

Dietary supplementation with canthaxanthin and 25-hydroxycholecalciferol has beneficial effects on bone and oxidative metabolism in European quail breeders

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ABSTRACT This study aimed to investigate the effect of supplementation with canthaxanthin (Cx) and 25-hydroxycholecalciferol (25-OH-D₃) on the production performance, egg quality, bone mineral content, blood biochemical parameters, and antioxidant status of European quail breeders. Two hundred and forty quail breeders were distributed in a completely randomized design with 5 diets and 8 replicates of 4 females and 2 males were used. All quail breeders received one of 5 diets: basal diet (containing 2,000 IU vitamin D₃) or the same diet supplemented with 3 ppm Cx and 34.5 µg 25-OH-D₃, 6 ppm Cx and 69 µg 25-OH-D₃, 9 ppm Cx and 103.5 µg 25-OH-D₃, or 12 ppm Cx and 138 µg 25-OH-D₃. Production performance and internal and external egg quality parameters were not influenced by diet. Eggshell dry weight decreased linearly with increasing supplementation levels, and eggshell ash and calcium content increased quadratically. Plasma phosphorus, calcium, and ionic calcium levels in females and plasma ionic calcium levels in males showed a positive quadratic response to dietary supplementation. Femoral

and tibiotarsal dry weight and calcium content were influenced by diet. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity in the liver of males and females and in the serum of females showed a positive quadratic relationship with Cx and 25-OH-D₃ levels, whereas the malonaldehyde concentration showed a negative quadratic relationship. DPPH scavenging activity in the serum of male quail increased linearly with supplementation. There was a positive quadratic effect on superoxide dismutase gene expression and a positive linear effect on glutathione peroxidase 7 gene expression, suggesting that dietary enrichment with Cx and 25-OH-D₃ might help protect spermatozoa against oxidative damage. The dietary supplement was pro-oxidative at high concentrations (above 9 ppm Cx). The results indicate that diets with adequate levels of Cx and 25-OH-D₃ have a beneficial effect on calcium and phosphorus metabolism as well as on the antioxidant defense system. We recommend supplementing European quail breeders in the laying period with 6 ppm Cx and 69 µg 25-OH-D₃.

Key words: antioxidant system, carotenoid, egg yolk, tibiotarsus, vitamin D₃

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INTRODUCTION

Canthaxanthin (Cx) and 25-hydroxycholecalciferol (25-OH-D₃) are often used as supplements in commercial poultry breeder diet. Their benefits include increased shell and yolk quality (Bar et al., 1980), egg viability, and breeder fertility (Surai et al., 2001b; Pappas et al., 2005). Dietary Cx is absorbed in the small intestine and

distributed throughout the body. In birds, it is deposited in the egg yolk (Rosa et al., 2016; Bonilla et al., 2017), liver (Surai et al., 2003), feathers (Ren et al., 2016a), muscles, and abdominal fat (Tunio et al., 2013).

Tissues that are rich in polyunsaturated fatty acids, such as egg yolk, sperm cells, and embryonic tissues, are susceptible to lipid oxidation (Surai et al., 1999). The effect of Cx on lipid oxidation reduction has been shown in the metabolism of chick and layers and thus in meat and yolk eggs by eliminating free radicals (reactive oxygen and nitrogen species) (Surai et al., 2001a, 2003; Surai, 2012a).

The vitamin D₃ metabolite 25-OH-D₃ is 2 to 4 times more available and 20% more absorbed than vitamin D₃ (Edwards et al., 1992). It plays an important role

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in calcium and phosphorus metabolism by acting on the absorption and recycling of calcium in the intestine (Berridge et al., 2003). Consequently, it can increase the concentration of circulating calcium for egg production (Sun et al., 2012), positively influencing the laying rate, shell quality, embryonic development, bone tissue growth, and hatching (Saunders-Blade and Kover).

We hypothesized that supplementation with Cx and 25-OH-D₃ can improve egg production, bone development, and antioxidant defense. Our objectives were to evaluate the effects of supplementation on production performance, internal and external egg quality, shell mineral matter, blood biochemistry, antioxidant status, and bone quality in male and female European quail breeders and identify the best concentration of Cx and 25-OH-D₃ for fertile egg production.

MATERIAL AND METHODS

The experiment was approved by the Animal Ethics Committee (CEUA) of the State University of Maringá (protocol no. 7846161115). Experiments were conducted at the Iguatemi Experimental Farm (FEI), State University of Maringá, Maringá, Brazil.

Animals, Housing, and Handling

Quail breeders aged 24 wk were selected with suitable body weight (female = 292.01 ± 17.82 g, male = 251.84 ± 19.07 g) and egg production capacity ($90 \pm 5\%$). Quail were distributed (4 females and 2 males) in 25×39 cm laying cages made of galvanized wire with water and feed ad libitum under a 17L:7D photoperiod. Males and females in the same cage received the same diet. Birds had 14 D of adaptation prior to the experiment. The experiment was carried out during spring in birds aged from 26 to 42 wk.

Experimental Design

A total of 240 meat-type quail breeders were distributed in a completely randomized design consisting of 8 replications of 5 treatments (diets). Each cage containing 4 females and 2 males represented an experimental unit. All quail breeders received one of the 5 following diets: basal diet (containing 2,000 IU vitamin D₃) or the same diet supplemented with 3 ppm Cx and $34.5 \mu\text{g}$ 25-OH-D₃, 6 ppm Cx and $69 \mu\text{g}$ 25-OH-D₃, 9 ppm Cx and $103.5 \mu\text{g}$ 25-OH-D₃, or 12 ppm Cx and $138 \mu\text{g}$ 25-OH-D₃.

The isoenergetic and isoproteic experimental diets were based on corn and soybean meal, formulated according to Rostagno et al. (2005). The vitamin D₃ requirements were met by using a vitamin and mineral premix and the commercial dietary supplement composed of Cx and 25-OH-D₃ (MaxiChick, DSM, São Paulo, Brazil) (Table 1).

Production Performance, Egg Quality, and Yolk Pigmentation

Production performance was evaluated by measuring daily feed intake, feed conversion, egg production, and egg mass during 5 production cycles of 21 D each. Feed and leftover feed were weighed weekly, eggs were collected daily, and dead birds were counted daily to correct the feed consumption.

In the last 3 D of each cycle, internal and external quality analyses of egg and yolk pigmentation were carried out using 3 eggs per experimental unit. Mean egg weight, specific gravity, yolk index, Haugh unit (Card and Nesheim, 1966), percentages of egg yolk, shell, and albumen, shell weight, shell thickness, and yolk pigmentation were determined.

