Image representing the control treatment (NRC, 1994), 80 and 90% of methionine plus cystine (ROSTAGNO *et al.*, 2005); B) Image representing the treatments with 100 and 110% of methionine plus cystine (ROSTAGNO *et al.*, 2005); C) Image representing the treatment with 120% of methionine plus cystine (ROSTAGNO *et al.*, 2005).
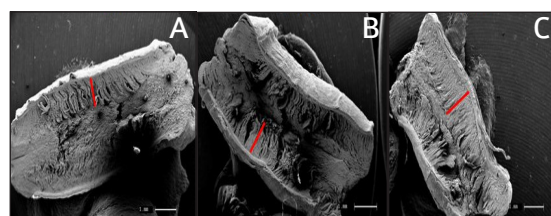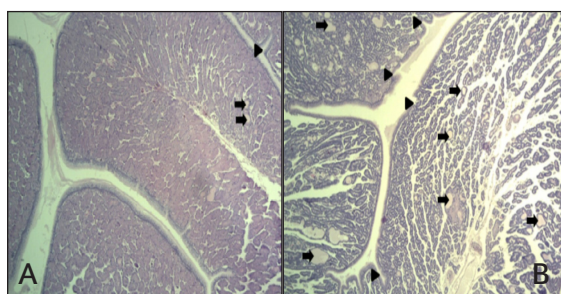

Source: Autor Himself

The image representing birds fed 120% methionine plus cystine (ROSTAGNO *et al.*, 2005) demonstrates the greater height of intestinal villi, which is shown by the red line in the image (Figure 3, C), demonstrating the beneficial effect of this amino acid in increasing the surface area for absorption of the small intestine.

Similar results were found by Smith *et al.* (1990), who observed an increase in the height of the intestinal villi and increased crypt depth in broilers fed higher levels of methionine plus cystine in the pre-starter diets. Similarly, Lima *et al.* (2012) also observed a greater width of intestinal villi in laying hens fed digestible tryptophan levels.

According to Gomide Junior *et al.* (2004), intestinal development is related to the consumption of nutrients with a consequent increase in the length of the intestine. Allied to this, the development of the intestinal mucosa corresponds to an increase in villus height and density as a result of the greater number of epithelial cells (enterocytes, goblet cells and enteroendocrine), which increases the area of contact with food and nutrients, and consequently, increasing absorption (MAIORCA *et al.*, 2002).

Figure 4 – Photomicrographs of the magnum of laying hens at 18 weeks old supplemented with the methionine plus cystine. A) Image representing the treatments, control (NRC, 1994), 80, 90 and 100% of methionine plus cystine (ROSTAGNO *et al.*, 2005); B) Image representing the treatments with 110 and 120% of methionine plus cystine (ROSTAGNO *et al.*, 2005). Hematoxylin-eosin (100x). Arrows: albumen stocks; Arrowheads: secondary folds of the magnum.

Source: Autor Himself

The magnum of 18-wk-old laying hens fed 110 and 120% of methionine plus cystine showed an increase in the number of functional glands secreting albumen (arrows), as well an increased presence of secondary folds in this organ (Figure 4, B), which increases the epithelial area of the magnum. These treatments also had lower liver glycogen stocks, so it is possible that

the energy in these groups is being allocated in to further develop the reproductive system rather than being stored in the liver.

These results indicate an earlier development of the reproductive tract of birds fed higher levels of dietary supplementation with sulfur amino acids. Allied to this, the increase in epithelial area facilitates the passage of the magnum during egg formation, since the epithelium of the organ is rich in goblet cells (Figure 4).

Similar results have been reported by Lima *et al.* (2012), who observed an increase in the production of albumen by the magnum of laying hens supplemented with digestible tryptophan levels. According to Novak *et al.* (2004), the synthesis of albumen in the magnum may be affected by the concentration of amino acids in the blood. Thus, it is possible that the highest concentration of sulfur amino acids in the blood of birds fed higher levels of methionine plus cystine allowed greater development of folds in the magnum, with a consequent increase in the number of cells secreting albumen

There was a significant quadratic effect ($P<0.05$) of methionine plus cystine levels on the relative weight of the spleen of laying hens at 18 weeks of age. On the other hand, there were no effects on the relative weight of the liver and abdominal fat pad by Dunnet test or regression analysis (Table 5).

