SHORT COMMUNICATION

Effects of boron supplementation alone or in combination with different vitamin D_3 levels on laying performance, eggshell quality, and mineral content and fatty acid composition of egg yolk in laying hens

Ö. Sizmaz[1,4], B.H. Koksal[2], A. Tekeli[3] and G. Yildiz[1]

[1] Ankara University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Disease,
06110 Ankara, Turkey
[2] Adnan Menderes University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Disease,
09016 Aydın, Turkey
[3] Van Yüzüncü Yıl University, Agriculture Faculty, Department of Animal Science, 65080 Van, Turkey

[4] Corresponding author:
e-mail: ozgeabacioglu@gmail.com

ABSTRACT. This study aimed to evaluate the effects of dietary boric acid with vitamin D_3 on laying performance, eggshell quality, and mineral content and fatty acid composition of egg yolk in hens. In the experiment, 160 late-phase laying hens were equally divided into four groups, 40 birds each (10 replicates of 4 hens each). The study included a control group and three treatment groups. In the control group, there was no additive, while the experimental groups contained: 1) vitamin D_3 3000 IU/kg + 120 ppm boric acid, HDB; 2) vitamin D_3 300 IU/kg + 120 ppm boric acid, LDB; and 3) 120 ppm boric acid, B; respectively. After 12 weeks of feeding, the egg mass was significantly reduced in HDB group in comparison to the control and feed efficiency was increased in HDB group in comparison to the control and B groups ($P < 0.05$). No significant differences were observed in eggshell quality among groups ($P > 0.05$). Egg yolk cholesterol content was decreased in LDB group in comparison to B group ($P < 0.05$). The concentration of C18:3 fatty acid was the highest ($P < 0.05$) in B group. In comparison to the control group, the C16:0 fatty acid concentration was lower in B group ($P < 0.05$). So, the combination of boric acid (120 ppm) and a high dose of vitamin D_3 (3000 IU/kg) is not recommended in laying hen feeding as it negatively influences laying performance parameters. On the other hand, although the supplementation of 120 ppm boric acid without additional vitamin D_3 increases egg yolk omega-3 fatty acid content, the cholesterol-reducing effect (but without fatty acid profile change) may be obtained only when boric acid is added simultaneously with a low level of vitamin D_3 (300 IU/kg), which can be used to produce functional food.

Introduction

Boron is a trace element naturally found in many foods and used as a nutritional supplement (Nielsen and Eckhert, 2020). There are approximately 230 different boron minerals in nature, while the boron sources used in animal feeding are mostly boric acid and borax types (Basoglu et al., 2017; Sokmen and Buyukakinci, 2018). Turkey is a country with large, high-quality boron reserves, which

account for 73.4% of the world's boron reserves (Kılıç et al., 2018; Sokmen and Buyukakinci, 2018).

Boron taken in the diet affects mineral metabolism (Ca, P, Mg), energy metabolism (triglycerides, glucose) and protein metabolism (amino acids) (Białek et al., 2019).

Much of the interest in boron in animal nutrition is due to the fact that this mineral may stimulate the growth of chicks deficient in vitamin D_3. Such results might suggest that boron affects vitamin D_3 metabolism or exerts a synergistic effect with vitamin D_3 to increase the performance parameters (Hunt and Nielsen, 1981).

We hypothesized that using boron in combination with different levels of vitamin D_3 could have a positive effect on the quality of eggshell regarding mineral content and the egg yolk fatty acid profile. The present study aimed to investigate the effects of supplementing boron as boric acid in laying hens on laying performance, external quality of egg and fatty acid profile of egg yolk, which may be important from the point of view of human nutrition. The idea of using boron minerals together with vitamin D_3 in laying hen nutrition and the possibility of the synergistic effect between the examined components prove the originality of this study.

Material and methods

The study was approved by the Animal Experiments Local Ethics Committee of the University of Ankara (Ethical Approve Number: 200515).

