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Internal quality of laying hen eggs fed on protease at different storage and stocking conditions

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ABSTRACT. Effects on diets with reduction of crude protein and methionine + cystine (RCP) and protease supplementation (PRO) for laying hens at different ages and their influence on the internal quality of eggs at different conditions (STO) and storage times (TM) are evaluated. Four hundred and twenty eggs from commercial Hy-Line W36 laying hens collected at 32, 44 and 58 weeks of age were used. Experimental design was completely randomized in a factorial scheme 3 x 2 x 2 x 2: RCP (control, enhancement of one and two times the enzyme protease), PRO (with and without protease), STO (room temperature and refrigerated) and TM (14 and 28 days), with seven replications of one egg per experimental unit. The factorial scheme 3 x 2 x 2 was used at 58 weeks of age, because TM could not be evaluated. The percentages of egg weight loss, Haugh unit, albumen percentage, percentage and yolk index were evaluated after each storage time. RCP and PRO had no effect on internal egg quality whereas eggs under refrigeration preserved their internal quality regardless of the different storage periods.

Keywords: egg yolk, egg white, enzyme, Haugh unit, nutrition, poultry.

Qualidade interna de ovos de poedeiras leves alimentadas com protease em diferentes condições de armazenamento e estocagem

RESUMO. O objetivo desse estudo foi avaliar os efeitos da redução de proteína bruta e metionina + cistina (RPB) e suplementação de protease (PRO) em dietas de poedeiras comerciais em diferentes idades, e sua influência sobre a qualidade interna de ovos armazenados em diferentes condições (ARM) e períodos de estocagem (EST). Foram utilizados 420 ovos das poedeiras comerciais da linhagem Hy-Line W36 colhidos com 32, 44 e 58 semanas de idade. O delineamento experimental foi inteiramente ao acaso em esquema fatorial 3 x 2 x 2 x 2 sendo os fatores: RPB (controle, valorização de uma e duas vezes da enzima protease), PRO (sem e com protease), ARM (ambiente e refrigerado) e EST (14 e 28 dias), com sete repetições de um ovo por unidade experimental. Com 58 semanas foi utilizado o esquema fatorial 3 x 2 x 2, pois não foi possível analisar o fator EST. Após cada período de estocagem foram avaliadas as características de perda de peso do ovo em porcentagem, Unidade Haugh, porcentagem de albúmen, porcentagem e índice de gema. A RPB e PRO não apresentaram efeitos na qualidade interna dos ovos. Ovos mantidos sob refrigeração tiveram a qualidade interna conservada, independente dos diferentes períodos de estocagem.

Palavras-chave: gema, clara, enzima, Unidade Haugh, nutrição, aves.

Introduction

Eggs are low-cost food which, due to their high nutritional value, provides all essential nutrients that the human body requires. Since they are of animal origin, they are highly perishable with continuous loss of quality. Quality is also affected by management, environment, feed and other factors directly linked to laying hens, such as strain, lineage, diseases and age (Barbosa, Sakomura, Mendonça, Freitas & Fernandes, 2009; Moreng & Avens, 1990).

Protein in laying hens´ diets is one of the most costly nutrients in diet preparation, albeit highly important for poultry´s performance. However, when used above the requirements, it not only raises costs but also increases nitrogen excretion in the environment (Brumano et al., 2010; Yu, Wu, Liu, Gauthier & Chiou, 2007). Due to low cost availability of synthetic amino acids, reduction in crude protein levels is possible without any decrease in performance and in egg quality. Further, the use of exogenous enzymes in diets has become a strategy in diet formulation featuring decrease in nutrient levels and their improvement. The enzyme protease completes the production of endogenous peptidases and

reduces the levels of the diet´s crude protein and energy (Cowieson & Adeola, 2005) since it guarantees a greater efficiency in the use of enzymes and neutralizes the ingredients´ antinutritional factors.

Deteriorated internal characteristics of eggs may be perceived by their physical quality, although several internal and external factors, such as nutritional value, taste, smell, yolk color, palatability and external aspect are difficult to determine. Loss of quality mostly occurs at high temperatures since their chemical and physical reactions increase. Protein in the thick albumin is degraded due to the activities of enzymes which hydrolyze the amino acid chains. The protein structure is destroyed and water bonded to the great protein molecules is released (Moreng & Avens, 1990).