Specific gravity was determined by using 1.058 to 1.086 g/mL NaCl solutions. Yolk and albumen height and yolk diameter were measured using a digital calliper (precision of 0.02 mm; Digimess, King tools, Montreal, Canada). The yolk index (%) was calculated by dividing the yolk height by yolk diameter.

Yolk color was measured using a portable colorimeter (CR-400, Konica Minolta, Osaka, Japan). Results are expressed as CIELAB coordinates L*, a*, and b*, where L* represents lightness (0 = black to 100 = white), a* represents redness (green = -100 to red = +100), and b* represents yellowness (blue = -100 to yellow = 100) (Faitarone et al., 2016).

The thickness of the washed and dried eggshells was determined using a Mitutoyo thickness meter (Quick Mini 700-118, Mitutoyo Corporation, Tokyo, Japan). Shells collected during cycles 4 and 5 were used to determine the dry weight, ash, and calcium content. Shells were weighed, oven-dried at 55°C for 72 h, and weighed again. Then, samples were oven-dried at 105°C for 24 h. Samples were weighed for a third time, calcined in a muffle furnace at 600°C for 6 h, and weighed after 30 min of cooling. Acidic mineral solutions were prepared following Association of Official Analytical Chemists (AOAC) procedures and were used for eggshell chemical analyses (dry weight and ash weight basis) as given in AOAC International (2005) and AOAC (1995). Shell calcium concentration was determined by atomic absorption spectroscopy (AA-175, Varian Inc., Palo Alto, CA).

Sample Collection and Storage

At the end of the experimental period, when birds were aged 42 wk, blood and viscera were collected from 8 females and 8 males per treatment. All females had egg inside the uterus in the final stage of eggshell formation. Whole blood samples were collected by venepuncture into EDTA containing tubes for plasma analysis (albumin, total protein, calcium, and phosphorus) and into tubes without anticoagulant for antioxidant analysis. Samples were centrifuged at $15,000 \times g$ for 15 min. Plasma and serum were frozen at -20°C until further use.

Table 1. Ingredients and chemical composition of the experimental diets.

Ingredients (%)	Diets ¹				
	Basal	3Cx	6Cx	9Cx	12Cx
Corn	51.76	51.76	51.76	51.76	51.76
Soybean meal (45% protein)	37.03	37.03	37.03	37.03	37.03
Limestone	5.85	5.85	5.85	5.85	5.85
Soybean oil	2.88	2.88	2.88	2.88	2.88
Dicalcium phosphate	1.29	1.29	1.29	1.29	1.29
Vitamin–mineral premix ²	0.40	0.40	0.40	0.40	0.40
Common salt	0.33	0.33	0.33	0.33	0.33
DL-methionine (98.5%)	0.20	0.20	0.20	0.20	0.20
L-lysine (76.5%)	0.06	0.06	0.06	0.06	0.06
Inert (kaolin)	0.20	0.15	0.10	0.05	0.00
MaxiChick ³	0.00	0.05	0.10	0.15	0.20
Estimated composition					
Crude protein (%)	21.00	21.00	21.00	21.00	21.00
Metabolizable energy (kcal/kg)	2.850	2.850	2.850	2.850	2.850
Calcium (%)	2.700	2.700	2.700	2.700	2.700
Chlorine (%)	0.248	0.248	0.248	0.248	0.248
Available phosphorus (%)	0.350	0.350	0.350	0.350	0.350
Potassium (%)	0.828	0.828	0.828	0.828	0.828
Sodium (%)	0.150	0.150	0.150	0.150	0.150
SID ⁴ lysine (%)	1.097	1.097	1.097	1.097	1.097
SID ⁴ methionine (%)	0.480	0.480	0.480	0.480	0.480
SID ⁴ methionine + cystine (%)	0.767	0.767	0.767	0.767	0.767
Canthaxanthin (mg/kg) ⁵	2.21	3	6	9	12
25-Hydroxycholecalciferol (µg/kg)	-	34.5	69.0	103.5	138.0
Vitamin D ₃ (IU/kg)	2,000	2,000	2,000	2,000	2,000

Abbreviations: Cx, canthaxanthin; 25-OH-D₃, 25-hydroxycholecalciferol.

¹Basal diet, diet containing 2,000 IU vitamin D₃; 3Cx, basal diet supplemented with 3 ppm Cx and 34.5 µg 25-OH-D₃; 6Cx, basal diet supplemented with 6 ppm Cx and 69.0 µg 25-OH-D₃; 9Cx, basal diet supplemented with 9 ppm Cx and 103.5 µg 25-OH-D₃; and 12Cx, basal diet supplemented with 12 ppm Cx and 138.0 µg 25-OH-D₃ per kilogram.

²Provided per kilogram of premix: vitamin A, 2,500,000 IU; vitamin D₃, 500,000 IU; vitamin E, 5,000 IU; vitamin B1, 625 mg; vitamin B2, 1,500 mg; vitamin B6, 1,250 mg; vitamin B12, 5,000 µg; vitamin K3, 750 mg; pantothenic acid, 3,000 mg; niacin, 6,000 mg; folic acid, 250 mg; biotin, 50.0 mg; choline, 75 g; butylated hydroxytoluene, 1,000 mg; zinc, 13 g; iron, 13 g; manganese, 15 g; copper, 3,000 mg; cobalt, 50 mg; iodine, 250 mg; selenium, 63 mg.

³MaxiChick (DSM, São Paulo, Brazil) is a dietary supplement composed of Cx (6 mg/kg) and 25-OH-D₃ (69 µg/kg).

⁴SID: standardized ileal digestible.

⁵Cx contents were analyzed in the basal diet and estimated for the other diets.

After blood collection, the birds were killed by cervical dislocation. Liver fragments and mucosa of the uterovaginal junction were collected, fixed in liquid nitrogen, and stored at -80°C (MDF-U53VA-PA, Panasonic, Osaka, Japan). Leg bones (femur and tibiotarsus) were dissected, wrapped in saline gauze, and frozen at -20°C until further analysis.

