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The Effects of Dietary L-Carnitine Supplementation on Semen Traits, Reproductive Parameters, and Testicular Histology of Japanese Quail Breeders

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Primary Audience: Animal Nutritionists, Feed Additive Suppliers, Researchers, Poultry Scientists

SUMMARY

The present study was conducted to determine the effects of supplemental dietary L-carnitine at different levels on semen traits, reproductive parameters, and testicular histology in male Japanese quail breeders. Forty-five 5-wk-old male Japanese quail breeders were fed the same basal diet that was supplemented with 0 (control), 250, or 500 mg of L-carnitine/kg of diet. There were no significant effects of dietary L-carnitine supplementation at different levels on BW, feed intake, testes weight, fertility rate, hatchability rate of set and fertile eggs, and malonaldehyde production (μg/mL of semen) of male Japanese quail breeders. However, the supplementation of dietary L-carnitine at levels of 250 or 500 mg/kg to a basal diet significantly increased sperm viability and decreased multinucleated giant cells per testes in mature male Japanese quail breeders. Additional studies are required to explore the antioxidant role that L-carnitine has in Japanese quail breeders.

Key words: L-carnitine, semen trait, fertility, hatchability, spermatozoa morphology, antioxidant, Japanese quail breeder

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DESCRIPTION OF PROBLEM

The scientific and popular literature contains many, often unsubstantiated, statements about the negative effects of animal breeding on performance. Selection for meat production in broiler chickens has often been linked with negative effects on reproductive traits. There are various data sets covering much of the last 30 yr to test whether selection and management of meat-producing chickens had a negative effect on fertility and hatchability. The analysis has indicated that chick output per broiler breeder female has shown a steady increase over the last 30 yr in the United Kingdom, Europe, and the United States [1]. The reproductive efficiency of heavy male poultry also needs to be improved [2]. Since the 19th century, many researchers have reported that lipids are a basic component of semen, contributing to the membrane structure of spermatozoa, the metabolism of the sperm cells, and their ability

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to capacitate and fertilize the female gamete [3]. In mammals, the lipid composition of sperm membranes plays an important role in the physicochemical modifications leading to fertilization [4]. In birds, the lipid composition of spermatozoa has an influence on fertility [5].

In many animal species, the major lipid components of spermatozoa are phospholipids, and they contain extremely high amounts of long chain polyunsaturated fatty acids (**PUFA**) [2, 3, 6, 7, 8]. The phospholipids of avian spermatozoa are characterized by very high proportions of long chain PUFA of the n-6 series, mainly arachidonic acid (C20:4n-6) and docosatetraenoic acid (C22:4n-6) from the major fatty acyl components [7]. In contrast, in mammalian spermatozoa, long chain PUFA of the n-3 series, in particular docosahexaenoic acid (C22:6n-3), are predominant [9, 10]. Although avian sperm cell membranes have lower amounts of PUFA than mammalian sperm cells, they have higher amounts of PUFA than other avian tissues and are therefore more susceptible to lipid peroxidation during in vitro handling and sperm storage, which is probably the primary cause of fertility dysfunction [7, 11, 12]. Reactive oxygen species (**ROS**) are important mediators of damage to sperm cell membranes. Exposure of cell membranes to ROS results in lipid peroxidation, causing membrane breakdown, decreased motility, abnormal morphology, and a lowered capacity for spermatocyte penetration [13, 14, 15].