Although fresh eggs are excellent, the maintenance of their chemical and physical characteristics on the market for commercialization is a challenge. Egg processing in Brazil, a tropical country, occurs on farms and the eggs reach the commercial outlets at room temperature when they should have been refrigerated at 10-13°C and relative air humidity of 70-85%, immediately after laying (Barbosa et al., 2009; Moreng & Avens, 1990). Further, since the refrigeration of eggs is not mandatory, it constitutes a negative point in egg quality. Eggs are normally refrigerated by the final consumer (Figueiredo et al., 2011). So that the benefits provided by eggs are enhanced, costs with feed should be decreased and storing strategies for a longer shelf period at each age should be undertaken, without diminishing their performance.

Current assay evaluated the effects of deceasing crude protein and methionine + cystine and protease supplementation in the diets of commercial laying hens at different ages on the internal quality of egg stored in different conditions and at different stocking periods.

Material and methods

Procedures in current assay were approved by the Committee for Ethics in Research – CEP/FZEA/USP, Process 14.1.541.74.0.

The experiment was conducted in the Laboratory of Poultry Breeding of the Faculty of Animal Science and Food Engineering of the São Paulo University (FZEA/USP), with 420 eggs from commercial Hy-Line W36 laying hens harvested at the end of Phase I (32 week old), Phase II (44 weeks old) and Phase III (58 weeks old) to evaluate their internal quality.

Eggs from laying hens, 32 and 44 weeks old, were distributed in a totally randomized experimental design within a 3 x 2 x 2 x 2 factorial scheme, covering the following factors: RCP – reduction of crude protein and methionine + cystine (control, valorization of one and two times the protease enzyme); PRO - protease (with or without); STO – storing conditions (at room temperature or under refrigeration); TM – stocking period (14 and 28 days), with a total of 24 treatments, with seven replications of one egg per experimental unit. The eggs of 58-week-old laying hens were distributed in a factorial scheme 3 x 2 x 2 for factors RCP, PRO and STO, with 12 treatments, seven replications of one egg per experimental unit.

Crude protein and methionine + cystine levels were valorized by preparing control diet (composition and calculated nutritional levels) without the inclusion of protease in each production phase. The periodically revised data base of digestibility assays provided by the manufacturing firm was used to determine the nutritional value of the enzyme in the substrate, following a decrease of levels through the calculation of the protease´s nutritional matrix (Table 1).

Table 1. Nutritional contribution of protease for the diets.

Valorized levels (%)	Phase I		Phase II		Phase III	
	1x matrix	2x matrix	1x matrix	2x matrix	1x matrix	2x matrix
Crude protein	0.64	1.28	0.57	1.14	0.46	0.92
Methionine + Cystine	0.04	0.08	0.04	0.08	0.03	0.06

Diets were prepared from corn and soybean meal, following the Handbook of Performance Standard of Hy-Line W36 (HY-LINE), except for crude protein and methionine + cystine levels, by adding 600 FYT kg^{-1} of feed of phytase and valorizing available calcium and phosphorus by 0.15%. Protease enzyme was Ronozyme® ProAct (CT), produced by submersed fermentation from *Bacillus licheniformis* with transcribed genes *Nocardiopsis prasina*. The protease´s activity is measured in PROT units. A unit is the released quantity of 1 µmol of p-nitroaniline from 1 µM substrate (Suc-Ala-Ala-Pro-Phe-p-Nitroaniline) per minute at pH 9.0 and 37°C. The enzyme consists of 9000 PROT kg^{-1} of feed of protease (Tables 2, 3 and 4).

Water and meals were provided *ad libitum*. The hens were provided with artificial illumination with 16 hours $light^{-1}$ day^{-1}, following Handbook of Performance Standard of Hy-Line W36 (HY-LINE). The intake of daily ration of the hens with regard to RCP and PRO was evaluated within each production phase.

Storing conditions, dealing with the maintenance of eggs at room temperature (without control), imitated their exposure in supermarkets, whereas the refrigerated eggs (controlled) simulated eggs at home

(Giampietro-Ganeco et al., 2012) during the stocking period between 14 and 28 days. Eggs were stored at room temperature on the bench of the Poultry Laboratory of the FZEA/USP, and the other eggs were refrigerated in a common fridge on the same premise. Means of temperature (T°C) and relative air humidity (URA) were registered daily, during 4 weeks, by a thermo-hygrometer close to the eggs stored at room temperature and under refrigeration (Table 5).