Blood Plasma Biochemistry Profile

Plasma samples from 4 females and 4 males per treatment were analyzed for total protein, albumin, phosphorus, and calcium by colorimetry (Bioplus 2000, São Paulo, Brazil) using the standard procedures of commercial kits (total protein, albumin, phosphorus UV, and calcium; Gold Analisa Diagnóstica Ltd., São Paulo, Brazil), according to the manufacturer's instructions. Ionic calcium was calculated using the following formula: $\text{ICa (mg/dL)} = 6 \times \text{calcium} - [(0.19 \times \text{total protein}) + \text{albumin}] / (0.19 \times \text{phosphorus}) + \text{albumin} + 6$.

Bone Analysis

Femoral and tibiotarsal breaking strength (peak weight needed to break a bone) and elasticity were

analyzed using a texturometer (CT3 Texture Analyzer, Ametek Brookfield, Middleborough, MA). Bones were thawed and subjected to a 3-point bending flexural test. The epiphyses were secured on the metal platform, and a 500 g weight was applied on the diaphysis at a descent rate of 5 mm/s. The breaking strength (kgf) and elasticity (mm) were measured immediately before bone rupture.

For analysis of mineral content, femur and tibiotarsus were weighed on a precision balance (AY-220, Shimadzu Corporation, Tokyo, Japan), placed in crucibles, calcined at 55°C for 72 h, and weighed again. Subsequently, samples were oven-dried at 105°C for 24 h, weighed, calcined in a muffle furnace at 600°C for 6 h, and weighed after 30 min of cooling. The ash content (% dry matter basis) was determined, and mineral solutions were prepared for the determination of calcium concentrations by atomic absorption spectroscopy (AA-175, Varian Inc.).

Cx Analysis

Canthaxanthin contents were analyzed in the basal diet by HPLC (CBO Laboratories, São Paulo, Brazil) and estimated for the other diets.

Antioxidant Analysis

Frozen liver samples were subjected to lipid extraction. Samples were macerated in liquid nitrogen, weighed, homogenized (200 µg) with 1.8 mL of methanol diluted 1:2 (v/v) in distilled water, vortexed for 10 s, and centrifuged (Z323 K, Hermle, Germany) at 3,000 × *g* for 20 min. Liver tissue (1.5 mL) was added to 1.5 mL of 10% trichloroacetic acid (TCA), vortexed for 10 s, and centrifuged at 3,000 × *g* for 20 min. The supernatant (deproteinized liver tissue) was collected and frozen at −20°C until the antioxidant assay (Chrzczanowicz et al., 2008).

Blood serum samples were thawed, and 200 µL of the serum was added to 200 µL of 10% TCA, vortexed for 10 s, and centrifuged at 9,500 × *g* for 10 min. The supernatant (deproteinized serum) was subjected to the free radical scavenging assay (Ren et al., 2016c).

Free Radical Scavenging Activity (% 2,2-diphenyl-1-picrylhydrazyl [DPPH])

DPPH (D9132, Sigma-Aldrich, St. Louis, MO) free radical scavenging activity was determined by the DPPH method based on Brand-Williams et al. (1995).

Thiobarbituric Acid Reactive Substances in Hepatic Tissue

The malondialdehyde concentration was determined by the thiobarbituric acid reactive substances method of Chrzczanowicz et al. (2008), with modifications. Briefly, 500 µL of deproteinized liver tissue extract was homogenized with 2.0 mL of a solution containing 1% thiobarbituric acid, 10% TCA, and 0.06% HCl and incubated in a water bath (100°C) for 15 min. After cooling for 5 min, the absorbance was read at 532 nm using a spectrophotometer (SP-22, Biospectro, Curitiba, Brazil).

Total RNA Extraction and Reverse Transcription

Vaginal mucosa samples were collected, frozen immediately under liquid nitrogen, and stored at −80°C until total RNA extraction. The analysis was performed according to the manufacturer's instructions. Briefly, 100 mg of tissue was mixed with 500 µL of Trizol (Invitrogen, Carlsbad, CA), manually homogenized with 200 µL of chloroform, and centrifuged at 12,000 × *g* and 4°C for 15 min. The liquid phase was transferred to a new tube, mixed with 500 µL of isopropanol, homogenized, and centrifuged at 12,000 × *g* and 4°C for 15 min. The supernatant was discarded, the precipitate was washed with 1 mL of 75% ethanol, centrifuged at 12,000 × *g* for 5 min, and the supernatant was discarded once more. The pellet was dried for 15 min and resuspended in RNase-free ultrapure water. The RNA concentration was determined at 260 nm using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific

Inc., Waltham, MA). RNA samples were then treated with amplification grade DNase I (Invitrogen).

cDNA synthesis was carried out using the SuperScript III First-Strand Synthesis SuperMix kit, according to the manufacturer's instructions. In an RNA-free sterile tube, 6 µL of total RNA, 1 µL of 50 µM oligo (dT), and 1 µL of annealing buffer were mixed. The sample was incubated for 5 min at 65°C and then placed on ice for 1 min. Then, 10 µL of 2× Reaction Mix and 2 µL of SuperScript III/RNaseOUT Enzyme Mix were added. The solution was incubated for 50 min at 50°C, followed by 5 min at 85°C. The reaction was stopped by cooling in an ice bath, and samples were stored at −20°C until use.

Quantitative Real-Time PCR Analysis

Real-time PCR was performed with the PowerUp SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA) on a StepOne Real-Time PCR system (Applied Biosystems). The primers for superoxide dismutase (SOD1) and glutathione peroxidase 7 (GPX7) were designed for *Coturnix coturnix* on the basis of the gene sequences (XM_015881247.1 and XM_015870585.1, respectively) deposited in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov), as follows: SOD1, F: 5'-GGAGTGGCAGAGGTTAGAAATAG-3' and R: 5'-AGGTCCAGCATTTCAGTTAG-3' (amplicon = 150 bp); GPX7, F: 5'-TGGTGCCTCCTTTCCTATGT-3' and R: 5'-GTTCCAGGTTGGTTCTTCTCCT-3' (amplicon = 106 bp). β-Actin gene was the endogenous control. A primer designed for *Gallus gallus* L08165 was used (F: 5'-GCCAACAGAGA-GAAGATGAC-3' and R: 5'-CACCAGAGTCCATCA-CAATAC-3', amplicon = 130 bp). Primer specificity and efficiency (90–100%) were verified. Samples were analyzed in duplicate in 20 µL (5 µL of cDNA + 10 µL of PowerUp SYBR Green PCR Master Mix + 3.4 µL of DNase- and RNase-free ultrapure water + 0.8 µL of forward and reverse primer), and a difference less than or equal to 5% was acceptable. Relative gene expression was calculated using the 2^{−ΔΔC_t} method (Livak and Schmittgen, 2001).