Animal management and experimental diets

In the study, 160 Hyline-White 98, 59-week-old layers were randomly allocated into four experimental groups according to dietary treatments. The trial was conducted in the experimental poultry house of Ankara University, Faculty of Veterinary Medicine (Turkey). In each treatment group, there were 10 replicates, each with four layers. The house was provided with programmable lighting and ventilation during the whole 12-week experimental period. Laying hens were housed in cages (50 × 59 × 60 cm) in an environmentally controlled room with a temperature between 20–22 °C according to good management practice. Hens were exposed to a 17-hour constant light schedule until the end of the experiment. Feed and water were provided *ad libitum* throughout the experiment, and the diets were in a mash form. The experimental diets based on maize-soyabean were formulated to be *iso*-caloric and *iso*-nitrogenous and to provide adequate

levels of all nutrients as recommended for laying hens by the National Research Council (NRC, 1994). The experimental treatments used were: CON – no additives; HDB – vitamin D_3 at a dose of 3000 IU/kg + 120 ppm boric acid; LDB – vitamin D_3 at a dose of 300 IU/kg + 120 ppm boric acid; and B – 120 ppm boric acid. Boric acid used in this study contained 17.5% boron. Although the inclusion of at least 2 ppm boron in poultry feed was recommended by NRC (1984), no matter which poultry category or type of production, such suggestions are not present in the requirements for poultry in NRC (1994). Thus, the only suitable suggestions for boron doses are taken from the experimental diets used in the previous studies (Sizmaz and Yildiz, 2016). Basal diet composition and nutritional values (analysed according to the reference methods (AOAC Interntional, 2000)) are shown in Table 1.

Table 1. Basal diet composition and nutritional level

Indices	Amount, %
Ingredients	
maize	60.80
soyabean meal	10.00
full-fat soyabean	12.00
meat bone meal	4.00
limestone	10.00
DCP	2.00
salt	0.35
DL-methionine	0.30
vit.-min. premix[1]	0.35
NaHCO$_3$	0.20
Analysed values	
CP	15.94
ME, kcal/kg	2 772.66
Ca	3.80
P	0.82
boron, ppb	27.86

DCP – dicalciumphosphate; CP – crude protein; ME – metabolizable energy; [1]vitamin and mineral premix provided per kg of diet, mg: retinol 3 600, cholecalciferol 200 000, tocopherol 50 000, menadione 10 000, tiamine 4 000, riboflavine 8 000, pyridoxine 5 000, cobalamine 25, niacine 50 000, panthotenic acid 20 000 mg, folic acid 20 000, biotine 250, ascorbic acid 75 000, choline 175 000, caroten 5 000, canthaxanthin 250 000, Mn 100 000, Zn 150 000, Fe 100 000, Cu 20 000, I 1 500, Co 500, Se 200, Mo 1 000, Mg 50 000

Sampling and handling

On the last day of each 3-week interval (after 3, 6, 9 and 12 weeks), two eggs from each replicate (20 eggs from each treatment) were randomly selected and analysed for external egg quality (eggshell strength, eggshell thickness and egg shape index).

At the end of the experiment, in total 40 eggs (one egg from each replicate; 10 eggs per treatment)

were randomly selected and later analysed for the cholesterol and fatty acid concentrations in yolk and mineral content in yolk and shell.

At the end of the experiment, 10 hens were chosen randomly from each treatment (one from each replicate) to collect the blood samples. Blood samples were taken from vena brachialis under the wing and then centrifuged at 3220 g for 8 min to collect serum. Then sera were analysed for the cholesterol concentration.

Laying performance

Each day for each cage, the following parameters were recorded: feed provided, feed left-overs, number of laid eggs, individual egg weight; to calculate daily feed intake per cage (feed provided − feed left-overs), daily egg production per cage (the total number of laid eggs divided by the number of hens in that cage and multiplied by 100), daily average egg weight per cage (the total egg weight divided by the total number of eggs collected from each cage) and daily egg mass per cage (daily average egg weight in that cage multiplied by daily egg production of the same cage).

Basing on the daily cage values, the following parameters were calculated for each treatment for each period (weeks 1–4, 5–8 and 8–12) and overall trial: average egg production (g/day/hen), average egg weight (g), average egg mass (g/day/hen), average feed intake (g/day/hen) and average feed efficiency (feed-to-egg ratio; g/g).

External egg quality

The egg shape index defined as the ratio of the vertical diameter to the horizontal diameter was measured (mm) with the use of calliper to the nearest 0.1 mm. Eggshell thickness was measured using a digital micrometer (Mitutoyo No. 1044N, 0.01–5 mm; Kawasaki, Japan); measurements at the blunt and sharp ends and the middle of the egg were averaged to determine the overall eggshell thickness. Eggshell breaking strength was measured using an egg-breaking tester (static compression device, Dr.-Ing. Georg Wazau Mess- + Pruftechnick, Berlin, Germany).