Eggs were collected at random at the end of each production phase, weighed (EW, g) one by one in a 0.001 precision scale and distributed at random in cellulose pulp trays with a capacity of 30 eggs. They were then conditioned in different storing conditions. After each stocking period, the eggs were analyzed for internal quality taking into account the following characteristics: loss of weight in percentage (LW, %); Haugh unit (UH); albumin percentage (AP, %), yolk percentage (YP, %) and yolk index (YG).

Table 2. Experimental diets of Phase I (21 - 32 weeks old) for commercial laying hens (mean intake calculated for 84 g hens^{-1} day^{-1}).

Ingredients (%)	Treatments					
	CP	CN1	CN2	CP+ protease	CN1+ protease	CN2+ protease
Corn (7.87%)	45.872	48.053	50.264	45.872	48.053	50.264
Soybean meal (45%)	33.748	31.953	30.124	33.748	31.953	30.124
Lime	10.752	10.752	10.753	10.752	10.752	10.753
Soy oil	6.691	6.305	5.910	6.691	6.305	5.910
Bicalcium phosphate	1.882	1.897	1.911	1.882	1.897	1.911
Common salt	0.489	0.489	0.489	0.489	0.489	0.489
Methionine[1]	0.340	0.309	0.278	0.340	0.309	0.278
L-Lysine	0.007	0.023	0.052	0.007	0.023	0.052
Choline chloride	0.030	0.030	0.030	0.030	0.030	0.030
Antioxidant[2]	0.013	0.013	0.013	0.013	0.013	0.013
Supplement[3]	0.150	0.150	0.150	0.150	0.150	0.150
Inert material[4]	0.020	0.020	0.020	0.008	0.008	0.008
Phytase	0.006	0.006	0.006	0.006	0.006	0.006
Protease	-	-	-	0.012	0.012	0.012
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated levels (%)						
EM (kcal kg^{-1})	2900	2900	2900	2900	2900	2900
Crude protein	19.05	18.41	17.77	19.05	18.41	17.77
Calcium	4.61	4.61	4.61	4.61	4.61	4.61
Pd	0.45	0.45	0.45	0.45	0.45	0.45
Sodium	0.21	0.21	0.21	0.21	0.21	0.21
Chlorine	0.34	0.34	0.34	0.34	0.34	0.34
Potassium	0.75	0.72	0.70	0.75	0.72	0.70
Digestible amino acids (%)						
Methionine	0.47	0.45	0.45	0.47	0.45	0.45
Methionine+cystine	0.80	0.76	0.72	0.80	0.76	0.72
Lysine	0.96	0.96	0.96	0.96	0.96	0.96
Tryptophan	0.22	0.21	0.20	0.22	0.21	0.20
Threonine	0.65	0.63	0.61	0.65	0.63	0.61

CP – Positive control; CN1 - Negative control with less one time the valorization of the enzyme protease; CN2 – Negative control with less two times the valorization of the enzyme protease; CP + protease – CP with 120 ppm protease; CN1 + protease – CN1 with 120 ppm protease; CN2 + protease – CN2 with 120 ppm protease. [1]methionine hydroxy analogue (MHA): 88% methionine. [2]Feed Guard®: Ethoxyquin, BHT, TBHQ and Citric Acid. [3]Supplementation of minerals and vitamins per kg of product: Copper (minimum) 0.024 g; Iron (minimum) 0.15 g; Manganese (minimum) 0.21 g; Zinc (minimum) 0.15 g; Iodine (minimum) 3.6 mg; Selenium (minimum) 0.6 mg; Vitamin A (minimum) 9,000 U.I; Vitamin D3 (minimum) 3,000 U.I; Vitamin E (minimum) 15 U.I; Vitamin K3 (minimum) 2.4 mg; Vitamin B1 (minimum) 2.1 mg; Vitamin B2 (minimum) 6 mg; Vitamin B6 (minimum) 3 mg; Vitamin B12 (minimum) 12 mcg; Niacin (minimum) 0.0375 g; Pantothenic acid (minimum) 0.0165 g; Folic acid (minimum) 0.6 mg. [4]Inert matter: washed sand.

