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Vitamin E and Selenium Given as Dietary Supplements Accumulate in Tissues and Semen and Improve Reproductive Parameters in Older Red Cornish

Rosalie Adina Bălăceanu[1], Victor G. Nimigean[2], Vanda Roxana E.S. Nimigean[2], Ştefania Raită[1], Laurenţ Ognean[3] and Nicolae Dojană[1]

[1] University of Agronomical Sciences and Veterinary Medicine of Bucharest, 050097 Bucharest, Romania
[2] Carol Davila University of Medicine and Pharmacy, 010221 Bucharest, Romania
[3] University of Agronomical Sciences and Veterinary Medicine of Cluj Napoca, 400372 Cluj Napoca, Romania

The reproductive performance of broiler breeder chickens noticeably decreases toward the end of their commercial lives. Herein, we determined the effects of vitamin E and selenium dietary supplementation on semen traits, egg fertility (defined as fertilization and hatching rates) of adult (49-week-old) and older (63-week-old) Red Cornish breeders. We found that both vitamin E and selenium were concentrated in the liver and adipose tissue of adult and older Red Cornish breeders, and were transferred to the semen and egg yolk, respectively, in proportion to the level of supplementation. Vitamin E supplementation, in particular, improved ejaculate volume, total sperm count, sperm motility, and viability in both adult and older roosters, whereas selenium improved sperm motility and viability in the adult roosters. Egg fertility increased following supplementation with either vitamin E or selenium. The hatching rate also improved by both supplements in proportion to the level of supplementation. No significant synergistic effects of vitamin E and selenium were found. The levels of egg fertility and sperm trait improvements diminished with the age of the birds and depended on vitamin E and/or selenium doses. Thus, as dietary vitamin E and selenium supplements improved semen quality and egg fertility in these older Red Cornish broiler breeders, such birds could be maintained in flocks to prolong their reproductive output.

Key words: aged Red Cornish breeder, antioxidant supplementation, reproductive performance

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Introduction

Red Cornish (RC) inbred chicken strains are used as male parental genetic lines to produce broiler hens. Genetic categories of RC inbred strains are subjected to high selection pressure, which has imparted undesirable side-effects such as reduced fertility and poor egg hatchability (Rauw *et al*., 1998). In 1973, Edens *et al*. showed that spermatozoa of high body weight (BW)-line breeders had lower endogenous respiration potential than those of a low BW-line. Later, Gowe *et al*. (1993) proposed a strategy to maintain the high fertility and hatchability of breeders in selection programs. Rauw *et al*. (1998) published a review regarding the side-effects of selection on production efficiency of broiler breeders, including decreased hatching rates and lower sperm concentration, ejaculate volume, and sperm motility. Studies by Oldenbroek and van der Waaij (2015) and Wang *et al*. (2018) confirmed that higher selection pressure altered the metabolism of these birds over time and made them more sensitive to stressors and antioxidant factor deficiencies. We considered the reproductive problems encountered by RC breeders that are unusual because of the low breeding potential of roosters, low laying rate, precocious aging, and increased sensibility to stressors. Many RC breeders are excluded from breeding programs owing to these problems.

In poultry farming, the possible beneficial effects of vitamin E and selenium (Se) dietary supplementation on semen function and hatchability have been investigated in young animals. Edens and Sefton (2009) found that Sel-Plex (containing Se mainly in the form of selenomethionine

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Correspondence: Dr. Rosalie Adina BĂLĂCEANU, DVM, Ph. D, Faculty of Veterinary Medicine, University of Agronomical Sciences and Veterinary Medicine of Bucharest, 050097 Bucharest, Romania.
(E-mail: rosalie_timeea@yahoo.com)

in yeast protein, Alltech, Inc., Nicholasville, KY, USA) improved sperm cell morphology in 26-weeks-old Cobb-500 broiler breeders. A study by Tabatabaei *et al*. (2010) on the correlation of rooster age with semen quality considered three age categories of indigenous broiler breeders, ranging from 26 to 45 week of age. Shanmugam *et al*. (2015) reported improvements in sperm motility, live sperm counts, and semen fertility in young (29-week-old) Dahlem Red roosters fed Se-enriched diets. Bealish *et al*. (2018) studied the effect of different Se sources and levels on semen in 32-week-old Silver Montazah roosters.