Composition of egg yolk and eggshell

To measure mineral content, cholesterol level and concentration of fatty acid of yolks, the eggs were boiled for 5 min, cooled, the shells were removed and the yolk was separated from the albumen. Yolks were weighed. Cholesterol from the yolk and layers' serum was measured using a commercial kit (Teco Diagnostic, Anaheim, CA, USA) with the

spectrophotometric method (UV-1208, Shimadzu, Kyoto, Japan) according to the manufacturers' recommendations.

Fatty acid methyl esters (FAMEs) were prepared according to the method of Metcalfe and Schmitz (1961). FAMEs profile and content were determined with the use of the gas chromatograph (GC; Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) equipped with flame ionization detector (FID) and column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness; SPTM-2380, Supelco, Bellefonte, PA, USA). The temperature values were: initial injector – 240 °C, FID – 240 °C and oven – 185 °C. The temperature of the oven was increased to 240 °C (5 °C per 1 min) and held for 1 min. The injection sample amount was adjusted as one μl with a 1:100 split ratio. The obtained results were referenced to the standard FAME mix C8-C24 (Supelco, Bellefonte, PA, USA). Amounts of fatty acids were expressed as a percentage (w/w) of total FAMEs.

Yolk and shell ash were determined by litting in the ash oven at 610 °C; and calcium (Ca) and phosphorus (P) contents were determined spectrophotometrically (UV-1208, Shimadzu, Kyoto, Japan) after microwave mineralization (MWS-2, Berghof, Eningen, Germany). Egg yolk and shell boron levels were measured at specific wavelengths for this element by an Inductively Coupled Plasma Mass Spectrometry (ICPMS, Thermo XSERIES2; Thermo Fisher Scientific, Waltman, MA, USA) after ashed in the microwave. The calibrations for the boron assays were conducted with a series of mixtures containing graded concentrations of standard solutions of boron (B ICP standard, Merck, Darmstadt, Germany).

Statistical analysis

Experimental data were analyzed using the SPSS software statistics package (V22.0; SPSS Inc., Chicago, IL, USA). The normality of data distribution was checked using the Kolmogorov-Smirnov test. One-way ANOVA was performed to investigate the differences between groups. Duncan test was used to determine statistically significant differences, with significance set at $P < 0.05$.

Results

The results of the productive performance of laying hens fed boron alone or in combination with two doses of vitamin D₃ are presented in Table 2. The obtained results showed that in comparison to the control group, supplementation of boric acid and vitamin D₃ did not affect the egg production, egg

Table 2. Effect of dietary boron alone or in combination with vitamin D_3 (low and high dose) on productive performance of laying hens

Indices	Treatments[1]				SEM	P-value
	CON	HDB	LDB	B		
Egg production, %						
week 1–4	80.74[a]	72.23[b]	76.52[ab]	79.08[a]	1.06	0.022
week 5–8	80.89	74.29	79.012	78.84	1.08	0.168
week 9–12	78.04	72.32	73.48	74.82	1.23	0.400
week 1–12	79.89	72.95	76.34	77.56	0.97	0.099
Egg weight, g						
week 1–4	63.71	61.20	63.43	63.36	0.51	0.277
week 5–8	64.93	61.50	63.75	63.88	0.50	0.097
week 9–12	65.21	65.16	67.13	64.10	0.55	0.274
week 1–12	64.62	62.62	64.77	63.78	0.37	0.137
Egg mass, g/day/hen						
week 1–4	51.48[a]	44.38[b]	48.49[ab]	50.03[a]	0.88	0.022
week 5–8	52.48[a]	45.72[b]	50.42[a]	50.36[a]	0.82	0.021
week 9–12	50.80	47.15	49.50	47.93	0.91	0.509
week 1–12	51.59[a]	45.75[b]	49.47[ab]	49.44[ab]	0.75	0.043
Feed intake, g/day/hen						
week 1–4	100.04	99.03	99.27	98.48	0.67	0.881
week 5–8	102.32	102.28	99.83	96.63	1.24	0.327
week 9–12	107.81	107.02	108.74	102.79	1.47	0.510
week 1–12	103.39	102.77	102.61	99.30	1.02	0.500
Feed efficiency, g of feed/g of egg						
week 1–4	1.96[b]	2.30[a]	2.07[ab]	1.98[b]	0.04	0.023
week 5–8	1.97[b]	2.28[a]	2.01[b]	1.93[b]	0.04	0.009
week 9–12	2.14	2.32	2.23	2.17	0.05	0.517
week 1–12	2.01[b]	2.28[a]	2.09[ab]	2.01[b]	0.04	0.036
Body weight change, g	31.88	18.6	15.49	−3.49	7.45	0.423