Table 3. Experimental diets of Phase II (33 – 44 weeks old) for commercial laying hens (mean intake for 95 g hen^{-1} day^{-1}).

Ingredients (%)	Treatments					
	CP	CN1	CN2	CP+ protease	CN1+ protease	CN2+ Protease
Corn (7,87%)	57.374	59.387	61.415	57.374	59.387	61.415
Soy meal (45%)	25.779	24.090	22.359	25.779	24.090	22.359
Lime	10.171	10.172	10.172	10.171	10.172	10.172
Soybean oil	4.302	3.937	3.571	4.302	3.937	3.571
Bicalcium phosphate	1.453	1.467	1.481	1.453	1.467	1.481
Common salt	0.437	0.437	0.437	0.437	0.437	0.437
Methionine[1]	0.241	0.216	0.208	0.241	0.216	0.208
L-Lysine	0.024	0.075	0.128	0.024	0.075	0.128
L-Tryptophan	-	-	0.010	-	-	0.010
Choline chloride	0.030	0.030	0.030	0.030	0.030	0.030
Antioxidant[2]	0.013	0.013	0.013	0.013	0.013	0.013
Supplement[3]	0.150	0.150	0.150	0.150	0.150	0.150
Inert material[4]	0.020	0.020	0.020	0.008	0.008	0.008
Phytase	0.006	0.006	0.006	0.006	0.006	0.006
Protease	-	-	-	0.012	0.012	0.012
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated levels (%)						
EM (kcal kg^{-1})	2900	2900	2900	2900	2900	2900
Crude protein	16.32	15.75	15.18	16.32	15.75	15.18
Calcium	4.27	4.27	4.27	4.27	4.27	4.27
Pd	0.36	0.36	0.36	0.36	0.36	0.36
Sodium	0.19	0.19	0.19	0.19	0.19	0.19
Chlorine	0.31	0.31	0.31	0.31	0.31	0.31
Potassium	0.64	0.61	0.59	0.64	0.61	0.59
Digestible amino acids (%)						
Methionine	0.39	0.39	0.39	0.39	0.39	0.39
Methionine+cystine	0.66	0.62	0.59	0.66	0.62	0.59
Lysine	0.79	0.79	0.79	0.79	0.79	0.79
Tryptophan	0.18	0.17	0.17	0.18	0.17	0.17
Threonine	0.56	0.54	0.52	0.56	0.54	0.52

CP – Positive control; CN1 - Negative control with less one time the valorization of the enzyme protease; CN2 – Negative control with less two times the valorization of the enzyme protease; CP + protease – CP with 120 ppm protease; CN1 + protease – CN1 with 120 ppm protease; CN2 + protease – CN2 with 120 ppm protease. [1]methionine hydroxy analogue (MHA): 88% methionine. [2]Feed Guard®: Ethoxyquin, BHT, TBHQ and Citric Acid. [3]Supplementation of minerals and vitamins per kg of product: Copper (minimum) 0.024 g; Iron (minimum) 0.15 g; Manganese (minimum) 0.21 g; Zinc (minimum) 0.15 g; Iodine (minimum) 3.6 mg; Selenium (minimum) 0.6 mg; Vitamin A (minimum) 9,000 U.I; Vitamin D3 (minimum) 3,000 U.I; Vitamin E (minimum) 15 U.I; Vitamin K3 (minimum) 2.4 mg; Vitamin B1 (minimum) 2.1 mg; Vitamin B2 (minimum) 6 mg; Vitamin B6 (minimum) 3 mg; Vitamin B12 (minimum) 12 mcg; Niacin (minimum) 0.0375 g; Pantothenic acid (minimum) 0.0165 g; Folic acid (minimum) 0.6 mg. [4]Inert matter: washed sand.

PP was obtained by the difference between initial and final weight of eggs at the end of the stocking period. The result was divided by initial weight to calculate data of weight loss in percentage.

The eggs were broken on a smooth and clean surface to analyze their internal quality. UH (Haugh, 1937) was determined by measuring albumin height with a caliper and then calculated by the equation: $UH = 100\log (H + 7.57 - 1.7W^{0.37})$, where H = height of albumin and W = weight of the entire egg. The albumin was weighed on a 0.001 precision scale and the value obtained for the final weight of the entire egg multiplied by 100 gives AP.