The physiological requirements of vitamin E and Se (5 UI/kg and 0.06 mg/kg diet, respectively) for laying hens are quite low but exceedingly difficult to determine because of their interrelationships with other dietary factors such as polyunsaturated fatty acids, antioxidants, sulfur amino acids, and Se as well as variations with strains and breeds (NRC, 1994). There is no information on the dietary requirements of vitamin E and Se for RC, which are known to exhibit low fertility (Soller *et al*., 1965).

The purpose of this study was to identify the effects of different levels of vitamin E and Se dietary supplementation on semen traits and egg fertility (defined as fertilization and hatching rates) in younger and older RC breeders.

Materials and Methods

Animals and Experimental Design

All procedures in this study were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes by Member States of the European Union. In total, 30 44-week-old male (4,422±122 g BW) and 150 44-week-old female (3,557±141 g BW) local inbred strain RC breeders were used. The birds were equally divided into one control and five experimental groups. The birds were housed in pens (2.2×1.2×2.4 m), each containing 5 roosters and 25 hens, and reared separately in a temperature- and humidity-controlled room. The birds had free access to forage and water and were fed on a standard commercial diet (main ingredients by %: wheat 41.3, barley 31.5, oats 10.9, soybean meal 5.9, grass meal 2.6, fish meal 5.6, dicalcium phosphate 0.6, limestone 0.8, vitamin and mineral premix 0.5) containing 23 mg/kg vitamin E and 0.11 mg/kg Se as calculated values. The daily diets of experimental groups were supplemented with vitamin E (DL-α-tocopherol acetate vitamer; Hoffmann La Roche, Basel, Switzerland) and Se (as Sel-Plex, Alltech, Inc.). Birds from the control group received no vitamin E or Se supplementation. Birds from groups 1 and 2 received Se at 0.3 and 0.5 mg/kg, respectively; those from groups 3 and 4 received vitamin E at 50 and 200 IU/kg, respectively; and those from group 5 received 200 IU/kg of vitamin E and 0.5 mg/kg Se.

Experimental monitoring began with birds at 44 weeks of age and lasted for 20 weeks. Hens were artificially inseminated every 5 days using 0.025 mL of semen from roosters receiving the same diet, 1:3 diluted in a commercial semen extender. Semen, eggs, and tissues were each sampled at 49, 53, and 63 weeks of age. Semen was sampled by abdominal massage according to Burrows and Quinn (1937) and was immediately analyzed. The semen samples were cryopreserved at −20℃ for subsequent determinations of vitamin E and Se contents. At least 104 eggs per group were incubated in a poultry incubator system each week (model ZH 9856, Tehno Ms, Bucharest, Romania). Eggs were transferred to a hatcher (Tehno Ms) on day 19 of incubation. Five birds (one rooster and four hens) from each group were killed in a slaughter house for liver and adipose tissue sampling at each time point. Samples were divided into aliquots in polyethylene tubes and frozen at −20℃ until analysis. At each time point, yolks from 12 collected eggs of each group were separately sampled, pooled and stored frozen (−20℃) in plastic cups until the analysis could be conducted on all samples.