Statistical Analysis

Data were analyzed using the GLM procedure in SAS University Edition (SAS Institute Inc., Cary, NC). The following statistical model was used in the analysis:

$$Y_i = \beta_0 + \beta_1 x_i + \beta_2 x_i^2 + e_i$$

where *Y* was the response criterion, β₀ was the intercept, β₁ was the linear coefficient of regression, β₂ was the quadratic coefficient of regression, *x_i* denoted the levels of diets (we used Cx values varying between 0 and 12 ppm in the analysis), and *e_i* was the residual error. The PROC MEANS procedure was used to calculate the means. Significant difference was defined as *P* ≤ 0.05. Cage was denoted as the experimental unit.

Table 2. Effect of dietary supplementation with Cx and 25-OH-D₃ on the performance, egg quality, and eggshell composition of quail breeders (26–42 wk of age).

Variable	Diets ¹					Mean	SEM	P-value	
	Basal	3Cx	6Cx	9Cx	12Cx			Linear	Quadratic
Production performance									
Egg production rate (%)	86.51	87.97	87.25	85.60	83.69	89.23	0.712	0.722	0.707
Feed intake (g/day)	28.85	28.65	28.84	28.79	28.81	28.91	0.151	0.941	0.914
FCR (kg feed/kg egg)	4.09	3.91	3.92	4.00	4.11	3.85	0.096	0.953	0.954
FCR (kg feed/dozen eggs)	0.627	0.578	0.656	0.644	0.631	0.625	0.012	0.847	0.996
Egg quality									
Egg weight (g)	12.86	12.64	12.79	12.48	12.79	12.71	0.086	0.584	0.381
Egg mass (g/day)	10.96	11.11	11.05	10.87	10.73	11.27	0.170	0.745	0.815
Specific gravity (g/mL)	1.067	1.069	1.066	1.067	1.15	1.067	0.003	0.878	0.884
Haugh unit (%)	89.61	89.90	89.48	90.02	89.73	89.74	0.260	0.891	0.922
Albumen/yolk ratio	0.458	0.460	0.451	0.463	0.461	0.458	0.002	0.704	0.580
Albumen (%)	61.24	61.84	61.42	61.21	61.33	61.41	0.144	0.606	0.651
Yolk index (%)	31.04	30.22	30.94	30.94	30.94	30.81	0.147	0.583	0.484
Eggshell (%)	7.72	7.94	7.64	7.85	7.73	7.78	0.039	0.710	0.654
Shell thickness (mm)	0.222	0.230	0.223	0.228	0.226	0.226	0.001	0.416	0.427
Eggshell composition									
Dry weight (%)	97.59	97.64	97.49	97.31	97.08	97.41	0.048	0.030	0.425
Ash (% dry weight basis)	79.64	86.25	83.33	82.54	79.69	82.47	0.876	0.012	0.003
Ca (% dry weight basis)	33.66	37.56	36.65	35.23	33.16	35.41	0.560	0.011	0.023
Regression equations									
				R ²	Vertex \hat{y} ²	Cx (ppm)		25-OH-D ₃ (μg)	
Eggshell dry weight	$\hat{y} = 97.680 - 0.044Cx$			0.86	-	-		-	
Eggshell ash	$\hat{y} = 80.589 + 1.484Cx - 0.134Cx^2$			0.68	85.12	5.40		62.10	
Eggshell Ca	$\hat{y} = 34.191 + 1.071Cx - 0.100Cx^2$			0.86	40.46	5.35		60.03	

Abbreviations: Cx, canthaxanthin; FCR, feed conversion ratio; 25-OH-D₃, 25-hydroxycholecalciferol.

¹Basal diet, diet containing 2,000 IU vitamin D₃; 3Cx, basal diet supplemented with 3 ppm Cx and 34.5 μg 25-OH-D₃; 6Cx, basal diet supplemented with 6 ppm Cx and 69.0 μg 25-OH-D₃; 9Cx, basal diet supplemented with 9 ppm Cx and 103.5 μg 25-OH-D₃; and 12Cx, basal diet supplemented with 12 ppm Cx and 138.0 μg 25-OH-D₃ per kilogram.

²Maximum or minimum \hat{y} values.

RESULTS

Supplementation with Cx and 25-OH-D₃ did not influence production performance (egg production rate, feed conversion, egg mass, feed intake, and egg weight) and the majority of egg quality parameters (specific gravity, Haugh unit, yolk index, percentage compositions of albumen, yolk, and shell, shell weight, and shell thickness) of European quail breeders (Table 2).

Analysis of egg composition revealed that eggshell dry weight decreased linearly ($P = 0.030$) and eggshell ash ($P = 0.003$) and calcium ($P = 0.023$) contents responded quadratically to Cx (and 25-OH-D₃) levels (Table 2).

Diet also had a significant effect on yolk color: lightness (L*) decreased quadratically ($P < 0.001$), redness (a*) increased quadratically ($P < 0.001$), and yellowness (b*) decreased linearly ($P = 0.042$) with Cx and 25-OH-D₃ supplementation (Table 3).

Dietary supplementation influenced the blood biochemistry and bone composition of female quail breeders. Plasma phosphorus ($P < 0.001$), calcium ($P < 0.001$), and ionic calcium ($P = 0.005$), femur dry weight ($P = 0.009$), ash ($P = 0.021$) and calcium ($P = 0.011$) contents, and tibiotarsus dry weight ($P = 0.014$) and calcium content ($P = 0.012$) showed a negative quadratic response to supplementation (Tables 4 and 5). Diet did not

Table 3. Effect of dietary supplementation with Cx and 25-OH-D₃ on quail egg yolk color.

Color parameter	Diets ¹					Mean	SEM	P-value	
	Basal	3Cx	6Cx	9Cx	12Cx			Linear	Quadratic
L*	59.44	55.59	52.99	51.67	51.60	54.42	0.494	<0.001	<0.001
a*	-3.44	6.53	13.37	17.08	17.65	10.25	1.289	<0.001	<0.001
b*	39.50	38.54	38.24	37.92	38.00	38.47	0.236	0.042	0.321
Regression equations									
				R ²	Vertex \hat{y} ²	Cx (ppm)		25-OH-D ₃ (μg)	
L*	$\hat{y} = 59.44 - 1.494Cx + 0.073Cx^2$			0.85	51.79	10.24		117.80	
a*	$\hat{y} = -3.436 + 3.845Cx - 0.174Cx^2$			0.96	17.81	11.05		127.08	
b*	$\hat{y} = 39.16 - 0.115Cx$			0.80	-	-		-	

Abbreviations: Cx, canthaxanthin; 25-OH-D₃, 25-hydroxycholecalciferol.