[1] treatemnts: CON – no additives, HDB – vitamin D_3 at a dose of 3000 IU/kg + 120 ppm boric acid, LDB – vitamin D_3 at a dose of 300 IU/kg + 120 ppm boric acid, and B – 120 ppm boric acid; SEM – standard error of mean; [a-b] – means with different superscripts within the same row are significantly different at $P < 0.05$

Table 3. Effect of dietary boron alone or in combination with vitamin D_3 (low and high dose) on external egg quality in laying hens

Indices	Treatments[1]				SEM	P-value
	CON	HDB	LDB	B		
week 3						
egg shape index	77.20	77.45	77.55	77.40	0.35	0.989
egg breaking strength, kg	1.68	1.59	1.43	1.61	0.08	0.749
eggshell thickness, µm	38.67	39.33	38.70	38.70	0.13	0.187
week 6						
egg shape index	78.90	78.75	78.45	79.39	0.34	0.818
egg breaking strength, kg	1.34	1.46	1.02	1.36	0.10	0.370
eggshell thickness, µm	37.60	38.23	37.60	37.48	0.26	0.739
week 9						
egg shape index	78.75	77.10	78.25	77.50	0.35	0.334
egg breaking strength, kg	1.39	1.55	1.49	1.48	0.10	0.946
eggshell thickness, µm	38.53	36.83	37.10	37.53	0.28	0.157
week 12						
egg shape index	78.90	78.15	78.65	78.85	0.38	0.899
egg breaking strength, kg	1.41	1.26	1.30	1.07	0.10	0.652
eggshell thickness, µm	37.27	36.87	37.30	36.40	0.33	0.765

[1] treatments: CON – no additives, HDB – vitamin D_3 at a dose of 3000 IU/kg + 120 ppm boric acid, LDB – vitamin D_3 at a dose of 300 IU/kg + 120 ppm boric acid and B – 120 ppm boric acid, SEM – standard error of mean, [a-b] – means with different superscripts within the same row are significantly different at $P < 0.05$

Table 4. Effect of boron alone or in combination with vitamin D_3 (low and high dose) on serum and yolk cholesterol level (mmol/l) and yolk fatty acid composition (% of total fatty acid methyl esters)

Indices	Treatments[1]				SEM	P-value
	CON	HDB	LDB	B		
Cholesterol						
serum	114.13	96.23	87.50	82.12	4.82	0.112
egg yolk	14.89[ab]	15.85[ab]	14.36[b]	16.70[a]	0.278	0.028
Egg yolk fatty acid						
C14:0	0.62	0.53	0.66	0.68	0.068	0.874
C16:0	25.98[a]	25.52[ab]	25.37[ab]	24.47[b]	0.178	0.041
C16:1	1.60	1.47	1.24	1.42	0.073	0.360
C18:0	7.77	7.93	7.87	7.56	0.134	0.786
C18:1	45.02	45.68	45.16	45.09	0.428	0.948
C18:2	18.62	18.46	19.34	20.26	0.425	0.454
C18:3	0.39[b]	0.41[b]	0.44[b]	0.73[a]	0.031	0.002

[1] treatments: CON – no additives, HDB – vitamin D_3 at a dose of 3000 IU/kg + 120 ppm boric acid, LDB – vitamin D_3 at a dose of 300 IU/kg + 120 ppm boric acid, and B – 120 ppm boric acid; SEM – standard error of mean, [a-b] – means with different superscripts within the same row are significantly different at $P < 0.05$

weight and feed intake in the whole trail regardless of examined periods; however, egg mass and feed efficiency were negatively affected in HDP group ($P < 0.05$) markedly in 1–4-week, 4–8-week and the whole trial periods (the egg mass was reduced and feed efficiency was increased in HDP group in comparison to CON and B treatment).

External egg quality parameters are shown in Table 3. There was no statistically significant difference in the egg shape index, egg breaking strength and eggshell thickness at the end of 3, 6, 9 and 12 week of the study ($P > 0.05$).