Semen Analysis

The ejaculates were collected in transparent glass graduated collection tubes; volumes were directly recorded in the tube immediately after collection at the lower margin of the semen meniscus and were expressed in microliter (μL). Sperm motility was assessed by a wet preparation technique using a Nihon Kohden optical microscope (Sapaco 2000, Bucharest, Romania) on a warmed plate. Briefly, the semen sample was diluted (1:200) in 37℃ Ringer's solution immediately after collection. One drop of the diluted semen (no more than 20 μL) was placed on a slide and covered with a 20×20 mm glass coverslip. Motility was estimated by direct observation of spermatozoa in at least five fields under 400× magnification and a lowered condenser to disperse the light. Motility was expressed as the percentage of all spermatozoa showing progressive movements, either linearly or in a large circle, regardless of speed (rapid or slow). Nonprogressive spermatozoa with other patterns of movement were not considered in this category. Sperm count was determined using a hemocytometer with a Nihon Kohden optical microscope. Fresh semen samples were diluted (1:200) and fixed using neutral Hancock's solution (62.5 mL of 37% formaldehyde, 150 mL of 1% saline, 150 mL of sodium phosphate buffer, and 500 mL of double-distilled water) and a Potain pipette. The results are expressed as the number of spermatozoa per milliliter (mL). Viability of the spermatozoa was evaluated by eosin–nigrosin staining (Merck, Darmstadt, Germany) according to Kondracki *et al*. (2017). The results are expressed as the percentage of viable spermatozoa (eosinophobic).

Vitamin E and Se Analyses

Vitamin E content was determined in the liver, subcutaneous adipose tissue, egg yolk, and semen by high-performance liquid chromatography (HPLC), as per the method described by Ubaldi *et al*. (2005). Briefly, the samples were saponified with 50% potassium hydroxide in ethanol plus 0.5% ascorbic acid. Extraction was performed using ether. The samples were evaporated to dryness, dissolved in methanol, and injected into the HPLC system (Dionex Ultimate 3000 HPLC, Dionex, Sunnyvale, CA, USA). A calibration curve was obtained using six known concentrations of the analyte at 0.5−30.0 mg/kg dissolved in methanol. The quantitative determination of vitamin E was conducted using a UV de-

tector at 294 nm wavelength. Se concentration was determined in the same tissue and semen samples used for vitamin E analysis by atomic absorption spectrophotometry, according to Pechová *et al*. (2005), using a Jiebo Instrument spectrophotometer (Wuxi Jiebo Instrument Co. Ltd., Jiangsu, P. R. China). The samples were subjected to wet microwave mineralization in a closed system of nitric acid (puriss. p.a. grade 65%, Fluka, Bucharest, Romania) and hydrogen peroxide (puriss. p.a. 30%, Fluka) using an MLS-1200 microwave ashing system (Milestone, Sorisole, Italy). The mineralization product was evaporated to a final volume of approximately 200 μL and quantitatively transferred into 5 mL water in a disposable plastic test tube. The different Se forms (Se^{II}, Se^{VI}) were reduced in the samples to tetravalent Se using hydrochloric acid (HCl) treatment (5 mL of 20% HCl added to 5 mL of mineralized sample water). Then, hydride was generated with 1% sodium borohydride (purum grade; Fluka) in 0.1% sodium hydroxide (NaOH; Lachema, Brno, Czech Republic). The absorbance of the resulting selenium hydride was measured at 196 nm wavelength. A linear calibration curve was generated using an atomic spectroscopy standard Se solution (Fluka) with concentrations of 10, 30, 50, and 100 μg/L.

Fertilization and Hatchability Determination

On day 8 of incubation, fertilized eggs were detected by candling and the fertilization rate was calculated as the percentage of total incubated eggs. The hatching rate of fertilized eggs was calculated as the percentage of chicks hatched, and the overall hatching rate was calculated as the percentage of chicks hatched from all eggs.

Statistical Analysis

Data are expressed as the mean and standard error of mean calculated using the general linear model (GLM) in the SAS statistical package (version 9.4; SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used to compare means between experimental and control groups. Tukey post-hoc tests were performed to determine significant differences between the experimental groups and the control group. The Kruskal–Wallis nonparametric test was applied to analyze the effects of diets on fertilization and hatching rates. Differences were considered significant at $P<0.05$. Correlations of age (independent variable) with the investigated (dependent) variables were determined using Pearson's r values.