YP was calculated by the relationship between yolk weight and the weight of the entire egg; result was multiplied by 100. Height and diameter of the yolk

were measured by a caliper. The characteristics of YP and YG were obtained by weight obtained in a 0.001 precision balance. YG was calculated by the ratio between height and mean of the yolk diameter (Moreng & Avens, 1990).

Data underwent analysis of variance, analyzed separately by age, and means of treatments were compared by Tukey´s test at 5% probability, with Statistical Analysis System® (SAS Institute, Cary, USA).

Table 4. Experimental diets of Phase III (45 - 58 weeks old) for commercial laying hens (mean intake for 95 g hens^{-1} day^{-1}).

Ingredients (%)	Treatments					
	CP	CN1	CN2	CP+ protease	CN1+ protease	CN2+ Protease
Corn (7,87%)	58.171	59.815	61.478	58.171	59.815	61.478
Soy meal (45%)	25.094	23.714	22.294	25.094	23.714	22.294
Lime	10.701	10.702	10.702	10.701	10.702	10.702
Soybean oil	3.853	3.554	3.253	3.853	3.554	3.253
Bicalcium phosphate	1.297	1.308	1.319	1.297	1.308	1.319
Common salt	0.437	0.437	0.436	0.437	0.437	0.436
Methionine[1]	0.211	0.192	0.185	0.211	0.192	0.185
L-Lysine	-	0.035	0.078	-	0.035	0.078
L-Threonine	-	-	0.004	-	-	0.004
L-Tryptophan	0.017	0.024	0.032	0.017	0.024	0.032
Choline chloride	0.030	0.030	0.030	0.030	0.030	0.030
Antioxidant[2]	0.013	0.013	0.013	0.013	0.013	0.013
Supplement[3]	0.150	0.150	0.150	0.150	0.150	0.150
Inert material [4]	0.020	0.020	0.020	0.008	0.008	0.008
Phytase	0.006	0.006	0.006	0.006	0.006	0.006
Protease	-	-	-	0.012	0.012	0.012
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated levels (%)						
EM (kcal kg^{-1})	2872	2872	2872	2872	2872	2872
Crude protein	16.05	15.59	15.12	16.05	15.59	15.12
Calcium	4.43	4.43	4.43	4.43	4.43	4.43
Pd	0.33	0.33	0.33	0.33	0.33	0.33
Sodium	0.19	0.19	0.19	0.19	0.19	0.19
Chlorine	0.31	0.31	0.31	0.31	0.31	0.31
Potassium	0.63	0.61	0.59	0.63	0.61	0.59
Digestible amino acids (%)						
Methionine	0.37	0.37	0.37	0.37	0.37	0.37
Methionine+cystine	0.63	0.60	0.57	0.63	0.60	0.57
Lysine	0.75	0.75	0.75	0.75	0.75	0.75
Tryptophan	0.19	0.19	0.19	0.19	0.19	0.19
Threonine	0.55	0.53	0.52	0.55	0.53	0.52

CP – Positive control; CN1 - Negative control with less one time the valorization of the enzyme protease; CN2 – Negative control with less two times the valorization of the enzyme protease; CP + protease – CP with 120 ppm protease; CN1 + protease – CN1 with 120 ppm protease; CN2 + protease – CN2 with 120 ppm protease. [1]methionine hydroxy analogue (MHA): 88% methionine. [2]Feed Guard®: Ethoxyquin, BHT, TBHQ and Citric Acid. [3]Supplementation of minerals and vitamins per kg of product: Copper (minimum) 0.024 g; Iron (minimum) 0.15 g; Manganese (minimum) 0.21 g; Zinc (minimum) 0.15 g; Iodine (minimum) 3.6 mg; Selenium (minimum) 0.6 mg; Vitamin A (minimum) 9,000 U.I; Vitamin D3 (minimum) 3,000 U.I; Vitamin E (minimum) 15 U.I; Vitamin K3 (minimum) 2.4 mg; Vitamin B1 (minimum) 2.1 mg; Vitamin B2 (minimum) 6 mg; Vitamin B6 (minimum) 3 mg; Vitamin B12 (minimum) 12 mcg; Niacin (minimum) 0.0375 g; Pantothenic acid (minimum) 0.0165 g; Folic acid (minimum) 0.6 mg. [4]Inert matter: washed sand.