¹Basal diet, diet containing 2,000 IU vitamin D₃; 3Cx, basal diet supplemented with 3 ppm Cx and 34.5 μg 25-OH-D₃; 6Cx, basal diet supplemented with 6 ppm Cx and 69.0 μg 25-OH-D₃; 9Cx, basal diet supplemented with 9 ppm Cx and 103.5 μg 25-OH-D₃; and 12Cx, basal diet supplemented with 12 ppm Cx and 138.0 μg 25-OH-D₃ per kilogram.

²Maximum or minimum \hat{y} value.

Table 4. Effect of dietary supplementation with Cx and 25-OH-D₃ on plasma levels (mg/dL) of P, Ca, and ionic Ca in 42-wk-old quail breeders.

Variable	Diets ¹					Mean	SEM ²	P-value	
	Basal	3Cx	6Cx	9Cx	12Cx			Linear	Quadratic
Females									
P	2.18	0.96	0.97	0.74	0.94	1.16	0.131	<0.001	<0.001
Ca	13.51	11.18	10.44	11.11	11.17	11.48	0.296	<0.001	<0.001
Ionic Ca	10.16	9.08	8.56	9.44	9.12	9.24	0.183	<0.001	0.005
Males									
P	5.36	5.13	5.22	5.24	5.10	5.21	0.033	0.456	0.742
Ca	4.81	4.73	4.69	4.81	4.30	4.67	0.049	0.530	0.226
Ionic Ca	2.18	1.80	1.54	1.35	0.96	1.57	0.069	0.003	0.916
Regression equations					R ²	Vertex \hat{y} ²	Cx (ppm)	25-OH-D ₃ (μg)	
Female P	$\hat{y} = 2.071 - 0.339Cx + 0.0021Cx^2$				0.90	0.70	8.07	92.80	
Female Ca	$\hat{y} = 13.319 - 0.749Cx + 0.049Cx^2$				0.72	10.46	7.64	87.88	
Female ionic Ca	$\hat{y} = 10.028 - 0.362Cx + 0.025Cx^2$				0.90	8.72	7.24	83.25	
Male ionic Ca	$\hat{y} = 2.143 - 0.096Cx$				0.90	-	-	-	

Abbreviations: Cx, canthaxanthin; 25-OH-D₃, 25-hydroxycholecalciferol.

¹Basal diet, diet containing 2,000 IU vitamin D₃; 3Cx, basal diet supplemented with 3 ppm Cx and 34.5 μg 25-OH-D₃; 6Cx, basal diet supplemented with 6 ppm Cx and 69.0 μg 25-OH-D₃; 9Cx, basal diet supplemented with 9 ppm Cx and 103.5 μg 25-OH-D₃; and 12Cx, basal diet supplemented with 12 ppm Cx and 138.0 μg 25-OH-D₃ per kilogram.

²Maximum or minimum \hat{y} values.

influence ($P > 0.05$) the bone breaking strength or elasticity of the femur of females (Table 5).

In males, dietary supplementation had no significant effect ($P > 0.05$) on plasma calcium and phosphorus levels (Table 4). Plasma ionic calcium decreased linearly ($P < 0.01$) with increasing levels of Cx and 25-OH-D₃. There was no influence on femur dry weight, ash and calcium contents, and femoral breaking strength and elasticity ($P > 0.05$). There was an influence on tibiotarsal ash that decreased linearly ($P = 0.048$) and on tibiotarsal calcium which increased quadratically ($P < 0.001$)

with Cx and 25-OH-D₃ levels. Tibiotarsal elasticity showed a negative quadratic relationship ($P = 0.042$) with Cx and 25-OH-D₃ levels (Table 6).

Dietary supplementation produced a significant effect on the antioxidant status of the liver and blood of 42-wk-old quail breeders (Table 7). The DPPH radical scavenging activity in the liver of males ($P < 0.001$) and females ($P = 0.007$) and in the serum of females ($P < 0.001$) showed a positive quadratic relationship with Cx and 25-OH-D₃ levels, whereas malonaldehyde (MDA) demonstrated a negative quadratic relationship

Table 5. Effect of dietary supplementation with Cx and 25-OH-D₃ on the bone quality of 42-wk-old female quail breeders.

Variable	Diets ¹					Mean	SEM	P-value	
	Basal	3Cx	6Cx	9Cx	12Cx			Linear	Quadratic
Femur									
Dry weight (%)	69.87	66.34	65.20	65.43	72.32	67.88	0.972	0.018	0.009
Ash (%)	40.75	37.08	37.56	37.99	41.77	39.17	0.747	0.037	0.021
Ca (%)	17.22	15.86	12.90	15.86	16.02	15.34	0.438	0.007	0.011
Strength (kgf)	3.06	3.48	3.40	4.17	3.56	3.46	0.120	0.390	0.340
Elasticity (mm)	1.17	0.98	1.03	0.89	0.88	0.98	0.031	0.181	0.566
Tibiotarsus									
Dry weight (%)	69.18	68.18	65.82	68.61	71.26	68.50	2.565	0.036	0.014
Ash (%)	36.85	37.75	36.91	38.58	37.94	37.47	1.235	0.630	0.870
Ca (%)	15.22	14.93	12.93	15.03	15.27	14.54	0.676	0.010	0.012
Strength (kgf)	4.74	5.24	4.84	5.44	5.82	5.18	0.244	0.990	0.720
Elasticity (mm)	0.55	0.50	0.40	0.50	0.45	0.48	0.037	0.091	0.180
Regression equations					R ²	Vertex \hat{y} ²	Cx (ppm)	25-OH-D ₃ (μg)	
Femur dry weight	$\hat{y} = 69.813 - 0.028Cx + 0.18Cx^2$				0.99	64.55	4.06	46.70	
Femoral ash	$\hat{y} = 40.569 - 1.301Cx + 0.116Cx^2$				0.95	36.92	5.61	64.53	
Femoral Ca	$\hat{y} = 17.356 - 0.986Cx + 0.075Cx^2$				0.66	14.12	6.57	75.55	
Tibiotarsus dry weight	$\hat{y} = 69.271 - 0.964Cx + 0.093Cx^2$				0.81	66.77	5.18	59.58	
Tibiotarsal Ca	$\hat{y} = 15.681 - 0.594Cx + 0.047Cx^2$				0.60	13.80	6.32	72.68	

Abbreviations: Cx, canthaxanthin; 25-OH-D₃, 25-hydroxycholecalciferol.