Results

Vitamin E and Se Contents

Vitamin E values were the highest in the adipose tissue and were up to 378% higher in the supplemented groups than in the control group ($P<0.01$). These values were proportional to the level of dietary supplementation (Table 1). Vitamin E was transferred to the egg yolk and semen where it reached 759% and 232% higher concentrations, respectively, in the supplemented groups than in the control group ($P<$

Table 1. **Vitamin E and Se contents in the tissues and semen of Red Cornish broiler breeders of different ages fed on vitamin E- and Se-supplemented diets**

Vitamin E (μg/g fresh tissue or mL)					Selenium (ng/g fresh tissue or mL)				
Gr[1]	49 wk	53 wk	63 wk	SD[2]	Gr[1]	49 wk	53 wk	63 wk	SD[2]
	Liver								
C	34.5±1.1	35.4±4.4	23.5±2.2	6.6	C	204.3±41.2	214.3±8.9	187.5±16.5	13.6
3	63.4±32.2**	55.4±12.1	24.0±22.0	20.5	1	518.3±33.2*	514.6±11.0*	214.5±32.9	174.3
4	108.1±32.6***	100.7±22.1*	61.0±18.5*	25.1	2	1010.6±86.5**	1021.6±19.0	571.0±8.0*	256.6
5	106.0±26.6	108.0±33.6	78.8±18.0	16.7	5	981.0±32.5	1006.8±13.5	576.0±7.8	271.3
	Adipose tissue								
C	41.2±4.4	38.4±10.9	34.6±7.6	3.5	C	411.2±26.4	352.2±16.4	241.0±7.4	86.2
3	113.1±5.3*	139.7±12.2*	63.0±8.8*	38.6	1	865.3±21.4*	550.0±19.7*	311.4±6.0*	277.8
4	160.0±8.0**	165.5±22.6**	80.0±7.9	47.7	2	1322.8±9.6	660.6±21.4	615.0±11.0	396.1
5	169.8±22.4	176.0±29.4	71.0±15.3	58.9	5	1287.0±24.5	790.4±29.0	598.0±19.8	355.5
	Egg yolk								
C	22.2±2.2	20.2±2.0	15.3±1.4	3.6	C	111.1±8.6	139.4±22.8	54.9±6.1	43.0
3	82.2±33.2**	97.5±26.5**	43.0±16.4*	27.8	1	198.0±27.5**	214.3±29.1*	114.0±3.4	88.2
4	148.0±20.4	153.3±40.1	50.0±22.6	58.0	2	220.0±31.5**	224.0±30.3**	103.0±2.2**	103.3
5	146.0±18.9	141.1±32.3	32.9±26.4	64.4	5	239.9±26.5	242.0±41.9	109.9±8.6	110.3
	Semen								
C	11.53±0.3	13.2±2.2	7.0±0.5	3.0	C	40.42±2.12	44.3±3.1	20.0±3.2	13.0
3	13.66±0.48	17.0±3.2*	16.7±2.3*	2.0	1	81.13±5.21*	54.5±5.3*	41.4±0.5	20.2
4	38.34±1.11**	36.0±6.5**	32.0±5.5**	3.0	2	81.00±4.22	132.1±8.8	38.5±11.5*	46.8
5	34.42±0.98	36.5±5.5	25.0±11.0	5.8	5	88.05±7.2	98.0±10.9	75.4±9.6	11.2

Gr=group.

[1] C, control group; 1–5, experimental groups. Birds from groups 1 and 2 received dietary Se at 0.3 and 0.5 mg/kg, respectively; birds from groups 3 and 4 received dietary vitamin E at 50 and 200 IU/kg, respectively; and birds from group 5 received 200 IU/kg of vitamin E and 0.5 mg/kg Se.

[2] SD, standard deviation.