Effects of boric acid added with different doses of vitamin D_3 on egg yolk and blood serum cholesterol level and egg yolk fatty acid composition are reported in Table 4. Serum cholesterol levels did not differ among treatments, while yolk cholesterol concentration was decreased in LDB group in comparison to B group ($P < 0.05$). Compared with the control group, palmitic acid (C16:0) content was reduced and linolenic acid (C18:3) increased in egg yolk from laying hens fed diet with only boron addition ($P < 0.05$).

The mineral concentration of eggshell and yolk is noted in Table 5. The data showed that there were no differences in comparison to the control ($P > 0.05$), except eggshell boron concentration ($P < 0.001$) which was increased in all experimental treatments.

Table 5. Effect of boron alone or in combination with vitamin D_3 (low and high dose) on egg yolk and eggshell mineral concentration and boron level (in DM)

Indices	Treatments[1]				SEM	*P*-value
	CON	HDB	LDB	B		
Egg yolk						
DM, %	45.69	45.32	44.98	45.14	0.370	0.908
ash, %	1.92	1.83	1.84	1.85	0.032	0.779
Ca, g/kg	1.54	2.47	2.08	1.86	0.17	0.268
P, g/kg	0.32	0.32	0.32	0.33	0.01	0.998
B, ppb	18.19	21.65	22.20	22.01	0.84	0.294
Eggshell						
DM, %	99.18	99.21	99.19	99.18	0.01	0.890
ash, %	51.49	51.70	51.44	51.55	0.09	0.772
Ca, g/kg	24.34	26.36	25.83	27.27	0.53	0.266
P, g/kg	0.11	0.12	0.11	0.10	0.00	0.328
B, ppb	82.50[c]	118.37[b]	125.76[a]	118.08[b]	3.67	0.000

DM – dry matter; [1] treatments: CON – no additives, HDB – vitamin D_3 at a dose of 3000 IU/kg + 120 ppm boric acid, LDB – vitamin D_3 at a dose of 300 IU/kg + 120 ppm boric acid, and B – 120 ppm boric acid; SEM – standard error of mean; [a-c] – means with different superscripts within the same row are significantly different at $P < 0.05$

Discussion

Boron supplementation into laying hen diets has become an area of interest in the egg production industry due to the significance of boron in the performance and metabolism of other minerals (Mizrak, 2008; Koksal et al., 2012). It is known that there is a positive relationship between boron and vitamin D_3 when this vitamin is deficient or at an appropriate level (Hunt and Nielsen, 1981). In our study, we observed the effect of supplementing a laying hen feed with 120 ppm of boric acid with either 300 or 3000 IU levels of vitamin D_3.

The current study showed that egg performance was similar in laying hens fed boric acid, which is consistent with the results of Olgun et al. (2009), who reported that supplementation of different levels of boron (0, 100, 200 and 300 ppm) with simultaneous Ca deficiency did not influence the productive performance except for egg mass. The authors stated that egg mass was decreased in 200 and 300 ppm boron added groups. In the present study, egg mass was reduced in HDB group in two first periods (1–4-week and 4–8-week) as well as in the whole trial. The incoherent results may depend on the differences in dosage of boric acid, age of the laying hens and the synergetic effect of the vitamin D_3 with boron.

The previous studies showed that the body weight, feed intake, feed conversion ratio, egg production and egg weight were not affected by dietary boron in laying hens (Kurtoglu et al., 2002; Sizmaz et al., 2014), whereas the present results also showed that boric acid alone did not influence these parameters; however, when combined with a high dose of vitamin D_3 boric acid negatively increased feed efficiency. Koksal et al. (2012) stated that 90 mg/kg dietary boric acid decreased the feed conversion ratio of laying hens in comparison to control in the 5–6th weeks of the trial. Hunt (1994) reported that interaction between boron (orthoboric acid) and vitamin D_3 did not affect the body weight and feed intake, but the feed intake increase was stated when boron was added alone. This discrepancy between the results could be due to the use of different boron sources and because the vitamin D_3 levels were adjusted to the required values.

The present results are supported by several previous reports in which the external egg quality was not affected by boron supplementation (Kurtoglu et al., 2002; Olgun et al., 2009). Egg breaking strength plays a key role among all eggshell quality parameters (Zhang et al., 2017). No positive effect of dietary boron addition on eggshell strength was observed in the current study regardless of vitamin D_3 addition, despite lower egg mass in HDB group. The reduced egg mass observed in this study is possibly featured to the interaction effect between boron and vitamin D_3 in layers, which warrants further study.