¹Basal diet, diet containing 2,000 IU vitamin D₃; 3Cx, basal diet supplemented with 3 ppm Cx and 34.5 μg 25-OH-D₃; 6Cx, basal diet supplemented with 6 ppm Cx and 69.0 μg 25-OH-D₃; 9Cx, basal diet supplemented with 9 ppm Cx and 103.5 μg 25-OH-D₃; and 12Cx, basal diet supplemented with 12 ppm Cx and 138.0 μg 25-OH-D₃ per kilogram.

²Maximum or minimum \hat{y} values.

Table 6. Effect of dietary supplementation with Cx and 25-OH-D₃ on the bone quality of 42-wk-old male quail breeders.

Variable	Diets ¹					Mean	SEM	P-value	
	Basal	3Cx	6Cx	9Cx	12Cx			Linear	Quadratic
Femur									
Dry weight (%)	75.66	71.77	71.20	74.37	74.31	74.47	1.032	0.215	0.218
Ash (%)	29.99	25.78	27.54	26.93	27.38	27.22	2.694	0.221	0.225
Ca (%)	17.79	18.07	18.50	16.04	17.52	17.52	0.678	0.221	0.513
Strength (kgf)	3.98	3.27	3.63	3.76	3.68	3.63	0.101	0.435	0.561
Elasticity (mm)	0.72	0.83	0.79	0.93	0.95	0.83	0.030	0.653	0.574
Tibiotarsus									
Dry weight (%)	81.43	74.71	78.42	77.78	78.87	78.22	1.090	0.218	0.230
Ash (%)	32.06	29.96	30.37	29.98	29.72	30.44	0.332	0.048	0.287
Ca (%)	17.13	21.55	19.13	17.69	15.47	18.26	0.496	0.011	0.001
Strength (kgf)	5.99	5.25	5.95	4.96	5.44	5.47	0.171	0.435	0.561
Elasticity (mm)	0.75	0.59	0.45	0.45	0.54	0.55	0.031	0.015	0.042
Regression equations					R ²	Vertex \hat{y} ²	Cx (ppm)	25-OH-D ₃ (μg)	
Tibiotarsus ash					$\hat{y} = 31.360 - 0.158Cx$	0.69	-	-	-
Tibiotarsus Ca					$\hat{y} = 17.866 + 0.939Cx - 0.0985Cx^2$	0.75	20.10	4.77	54.85
Tibiotarsal elasticity					$\hat{y} = 0.759 - 0.079Cx + 0.005Cx^2$	0.29	0.447	7.90	90.85

Abbreviations: Cx, canthaxanthin; 25-OH-D₃, 25-hydroxycholecalciferol.

¹Basal diet, diet containing 2,000 IU vitamin D₃; 3Cx, basal diet supplemented with 3 ppm Cx and 34.5 μg 25-OH-D₃; 6Cx, basal diet supplemented with 6 ppm Cx and 69.0 μg 25-OH-D₃; 9Cx, basal diet supplemented with 9 ppm Cx and 103.5 μg 25-OH-D₃; and 12Cx, basal diet supplemented with 12 ppm Cx and 138.0 μg 25-OH-D₃ per kilogram.

²Maximum or minimum \hat{y} values.

($P < 0.001$). DPPH scavenging activity in the serum of male quail showed a linear increase ($P < 0.001$) with increasing supplementation (Figure 1).

The results of SOD1 and GPX7 expression in the vaginal mucosa of quail breeders are presented in Figure 2. There was a quadratic effect of diet on relative SOD1 expression. It was estimated from experimental data that the highest SOD1 expression levels would be achieved with supplementation of 5.27 ppm Cx and 60.60 μg 25-OH-D₃. GPX7 expression increased linearly with supplementation levels.

DISCUSSION

Supplementation of quail breeder diets with Cx and 25-OH-D₃ was found to have no effect on production performance and on the majority of egg quality parameters. In previous studies (Khatun et al., 1999; Zhang et al., 2011; Rosa et al., 2012; Cho et al., 2013), Cx levels of up to 6 ppm did not alter the laying rate, egg mass, or feed conversion ratio of laying hens fed diets based on

corn and soybean. Contrary to these results, Cho et al. (2013) and Zhang et al. (2011) reported an increase in the laying rate in hens fed diets based on sorghum and enriched with 2 ppm Cx. Such differences may be attributed to the replacement of corn with sorghum. As sorghum contains lower concentrations of essential nutrients than corn, the effect of Cx might have been more pronounced in chickens fed sorghum-based diets.

The lack of any effect of supplementation on external egg quality (egg weight, eggshell weight, and shell thickness) corroborates the results of previous studies. Cho et al. (2013) found no effect of 2.1 ppm Cx on the shell weight and thickness of 280 ISA Brown layers, and Käppeli et al. (2011) reported that laying hens aged between 49 and 66 wk fed a control diet containing 3,000 IU vitamin D₃ or a test diet containing 1,500 IU vitamin D₃, 3 ppm Cx, and 178.5 μg 25-OH-D₃ did not differ in shell weight or thickness. Also in contrast to our results, Ren et al. (2016a) showed that supplementation with Cx (6 ppm) and 25-OH-D₃ (69 μg) increased ($P < 0.05$) shell thickness at 38 and 77 wk. The differences between our

Table 7. Effect of dietary supplementation with Cx and 25-OH-D₃ on the antioxidant status of 42-wk-old female and male quail breeders.

Regression equations	P-value	R ²	Vertex \hat{y} ¹	Cx (ppm)	25-OH-D ₃ (μg)
Females					
Serum DPPH inhibition (%)	$\hat{y} = 69.99 + 2.134Cx - 0.1184Cx^2$	<0.001	0.64	79.64	9.04
Liver DPPH inhibition (%)	$\hat{y} = 44.22 + 1.609Cx - 0.135Cx^2$	0.007	0.62	49.01	5.96
Liver MDA (mg/kg)	$\hat{y} = 0.245 - 0.0224Cx + 0.0023Cx^2$	<0.001	0.83	0.189	4.83
Males					
Serum DPPH inhibition (%)	$\hat{y} = 64.95 + 0.652Cx$	<0.001	0.86	-	-
Liver DPPH inhibition (%)	$\hat{y} = 51.00 + 1.256Cx - 0.124Cx^2$	<0.001	0.97	54.18	5.06
Liver MDA (mg/kg)	$\hat{y} = 0.213 - 0.0239Cx + 0.00247Cx^2$	<0.001	0.98	0.156	4.82

Abbreviations: Cx, canthaxanthin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; 25-OH-D₃, 25-hydroxycholecalciferol; MDA, malonaldehyde.