Values are the mean±standard error of mean; $n \geq 5$ for each mean. Significance compared with group C: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 2. **Semen ejaculate volume and sperm count of Red Cornish broiler breeders of different ages fed on vitamin E- and Se-supplemented diets**

Group[1]	Ejaculate volume (μL)				Sperm count (10^9/ejaculate volume)			
	49 wk	53 wk	63 wk	SD[2]	49 wk	53 wk	63 wk	SD[2]
C	390±44	371±32	250±76	75.9	0.87±0.10	0.86±0.09	0.22±0.06	0.37
1	354±32	375±54	265±32	58.3	0.80±0.42	0.75±0.33	0.32±0.33	0.33
2	407±76	333±78	289±30	69.0	1.13±0.32*	1.05±0.40	0.39±0.62	0.48
3	524±23*	588±33*	345±55*	125.9	1.30±0.32*	1.15±0.03*	0.31±0.70*	0.53
4	619±42**	610±54**	449±87*	95.6	1.56±0.30*	1.57±0.13*	0.54±0.80*	0.59
5	605±32	604±40	460±7	83.4	1.52±0.03	1.36±0.15	0.54±0.89	0.52

[1] C, control group; 1–5, experimental groups. Birds from groups 1 and 2 received dietary Se at 0.3 and 0.5 mg/kg, respectively; birds from groups 3 and 4 received dietary vitamin E at 50 and 200 IU/kg, respectively; and birds from group 5 received dietary 200 IU/kg vitamin E and 0.5 mg/kg Se.
[2] SD, standard deviation.
Values are the mean±standard error of mean; $n=5$ for each mean. Significance compared with group C: * $P<0.05$; ** $P<0.01$.

Table 3. **Sperm motility and viability of Red Cornish broiler breeders of different ages fed on vitamin E- or Se-supplemented diets**

Group[1]	Motility (%)				Viability (%)			
	49 wk	53 wk	63 wk	SD[2]	49 wk	53 wk	63 wk	SD[2]
C	57.7±2.2	55.7±4.2	42.6±3.3	8.1	59.7±6.7	56.6±5.4	44.0±6.6	6.6
1	60.6±3.2	58.1±5.2	40.0±5.0	17.3	74.0±8.5	57.9±6.9	49.0±8.9	12.8
2	76.3±6.2*	63.6±4.2*	42.0±5.5	13.0	77.3±3.0*	66.6±6.3	40.5±6.5	19.0
3	68.2±4.1*	64.7±7.7*	58.0±7.4*	5.0	67.7±4.3*	66.6±8.4*	57.0±7.6	5.5
4	67.3±3.3*	73.8±6.7*	52.0±7.4*	10.8	83.0±3.8**	74.3±10.3**	52.1±8.5*	10.5
5	69.0±2.4	70.0±6.2	52.8±4.4	5.9	83.2±4.0	71.2±8.3	60.0±6.0	11.5

[1] C, control group; 1–5, experimental groups. Birds from groups 1 and 2 received dietary Se at 0.3 and 0.5 mg/kg, respectively; birds from groups 3 and 4 received dietary vitamin E at 50 and 200 IU/kg, respectively; and birds from group 5 received dietary 200 IU/kg vitamin E and 0.5 mg/kg Se.
[2] SD, standard deviation
Values are the mean±standard error of mean; $n=5$ for each mean. Significance compared with group C: * $P<0.05$; ** $P<0.01$.

0.001 for both). Se was found to accumulate in the adipose tissue and liver (values up to 395% higher in the supplemented groups than in the control group, $P<0.01$). Se was also transferred to the egg yolk and semen where its concentrations were 198% and 298% higher, respectively, in the supplemented groups than in the control group ($P<0.001$ for both). The 63-week-old birds significantly retained the ability to concentrate vitamin E and Se in the adipose tissue, liver, egg yolk, and semen. The combined supplementation with vitamin E and Se (group 5) had no effect on their accumulation in the tissues, egg yolk and semen. Correlation coefficients of vitamin E and Se contents with the age of birds ranged from −0.79 to −0.99 for the adipose tissue, egg yolk, and liver and from 0.66 to −0.93 for the semen.

Semen Traits

In comparison with the control group, groups 3 and 4 supplemented with vitamin E (Tables 2 and 3) showed a significant increase ($P<0.05$) in ejaculate volume, total sperm count, motility, and sperm viability. Semen traits in these 63-week-old roosters were significantly different from those of the control group roosters. The effect of Se supplementation on ejaculate volume was not significant. However, it did result in some (age-limited) improvements in sperm count, motility, and viability; these factors significantly improved only in 49-week-old roosters, whereas sperm motility improved in 49- and 53-week-old roosters but not in 63-week-old roosters. The correlation coefficients between semen trait and rooster age were negative (r values between −0.85 and −0.99).