Table 5. Mean temperature (T°C) and relative air humidity (URA) maximum and minimum for the stocking period per age.

Periods (weeks)	T°C max (°C)	T°C min (°C)	URA max (%)	URA min (%)
Room temperature				
32 – 36	26.69 ± 1.28	14.91 ± 1.00	66.29 ± 1.78	26.61 ± 5.61
44 – 48	30.44 ± 1.70	18.16 ± 1.77	53.20 ± 14.48	26.47 ± 9.06
58 – 62	31.31 ± 1.69	21.54 ± 0.63	76.59 ± 5.28	37.41 ± 6.05
Refrigerated				
-	11.00 ± 2.35	1.43 ± 0.51	51.36 ± 6.39	23.21 ± 6.68

Results and discussion

There was no difference ($p > 0.05$) between treatments in results of intake of daily diets per hen for all the production phases analyzed (not shown). Means were lower than those given by the handbook but performance and egg quality of the hens were not affected. Therefore, poultry exigencies were met with even with reduced levels of crude protein and methionine+cystine, with or without the inclusion of protease.

Tables 6, 7 and 8 presents results of the internal quality of the eggs for weight in natura, weight loss (in percentage), Haugh´s Unit, albumin percentage, percentage and index of yolk, according to age.

Table 6. Rates for egg weight in natura (EW, g), weight loss, in percentage (LW, %), Haugh´s Unit (UH), percentage of albumin (AP, %), percentage of yolk (YP, %) and yolk index (YG) of eggs of 32-week-old commercial laying hens.

Factors	EW (g)★	LW (%)★	UH	AP (%)★	YP (%)★	YG★
RCP						
Control	60.56	3.04	75.83	54.94	30.22	0.36
1x matrix	60.28	2.89	79.87	54.29	30.37	0.37
2x matrix	60.00	3.17	79.13	54.17	30.08	0.36
PRO						
without	60.90	2.99	78.40	54.51	30.47	0.36
With	59.67	3.09	79.16	54.42	29.98	0.36
STO						
Room temperature	60.86	4.58	65.84b	52.76	32.90	0.31
Refrigeration	59.71	1.56	91.09a	56.04	27.81	0.41
TM						
14 days	60.56	2.03	82.02a	54.26	30.39	0.39
28 days	60.00	4.11	75.45b	54.65	30.06	0.34
CV (%)	5.72	11.32	9.45	4.85	7.23	8.74

Means followed by different letters in each column and in each factor differ by Tukey´s test ($p < 0.05$). ★There were significant interactions ($p < 0.05$) between factors and the development of treatments is given in the text.

Table 7. Rates for egg weight in natura (EW, g), weight loss, in percentage (LW, %), Haugh´s Unit (UH), percentage of albumin (AP, %), percentage of yolk (YP, %) and yolk index (YG) of eggs of 44-week-old commercial laying hens.

Factors	EW (g)★	LW (%)★	UH★	AP (%)	YP (%)	YG★
RCP						
Control	60.22ab	3.36	77.81	52.71	32.38	0.29
1x matrix	61.63a	3.70	76.50	53.27	31.87	0.30
2x matrix	59.94b	3.63	75.63	51.95	32.12	0.29
PRO						
without	61.68a	3.59	75.96	53.08	32.13	0.29
With	59.36b	3.53	77.21	52.23	32.12	0.29
STO						
Room temperature	60.26	5.73	60.58	50.63b	34.90a	0.22
Refrigeration	60.79	1.71	89.95	54.39[a]	29.38b	0.37
TM						
14 days	60.79	2.64	78.88	53.16a	31.89b	0.31
28 days	60.26	4.56	74.15	52.15b	32.38a	0.28
CV (%)	6.03	12.01	6.84	5.51	6.37	9.17

Means followed by different letters in each column and in each factor differ by Tukey´s test ($p < 0.05$). ★There were significant interactions ($p < 0.05$) between factors and the development of treatments is given in the text.

The stocking factor for the analysis of eggs of 58-week-old laying hens was not taken into account since there was total loss of albumin and yolk quality in eggs maintained for 28 days at room temperature.

Consequently height and diameter could not be measured. Therefore, eggs stored at room temperature and refrigerated eggs for 28 days were not taken into account.