With the diet manipulation in laying hens, it is possible to modify the polyunsaturated fatty acid (PUFA) profile, including the higher content of omega-3 fatty acids (such as α-linolenic acid (C18:3), eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic (DHA, C22:6)) in the egg yolk (Sizmaz and Yildiz, 2016; Aguillón-Páez et al., 2020). Laying hens are more effective in converting α-linolenic acid into the long omega-3 fatty acids, with a preference for DHA to EPA, than mammalian species (Hayat et al., 2009; Manor et al., 2019). However, late-phase laying hens may have not the adequate capacity to desaturate and elongate α-linolenic acid to EPA and DHA, thus may require dietary supplementation (Ackman, 1989). Similarly, in the study by Scorei and Rotaru (2011), there was an increase in omega-3 fatty acids by the boron supplementation. Nielsen (2002) reported that the intake of omega-3 fatty acids would change the response to boron deprivation. In the present study, palmitic acid (C16:0) content was reduced and α-linolenic acid increased in egg yolk of laying hens fed diet with boron addition. It is challenging to explain the mechanism by which boron mineral

changes the egg yolk fatty acid levels and requires further studies. Besides modifying the fatty acid profile, many studies are showing that the addition of boron decreases the level of egg yolk cholesterol (Grossu et al., 2005). However, in the present study, no such effect of boron alone was stated. Only in the group fed diet with the combination of boron and a low level of vitamin D_3 such an effect was observed. However, studies examining the effects of boron (Kaya and Macit, 2018) and vitamin D_3 (Yao et al., 2013) on fatty acids and cholesterol are very limited. It should be also stressed that although serum cholesterol level did not alter, yolk cholesterol concentration decreased in boric acid with vitamin D_3 supplementation group, which is consistent with the previous studies in which boron in combination with humate (Koksal et al., 2012) or plant extract mixture did not alter serum cholesterol concentration (Sizmaz et al., 2014).

The concentrations of examined minerals (Ca and P) in yolk and shell were comparable, while boron level in the eggshell was higher in hens fed boron with and without vitamin D_3 than in those fed control diet. No such relationship was stated for egg yolk boron concentration that was similar in all treatments. Results of several experiments suggest that the dietary boron modifies the mineral metabolism in poultry, which exerts stimulatory effects on performance and egg quality (Hunt and Nielsen, 1981; Hunt, 1994). However, Küçükyılmaz and Erkek (2012) emphasized that the additional boron in Ca deficiency did not affect the shell boron content. Dietary boron increased the mineral metabolism characteristics with the retention and absorption of minerals such as Ca and P. Moreover, the egg yolk and eggshell mineral content in a deficiency of Ca and vitamin D_3 were improved by additionally boron to the ration (Hunt and Nielsen, 1981; Hegsted, 1991). Similar to the present study, some trial findings argued that the yolk mineral content and ash were not affected with supplementary boron in broiler breeder (Olgun et al., 2009; Küçükyılmaz and Erkek, 2012). Considering that a maximum of 100 mg of boron daily in the diet is recommended and its excess may be toxic (Mizrak, 2008), increasing boron in the diet of laying hens without increasing the amount of boron in the egg yolk will not have a negative impact on human health. Moreover, it should be stressed that regardless of the increase in boron content in eggshell, the egg breaking strength and eggshell thickness were not different, so it can be concluded that with or without vitamin D_3 addition boron does not influence external egg quality parameters.

Conclusions

Overall, it was demonstrated that the addition of boron with or without vitamin D_3 at two different levels (high and low) did not influence egg production or external egg quality parameters (eggshell thickness or egg braking strength); however, it is necessary to pay attention to the addition of vitamin D because its high dose (3000 IU/kg) may worsen the other production parameters such as egg mass and feed efficiency. On the other hand, although the supplementation of boric acid at a dose of 120 ppm without additional vitamin D_3 increases egg yolk omega-3 fatty acid content, the cholesterol-reducing effect (but without fatty acid profile changes) may be obtained only when boric acid is added simultaneously with a low level of vitamin D_3 (300 IU/kg), which can be used to produce functional food.

Acknowledgement

Conflict of interest

The authors declare that there is no conflict of interest.

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