¹Maximum or minimum \hat{y} values.

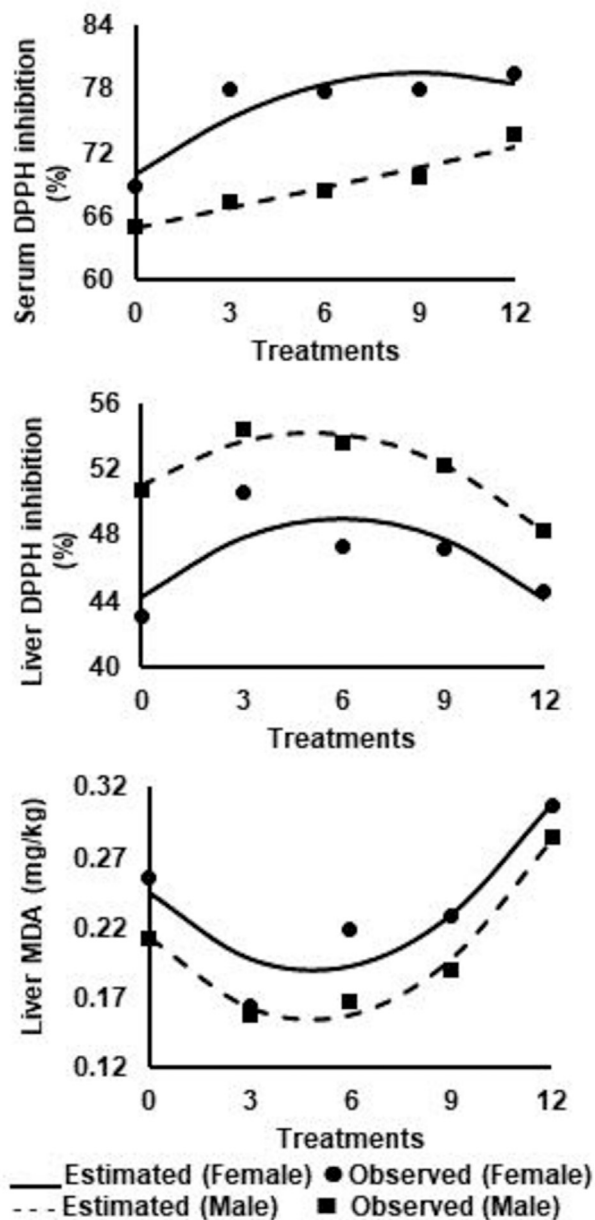


Figure 1. Effect of dietary supplementation with canthaxanthin and 25-hydroxycholecalciferol on the serum and liver antioxidant activity and thiobarbituric acid reactive substances (TBARS) method. Abbreviation: MDA, malonaldehyde.

results and those presented in the literature may be due to differences in quail age. Significant effects of 25-OH-D₃ supplementation were only observed in older birds (>70 wk) in the referred studies. Older birds have a lower ability to absorb calcium than younger birds (Elaroussi et al., 1994). Therefore, the high availability and rapid absorption of 25-OH-D₃ may have enhanced calcium deposition in the eggshell.

In our study, Cx combined with 25-OH-D₃ had an effect on serum calcium and phosphorus levels. Käppeli et al. (2011) described the effects of vitamin D₃ (3,000 IU) and the association with 35 to 37.5 µg of 25-OH-D₃ in laying hens; however, they did not observe the same behavior in serum biochemistry results. Blood and bone samples

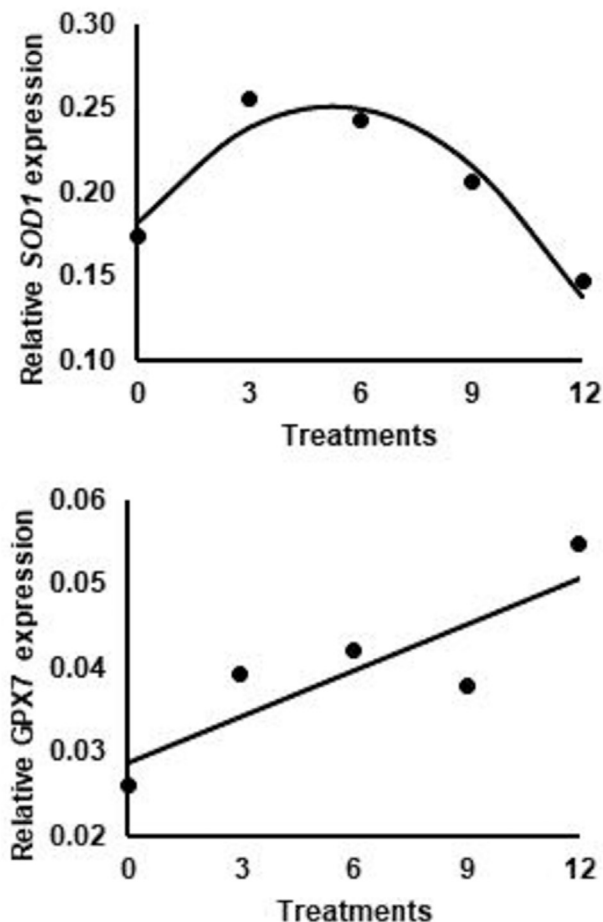


Figure 2. Graphics of the real-time PCR results of the effect of dietary supplementation with canthaxanthin (Cx) and 25-hydroxycholecalciferol (25-OH-D₃) on the relative expression of superoxide dismutase (SOD1) and glutathione peroxidase 7 (GPX7) genes in the vaginal mucosa of 42-week-old quail breeders. Note the quadratic effect of diet on the relative SOD1 expression and the crescent linear effect on the relative GPX7 expression in vaginal mucosa. Relative SOD1 expression = $0.1815 + 0.02615Cx - 0.00248Cx^2$ ($R^2 = 0.92$, $P = 0.004$, $SEM = 0.0095$, $y_{max} = 5.27$ ppm Cx and 60.61 µg 25-OH-D₃). Relative GPX7 expression = $0.0288 + 0.001818Cx$ ($R^2 = 0.72$, $P = 0.007$, $SEM = 0.0021$).

were collected from females during eggshell formation when minerals are removed from the bones, transported through the bloodstream to the uterus, and deposited in the eggshell. This physiological process was confirmed by blood biochemistry and eggshell calcium analyses. Plasma calcium, ionic calcium, and phosphorus decreased quadratically with supplement concentration, whereas the eggshell calcium content increased quadratically. These results suggest that 25-OH-D₃ stimulated the transfer of calcium from blood to the eggshell.