Fertility

Egg fertilization rates (Table 4) improved by vitamin E- and Se-supplemented diets. The improvement was significant ($P<0.01$) in groups 2 and 4, including eggs from 63-week-old birds. Fertilization rates decreased with age in all groups, including the controls (r values between −0.72 and −0.99).

Hatching Rates

The hatching rates of fertilized eggs (Table 5) were not significantly higher in the supplemented groups than in controls, except in group 4 ($P<0.05$; mean of 73.3% in this group). The hatching rate of all eggs followed the profile of egg fertility, and was significantly higher in groups 2 (mean 53.3%), 3 (mean 52.6%), and 4 (mean 61.2%). Significant differences between the vitamin E- and Se-supplemented

Table 4. **Egg fertilizability in Red Cornish broiler breeders of different ages fed on vitamin E- and Se-supplemented diets**

Group[1]	49 wk		53 wk		63 wk		SD[2]
C	(136)	64.4±5.5	(133)	61.3±8.7	(106)	51.3±11.4	6.8
1	(139)	66.8±4.5	(133)	63.9±7.5	(108)	53.0±9.8	7.2
2	(138)	78.4±6.5*	(133)	78.8±6.6*	(111)	58.8±6.4*	11.4
3	(139)	56.0±2.5	(124)	60.7±7.9	(106)	50.5±4.5	5.1
4	(137)	70.2±3.2*	(129)	78.9±4.3*	(104)	57.7±8.8*	5.8
5	(148)	74.6±3.8*	(125)	76.6±7.9*	(109)	67.5±9.0*	4.7

[1] C, control group; 1−5, experimental groups. Birds from groups 1 and 2 received dietary Se at 0.3 and 0.5 mg/kg, respectively; birds from groups 3 and 4 received dietary vitamin E at 50 and 200 IU/kg, respectively; and birds from group 5 received dietary 200 IU/kg vitamin E and 0.5 mg/kg Se. The values represent the mean±standard error of mean of fertilized eggs (detected by candling on day 8 of incubation) in five subgroups of 4−6 hens each artificially inseminated by their roosters. Numbers in parenthesis indicate the number of incubated eggs, collected in the last 10 days before incubation.
[2] SD, standard deviation.
Significance compared with group C: * $P<0.05$.

Table 5. **Hatchability in Red Cornish broiler breeders of different ages fed on vitamin E- and Se-supplemented diets**

Group[2]	Fertilized eggs[1]				Total incubated eggs			
	49 wk	53 wk	63 wk	SD[2]	49 wk	53 wk	63 wk	SD[2]
C	64.7±3.3	62.0±5.3	60.0±5.5	2.3	52.7±2.0	49.4±1.3	38.8±1.3	6.8
1	64.3±3.0	59.9±4.3	60.5±7.5	2.1	49.8±3.2	54.6±2.1	45.6±1.9	4.1
2	59.3±5.2	60.1±4.5	60.6±7.6	0.7	57.8±3.7*	55.5±2.2*	46.6±2.9*	5.6
3	61.2±2.3	62.2±5.4	60.8±6.0	0.5	50.7±3.0*	60.2±2.0*	46.9±2.1*	6.8
4	74.0±1.9**	73.1±11.0*	69.8±10.9*	2.0	66.6±5.3**	64.5±1.9*	52.6±2.0*	7.2
5	69.0±3.2	67.6±6.2	73.2±9.4	3.0	68.5±3.4	65.6±2.9	53.0±1.0	7.9

[1] Fertilized eggs were detected by candling on day 8 of incubation.
[2] C, control group; 1−5, experimental groups. Birds from groups 1 and 2 received dietary Se at 0.3 and 0.5 mg/kg, respectively; birds from groups 3 and 4 received dietary vitamin E at 50 and 200 IU/kg, respectively; and birds from group 5 received dietary 200 IU/kg vitamin E and 0.5 mg/kg Se. The values represent the mean±standard error of mean; $n\geq5$ for each mean.
[2] SD, standard deviation.
Significance compared with group C: * $P<0.05$; ** $P<0.01$.

groups and control group were further maintained in 63-week-old birds. Both fertile and all egg hatching rates were moderately correlated with age (r between −0.50 and −0.99).