Table 8. Rates for egg weight in natura (EW, g), weight loss, in percentage (LW, %), Haugh´s Unit (UH), percentage of albumin (AP, %), percentage of yolk (YP, %) and yolk index (YG) of eggs of 58-week-old commercial laying hens.

Factors	EW (g)	LW (%)⋆	UH⋆	AP (%)	YP (%)	YG
RCP						
Control	62.17	3.02	76.10	53.57	31.57	0.29
1x matrix	61.48	2.90	78.05	54.34	30.75	0.28
2x matrix	60.82	3.25	75.99	53.93	30.98	0.27
PRO						
without	62.79a	2.93	76.46	54.78a	30.50b	0.27b
With	60.18b	2.78	76.94	53.15b	31.70a	0.29a
STO						
Room temperature	60.15b	4.10	61.61	51.40	34.36a	0.19b
Refrigeration	62.75a	1.45	90.73	56.15	28.22b	0.36a
CV (%)	6.30	16.86	8.61	4.69	6.40	8.09

Means followed by different letters in each column and in each factor differ by Tukey´s test ($p < 0.05$). ⋆There were significant interactions ($p < 0.05$) between factors and the development of treatments is given in the text.

Weight of egg *in natura*

There were significant interactions ($p < 0.05$) with regard to the factors storing conditions and protease, in which *in natura* eggs of laying hens fed on protease had low weight, regardless of storing conditions. There were interactions ($p < 0.05$) between the factors decrease of crude protein and methionine + cystine and protease. Lower weight occurred with the evolution of interactions eggs of laying hens plus protease and diet valorization.

Further, eggs of 44-week-old laying hens revealed differences ($p < 0.05$) when there was a decrease of crude protein and methionine + cystine and protease. Eggs with greater weight were provided by laying hens fed on control diet and one time the valorization of protease and without the inclusion of the enzyme.

There were differences ($p < 0.05$) for the factors protease and storing conditions for eggs derived from 58-week-old laying hens. Eggs of laying hens fed on diets without protease weighed more; similarly, eggs stored under refrigeration.

Weight loss of the egg

There were significant interactions ($p < 0.05$) between storing and stocking conditions in eggs from 32- and 44-week-old laying hens. The eggs stored under refrigeration had the lowest percentage in weight loss when compared to eggs at room temperature, regardless of the storing period. The lowest loss rate occurred on the 14th day (not shown). High temperatures enhance weight loss in eggs stored at room temperature due to an increase in transpiration which causes great loss of CO_2 and H_2O to the environment and thus a decrease in the initial weight. Weight loss increases as storing period is prolonged.

Barbosa et al. (2009) also reported similar results when they stocked eggs up to 35 days, derived from different poultry strains and at different storing conditions. There was a linear PP decrease related to PA reduction during the storing period. Similarly, Freitas et al. (2011) registered lower PP rates when they stored egg in a fridge (10°C) and in a cold chamber (3°C).

There were significant interactions involving storage conditions and protease in eggs from 58-week-old laying hens (not shown). Means revealed that at room temperature eggs from diets supplemented with protease had a lower weight loss (3.94%) when compared to eggs from diets without any supplementation (4.26%).

Haugh Unit

Classification by the United States Department of Agriculture (USDA) which prepared the USDA Egg-Grading Manual was employed to discuss UH data. The handbook which classified UH rates according to the principle that the higher UH, the better is the egg, provides the following classification: AA (excellent) = rates above 72; A (high) = rates between 60 and 71; B (inferior) = rates lower than 60. Ranking is simple and is greatly used by the poultry industry since it was introduced by Haugh in 1937.

There were differences ($p < 0.05$) for storing conditions and stocking for eggs from 32-week-old laying hens. The factor storing conditions had a positive effect for refrigerated eggs, with excellent ranking, whereas eggs at room temperature ranked as high quality eggs. In the case of the factor stocking, in spite of the fact that eggs stocked during 14 days had a better UH average, average rates were classified as excellent for both periods.

There were significant interactions in the factors storing and stocking conditions. Eggs at room temperature had lower UH rates (14 days = 69.37; 28 days = 62.14), classified as high quality, whereas eggs under refrigeration (14 days = 94.38; 28 days = 87.82) were classified as excellent, regardless of the stocking period. The two types of storage decreased egg quality over time (result not shown).