Ash and calcium contents in dry matter of eggshell increased until around 60 µg of 25-OH-D₃. However, eggshell thickness was not influenced by diet, suggesting that dense calcium crystals were formed in the eggshell, conferring greater hardness and resistance with high doses of 25-OH-D₃. This hypothesis was supported by the findings of Ren et al. (2016a), who assessed the effect of Cx and 25-OH-D₃ supplementation, with or without vitamin D₃, in duck breeders. The authors observed fewer broken eggs from breeders supplemented with Cx and 25-OH-D₃.

Male and female quail consume the same feed, which is generally formulated to meet the nutritional requirements for egg production, that is, high calcium (2.7%) and phosphorus (0.53%) concentrations. Analysis of the plasma ionic calcium showed that concentration was 1.57 mg/dL in males and 9.24 mg/dL in females; the difference observed in concentration was probably due to the action of hormones on calcium homeostasis in males. Males showed higher calcium deposition in the tibiotarsus, which increased the resistance to rupture—an important aspect of bone quality. [Ren et al. \(2016b\)](#) also observed that 25-OH-D₃ improved tibia quality in ducks.

Canthaxanthin is deposited in the yolk and can influence the various metabolic processes that occur in embryo development, hatching, and the first week of life ([Ren et al., 2016c](#)). Supplementation altered the color of the egg yolk from yellow to orange-red. We did not evaluate the concentration of carotenoids, but the results of redness (Minolta a*) and lightness (Minolta L*) suggested an increased concentration of Cx in the yolk. [Rosa et al. \(2016\)](#) observed that the carotenoid concentration in yolk increased from 1.98 to 27.98 mg/kg when Cobb-500 breeders were supplemented with 6 ppm Cx at 54 to 64 wk. High egg Cx levels were associated with reduced susceptibility to oxidative stress in the yolk, and enhanced embryonic development and progeny performance.

Canthaxanthin concentrations were probably also increased in the vaginal mucosa, liver, and male genitals, positively influencing sperm production. 25-hydroxycholecalciferol plays an important role in embryonic skeleton formation, and Cx eliminates free radicals in the yolk. Together, they positively influence embryonic development and metabolism. Antioxidant protection is especially important during hatching, a period of high metabolic stress, when chicks must expend energy to break the shell and come into contact with oxygen, initiating oxidation processes that occur naturally in the first week of life ([Surai, 2002a](#)). Canthaxanthin and 25-OH-D₃ stimulated the elimination of free radicals in blood serum, as measured by the DPPH assay. In female liver tissues, MDA concentration reduced with supplementation, supporting our hypothesis of deposition of Cx in various tissues. The progeny of broiler breeders (30-wk-old) fed Cx-supplemented diets showed higher α -tocopherol levels and reduced lipid peroxidation ([Surai et al., 2003](#)).

In this study, the low concentration of MDA in liver tissue and the high radical scavenging capacity in liver tissue and blood serum show that Cx and 25-OH-D₃ supplementation improves the antioxidant status of breeders and may have influenced the viability and fertility of breeders and progeny. Decreased levels of free radicals and MDA are associated with an increased expression of antioxidant enzymes (GPx-7, SOD1, and catalase). Canthaxanthin and 25-OH-D₃ enhanced the relative expression of GPX7 and SOD1 in the vaginal mucosa of quail breeders, suggesting a possible beneficial effect on fertility. Uterovaginal sperm host glands

protect spermatozoa from oxidative damage, increasing their survival in the female reproductive tract and thereby playing a fundamental role in reproduction ([Lake, 1975](#); [Bakst, 1993](#)). Supplemented quail breeders also showed lower MDA levels and improved antioxidant activity in the liver and blood, effects that might have been due to increased GPX7 and SOD1 expression in these tissues.

However, it must be noted that the relationship between hepatic MDA levels and supplement concentration in diets was quadratic. In females, MDA levels were estimated to increase at dietary concentrations above 4.82 ppm Cx and 55.55 μ g 25-OH-D₃/kg feed. For males, the inflection point was estimated at 5.06 ppm Cx and 58.18 μ g 25-OH-D₃/kg feed. These results show that Cx and 25-OH-D₃ can be pro-oxidative at high doses. [Saha et al. \(2016\)](#), by studying the influence of vitamin D₃ on the antioxidant system, found that excessive serum levels of vitamin D₃ lead to increased free radical production and lipid oxidation.

Supplementation of Cx + 25-OH-D₃ decreased MDA concentration in the liver and serum of European quail breeders. These results probably improved the protection against oxidative stress by enhanced antioxidant capacity to scavenge free radicals present during the metabolism of liver and serum in animals. When oxidative stress is reduced by the action of antioxidants, the mitochondrial biogenesis and their functions are improved ([Chen et al., 2019](#)). Canthaxanthin has a synergistic action with vitamins (such as vitamins C and E) in the neutralization of free radicals ([Urso et al.](#)). By preserving the nutritional quality of the egg yolk, these antioxidant molecules may improve hatchability, fertility, livability ([Rosa et al., 2012](#)), as well as chick quality and immune system ([Johnson-Dahl et al., 2016](#)).

Quail breeders supplemented with Cx and 25-OH-D₃ showed improved calcium metabolism, as evidenced by calcium contents in the eggshell, serum, femur, and tibiotarsus. During egg formation, supplemented breeders showed a higher calcium loss from bones and blood and increased deposition of calcium in eggshells. Plasma calcium levels in males were not influenced by diet and were much lower than those found in females. In contrast, calcium in the tibiotarsus of males increased in diets.

Supplementation of quail breeders with Cx and 25-OH-D₃ had a beneficial effect on the antioxidant system and calcium and phosphorus metabolism. On the basis of these results, we recommend dietary enrichment with 6 ppm Cx and 69 μ g 25-OH-D₃/kg feed for European quail breeders in the laying period.

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