Discussion

An important finding of this study was the identification of elevated concentrations of vitamin E and Se in the adipose and liver tissues and semen of dietary supplemented older birds. The liver is able to store some fat-soluble vitamins, but the adipose tissue contains the largest stores (Landrier *et al*., 2012). According to our results, the older birds retained the ability to absorb high quantities of vitamin E and concentrate it in the liver and adipose tissues at levels similar to those reported by Jensen *et al*. (1990) in pig liver and by Surai (2000) in 1-day-old chicken liver. Consistently, Se was highly absorbed and its concentration was higher in the adipose tissue than in the liver and higher in younger than in older birds. This phenomenon was again proportional to Se supplementation level, as described by Shi *et al*. (2014) for Se accumulation in rooster testes. The capacity to absorb and accumulate Se in tissues was demonstrated by Surai (2000) in 1-day-old chicken, by Bañuelos and Mayland (2000) in lambs and cows, Toman *et al*. (2009) in rats, and Woods *et al*. (2020) in 35-day-old broilers. Here, we found that this capacity seemed to decrease with age, but 63-week-old birds still retained the ability to store vitamin E and Se in the liver and adipose tissues and then transfer them to the semen and egg yolk, respectively. Vitamin E and Se tissue stores provide a source needed to meet the daily requirements of these birds. Furthermore, vitamin E and Se are transferred to eggs and developing embryos (Surai, 2000), which reinforces the need to ensure they are at appropriate levels in the diet of older breeders. There is little information regarding the transfer of vitamin E to seminal plasma in older roosters (Surai *et al*., 1997; Danikowski *et al*., 2002). Semen transfer and concentration of vitamin E, which we have shown to still occur in older roosters, help improve the antioxidant capacity of the semen. This explains the improvement in the semen

traits of older groups fed vitamin E supplement. According to our results, Se is also transferred and concentrated in the seminal plasma of older roosters. Seminal plasma transfer and concentration of Se play a physiological role because Se-dependent glutathione peroxidase (Se-GSH-Px) is found in large quantities in the semen, as demonstrated in adult avian species (Surai *et al*., 1998), boars (Lasota *et al*., 2004), and bulls (Sławeta *et al*., 1988). Se transferred and accumulated in the egg yolk will be the sole source for the embryo until it hatches and feeds.

The decrease in vitamin E and Se concentrations in the semen of old roosters may be a characteristic of their aging process and could help explain the decrease in reproductive performance of older birds.

Another important finding of this study was the ability of vitamin E to significantly improve semen traits in these older RC roosters. Improvements in semen characteristics by dietary supplementation with vitamin E have been reported by Surai (2000) in 25-week-old Cobb broiler breeders, by Franchini *et al*. (2001) in young (18-week-old) Ross 308 male broiler breeders, and by Abedi *et al*. (2016) in Japanese quail, reflecting its role as a protector of cell membrane lipid bilayer (Sahin *et al*., 2002). The statistical significance of these effects on semen traits in 53- and 63-week-old roosters reveals two aspects: (1) the decline in semen traits of RC older roosters was related not only to age but also to the prooxidant–antioxidant system, which includes vitamin E and Se; and (2) application of such dietary supplements mediates beneficial biological effects on the semen, and improve the reproductive performance of young and older broiler breeder roosters. The levels of dietary supplementation should always be moderate (Audet *et al*., 2004; Biswas *et al*., 2009; Rengaraj and Hong, 2015).