Table 5 – Relative weight (%) of the liver (LIV), spleen (SPL) and abdominal fat pad (AF) of laying hens with 18 weeks old

Treatment	LIV	SPL[1]	AF
NRC (1994)	2.36	0.22	3.24
Rostagno *et al.* 2005			
80%	2.40	0.22	3.60
90%	2.28	0.28	4.16
100%	2.20	0.28	4.38
110%	2.12	0.26	3.74
120%	2.30	0.24	4.12
Regression	ns	Q*	ns
C.V. (%)	4.52	11.02	10.91
Requirement[2]	–	0.398	–

C.V.(%): Coefficient of variation; ¥: Different means by Dunnett test at 5% probability; Q*: quadratic effect at 5% probability; ns: not significant. [1]$\hat{Y} =-0.000129x^2 + 0.025914x - 1.024000$, $R^2= 0.865$. [2]Requirement of methionine plus cystine (%) according to derivation of polynomial equation

The estimated level of methionine plus cystine for the relative weight of the spleen of replacement pullets at 18 weeks old is 100.44%, a level corresponding to 0.398% in the diet (Table 3).

The increase in spleen weight of birds supplemented with levels of methionine plus cystine may be attributed to greater modulation of the immune

system, since this organ is characterized as one of the major glands responsible for the immune response activity of birds, along with the Bursa Fabricius. The relationship between immune responses and tissue availability of sulfur amino acids and their metabolites is still unknown (GRIMBLE, 2006). This fact may be attributed to the various interrelationships of sulfur amino acids in the modulation of the birds immune response.

The active participation of methionine as a donor of methyl groups is one way of immune stimulation due to supplementation with sulfur amino acid. According to Wu *et al.* (2006), in the form of S-adenosylmethionine, methionine donates methyl groups that participate in the methylation of DNA and proteins, as well as in the regulation of gene expression. In addition to that, methionine acts as a substrate for the synthesis of choline, as well as phosphatidylcholine and acetylcholine, which are essential to neuron function and metabolism of leukocytes (KIM *et al.*, 2007).

Thus, the deficiency of sulfur amino acids in the diets of laying hens can reduce the activity of the immune system, given the involvement of these amino acids with the immune response. The supplementation of sulfur amino acids in the diets of laying hens can improve the effect of immunological responses and consequently increase the weight of spleen. However, it is necessary to avoid an excess of dietary amino acids due to the possible negative effects of amino acid imbalance, with a consequent reduction in the intake and utilization of other amino acids.

Relative liver weight and abdominal fat pad weight of birds were not affected by the different diet formulation basis (total amino acids vs. digestible amino acids) or the levels of methionine plus cystine. According to Bertechini (2012), the development of vital organs and visceral growth of replacement pullets occur mainly between 1 and 5 weeks of age. It may explain the lack of significant difference between treatments for the relative liver weight of the birds at 18 weeks of age. It should be noted, however, that liver weights of birds fed treatments with 100 and 110% methionine plus cystine were lower when compared with the other treatments, although not significantly (P>0.05). This result can be explained by the reduced accumulation of hepatic glycogen in birds of these treatments, represented by the lowest positive staining for periodic acid Schiff (Figure 2).

5 Conclusions/considerations

1. It is recommended levels above of 0.475% methionine plus cystine and methionine plus cystine:lysine ratio greater than 98 for replacement birds between 13 and 18 weeks;
2. Supplementation with methionine plus cystine in the diet of laying hens improves the development and activity of the magnum, as well as increasing the width of intestinal villi;
3. Birds fed diets with levels of 100 and 110% of methionine plus cysteine showed less accumulation of liver glycogen;
4. The levels of methionine plus cystine alter the relative weight of spleen.

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