There were significant interactions ($p < 0.05$) between the factors storing and stocking conditions for eggs of 44-week-old laying hens. Results were similar to those with 32-week-old hens (not shown). Barbosa et al. (2009) also reported that due to interaction decrease in UH was worse for eggs at room temperature (without control). The same

authors showed a decrease of 1.48 in UH for each day of stocking of eggs of the strain Hy-Line.

The factor storing conditions and protease had significant interactions ($p < 0.05$) for eggs of 58-week-old laying hens. Eggs at room temperature, classified as high quality, were jeopardized when compared to refrigerated eggs with excellent quality, whereas the inclusion or not of protease in the diets failed to show any effect (not shown). The development of the stocking period coincided with the decrease of the albumin height and, consequently, of UH rates. Albumin weight loss caused loss of weight in the eggs.

Eggs stored under refrigeration kept good mean UH rates throughout all the ages evaluated. Similarly, Figueiredo et al. (2011) demonstrated the positive effects of refrigeration for the conservation of the internal quality of eggs during stocking.

Albumin percentage

There were interactions between the factors decrease of crude protein and methionine + cystine and storing conditions for egg of 32-week-old laying hens. Refrigerated eggs had a higher PA especially when combined with the control diet (not shown). In the case of eggs of 44-week-old laying hens, there were differences ($p < 0.05$) for the factors storing and stocking conditions. In fact, the highest AP was obtained by refrigerated eggs and eggs stocked for a lesser period. In the case of eggs of 58-week-old laying hens, there were significant effects ($p < 0.05$) for the factor protease, in which diets without the enzyme had the highest AP rate, probably due to their great weight in natura.

Figueiredo et al. (2011) reported that albumin in eggs kept at room temperature lost water fast, with the consequent decrease of albumin weight and loss of weight of the egg. On the other hand, Freitas et al. (2011) failed to report any difference in AP and YP for eggs at room temperature (26°C), fridge (10°C) and cold chamber (3°C) in different storing periods (7, 14 and 21 days).

In current analysis, storing conditions at room temperature for longer stocking periods revealed an increase in AP due to the fluidification of the albumin and thus loss of egg quality (Moreng & Avens, 1990). The dissociation of carbonic acid (H_2CO_3), one of the main components of the albumin buffer system, occurs and forms H_2O and CO_2 throughout the storing period. The reaction is accelerated at higher temperatures since physical and chemical reactions occur that degrade the structure of protein in the thick albumin. The product of the reactions is the water bonded to the great protein molecules that cross to the yolk by osmosis (Barbosa et al., 2009).

Yolk percentage

There were significant interactions ($p < 0.05$) between the factors storing conditions and protease in the eggs of 32-week-old laying hens. Eggs refrigerated had a low YP, regardless of inclusion (28.04%) or not (27.56%) of protease in the diets of laying hens (not shown).

The factor storing conditions for eggs of 44- to 58-week-old laying hens was significant, with higher YP rates for eggs kept at room temperature. The stocking factor for eggs of 44-week-old laying hens and the factor protease for eggs of 58-week-old laying hens showed differences through a higher YP rate in the 28-day analysis and for eggs from diets with protease, respectively.

Increase of yolk percentage was caused by the acceleration of physical and chemical reactions that degrade the structure in the thick albumin, with water (one of the reactions´ product) crossing to the yolk by osmosis and thus weakening the vitelline membrane. In fact, it becomes flattened and bigger when broken on a plane and smooth surface, causing an increase in weight and percentage.

Yolk index

There were significant interactions for storing and stocking conditions for eggs of 32- and 44-week-old laying hens, with better results when eggs were refrigerated for 14 days of stocking and compared to those maintained at room temperature for 28 days (not shown).

The factors storing conditions and protease were significant for eggs of 58-week-old laying hens. Average highest rates occurred when the laying hens were fed on diet supplemented with protease and when eggs were stored under refrigeration.

Conclusion

Decrease of crude protein and methionine + cystine and supplementation by protease in the diets of laying hens do not influence diet intake and the internal quality of the eggs.

During the stocking period, the maintenance of eggs at room temperature triggers a great loss of weight due to the fluidization of the albumin with greater yolk percentage, liabilities for the yolk index and Haugh´s unit.

The above situation is very similar to the commercial conditions. Although damage is unavoidable over time, storing of eggs under refrigeration is a highly relevant factor for the conservation of the internal quality of the eggs.

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