Se protects spermatozoa against oxidative damage, and its deficiency causes a decrease in sperm concentration, sperm motility, and sperm capacity in animals including poultry species (Rengaraj and Hong, 2015). The stimulatory effect of Se supplements on the motility of spermatozoa in young roosters was especially noted, in contrast to the results reported by Maysa *et al*. (2009) in adult roosters, Moslemi and Tavanbakhsh (2011) in infertile men, and Audet *et al*. (2004) in boars under normal and intensive semen collection conditions. This effect of Se on sperm traits is not in agreement with the high Se concentrations found here in RC semen. The beneficial effect of Se on semen properties was very limited in 63-week-old roosters. Se effects are related to Se-GSH-Px (Lasota *et al*., 2004), which exhibits lower activity in 2-year-old Green-legged Partridge rooster semen (Partika *et al*., 2012) than in 25-week-old rooster semen (Surai *et al*., 1998). Further determination of Se-GSH-Px activity in older RC rooster semen could help us understand the limited effect of Se supplementation on semen traits in older RC roosters.

Considering that fecundity and hatchability are parameters influenced by both the rooster and hen, we did not separate these two partners in this experiment and administered vitamin E and Se to both male and female birds. Egg fertility (defined here as fertilization plus hatching rates) is a function of semen and egg traits and inherent hen fecundity (Morrow *et al*., 2002; Rengaraj and Hong, 2015; Negoiţă *et al*., 2017). The improvement in semen quality measures (sperm count, motility, and viability) described above for the vitamin E- and Se-supplemented groups, including the 63-week-old birds, indicates improvement in fertility of these birds. Direct involvement in the intermediary metabolism of the bird, protection of uterovaginal sperm depots, and transfer through the egg and developing embryo are other ways by which dietary vitamin E and Se can influence egg fertility (Surai, 2000; Rengaraj and Hong, 2015).

The hatching rate of fertile eggs provides data on embryonic mortality, whereas that of total eggs provides information on both semen traits and embryonic mortality (Fairchild *et al*., 2002; Iqbal *et al*., 2016). Hatching rates are influenced by a complex array of factors, including nutrition, bird and egg traits, incubation/incubator, and environment (King'ori, 2011). Higher deposits of vitamin E and Se in tissues resulted in higher overall egg hatching rates in groups 2, 3, and 4 in our study. Improvements in hatching rates in vitamin E-supplemented birds were reported by Lin *et al*. (2004) in 46-week-old Taiwan native chickens and by Abedi *et al*. (2016) in 35-week-old Japanese quail. The identification of vitamin E as a fat-soluble vitamin and its storage in association with body lipids suggest its involvement in various phases of lipid metabolism (Alfin-Slater and Morris, 1963; Negoiţă *et al*., 2017). Improved hatching rates were reported by Renema (2004) in 37-week-old broiler breeders supplemented with Se. Wilaison and Mori (2009) indicated that Se improved the hatching rates of Japanese quail and that a constant supply of dietary sodium selenate is essential to maintain cellular Se-GSH-Px activity. Higher hatching rates in older birds indicate the usefulness of applying vitamin E and Se supplements. We found that the hatching rates decreased with age in RC hens, but their weak direct correlation with age is related to different influencing factors, including egg fertility, embryonic mortality (Fairchild *et al*., 2002), and egg traits (Negoiţă *et al*., 2017).

The substantial decrease in vitamin E and Se concentrations in the egg yolk, semen and tissues between 53 and 63 weeks of age in control group may be indicative of the weakening of antioxidant systems as well as the dietary intake insufficiency during these ages. Vitamin E and Se concentrations in the adipose tissue, egg yolk, liver, and semen were significantly higher in the long-term supplemented groups than in the control groups. Moreover, increased antioxidant concentrations in the tissues of 53- and 63-week-old birds were associated with a significant increase in egg fertility.

In conclusion, vitamin E and Se dietary supplements accumulated in the adipose tissue, egg yolk, liver, and semen of older RC breeders and helped prolong their reproductive performance. The long-term supplementation improved semen traits and egg fertility both in younger and older RC breeders toward the end of their commercially productive life. Further determination of Se-GSH-Px activity in the semen of

older roosters may improve our understanding of the limited effects of Se supplements on semen traits in older RC roosters.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed equally to this work and are therefore all considered main authors.

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