The presence of high concentrations of long chain PUFA in the lipid structure of sperm cells requires efficient antioxidant systems to protect against peroxidative damage and sperm dysfunction [12, 16]. To counteract the toxic effects of ROS and to prevent significant free radical injury, spermatozoa and seminal plasma have an array of antioxidant mechanisms [15, 17]. Antioxidant systems of cells include some natural antioxidants (vitamin E, ascorbic acid, carotenoids, glutathione, ubiquinones, uric acid, and carnitine) and antioxidant enzymes [catalase, superoxide dismutase (**SOD**), glutathione peroxidase, and glutathione reductase] [7, 15, 18]. Lipid peroxidation overcomes the antioxidant defense mechanisms utilized by sperm cells when ROS are constituted [19], and lipid peroxides accumulate immediately in the plasma membrane. Thus, the level of sperm lipid peroxidation can be used as a biochemical index of semen quality [19, 20]. Some natural antioxidants such as vitamins E, A, and C and glutathione are able to chain-break ROS-induced lipid peroxidation in the seminal plasma or in the sperm membrane [21, 22]. Carnitine is an antioxidant that protects sperm membranes from toxic O_2 metabolites [20]. Acetylation of carnitine, especially acetylcarnitine complex, seems to exert a repairing effect by the removal of elevated intracellular toxic acetyl-coenzyme A or the replacement of fatty acid in membranes [23]. In addition, high concentrations of carnitine are present in both seminal plasma and spermatozoa, where carnitine functions to reduce the availability of lipids for peroxidation by transporting PUFA into the mitochondria for β oxidation to generate ATP for sperm motility [20, 22, 24, 25]. The concentration of carnitine and acetylcarnitine increases continuously during the epididymal passage when sperm motility and fertilizing ability develop [26]. Carnitine and acetylcarnitine act as the first and second scavenger agent to remove acetyl-CoA from the cell and may replace PUFA in the seminal plasma membrane phospholipids [23]. Previous studies have shown that seminal free L-carnitine concentration correlates with sperm count and motility in humans [27]. Carnitine increases the activity and levels of antioxidant enzymes like SOD in aging rats [18]. Carnitine can also work together with SOD to preserve the lipid membrane surrounding sperm, thus reducing lipid peroxidation and protecting cells from peroxidation damage [28].

A dietary supplement of 250 to 500 ppm L-carnitine may be recommended to achieve significant improvements in sperm count and quality for roosters [29]. In addition, studies have shown that the higher the inclusion rate of dietary L-carnitine, the higher the concentration in the yolk [30]. This L-carnitine in the yolk might be beneficial for both the developing embryo and the hatched day-old chick due to increased energy production with supplemental L-carnitine, because both the embryo and the day-old chick rely to a great extent on fat as an energy source. As a result, broiler chicks from L-carnitine-enriched eggs show a greater vitality and better feed conversion [31]. Various feeding studies have indicated that supplementary dietary L-carnitine has helped birds to achieve the targeted egg weight earlier at the beginning of the egg production

cycle [32]. Because of decreased lipid peroxidases when aged rats have been supplemented with L-carnitine, it was hypothesized that breeder birds and roosters fed diets supplemented with L-carnitine would show an improvement in semen traits and fertility parameters by preventing lipid peroxidation of sperm membranes. Thus, the objective of the present study was to determine the effects of different levels of dietary L-carnitine supplementation on semen traits, reproductive parameters, and testicular histology in male Japanese quail breeders (*Coturnix coturnix japonica*).

MATERIALS AND METHODS

Experimental Design

One hundred fifty unsexed 1-d-old Japanese quail chicks (*Coturnix coturnix japonica*) obtained from a commercial hatchery were kept in individual wire cages equipped with nipple drinkers under uniform environmental conditions from hatch until 5 wk of age. They were fed a starter-grower diet (24% CP and 2,900 kcal of ME/kg) from hatch to 5 wk of age. The Japanese quail chicks were allowed drinking water ad libitum and were provided continuous lighting exposure to 5 wk of age. At 5 wk of age, 45 male Japanese quails were randomly allotted to 3 treatment groups of similar mean weight each of which included 15 male Japanese quail breeders from 5 to 20 wk of age. These quails were offered the same basal diet that was supplemented with 0 (control), 250, or 500 mg of L-carnitine/kg of diet in the form of Carniking [33]. The L-carnitine was incorporated into diets in the place of corn. The basal diet contained 8.68 mg of L-carnitine/kg. The L-carnitine content of this basal diet was calculated based on the L-carnitine content in the ingredients used [34]. The composition and the calculated nutrient content of the quail breeder basal diet are presented in Table 1. Before experimental diet formulation, feed ingredients were analyzed for their CP, CF, crude fat, starch, and total sugar according to the methods of the Association of Official Analytical Chemists [35]. Metabolizable energy of feed ingredients was calculated based on analyzed values of feedstuffs [36]. The experimental diets were formulated to meet minimum nutrient requirements of Japanese quail breeder, as established by the NRC [37]. All experimental groups received a quail breeder diet (20.09% CP and 2,907 kcal of ME/kg) from 5 to 20 wk of age. The experimental diets in mash form and drinking water were provided ad libitum. Quails were kept at 22 to 24°C and subjected to a 14L:10D photoperiod until the end of the experiment.

Table 1. Composition (%) and analyzed nutrient content of basal diet

Ingredients	Breeder from 5 to 20 wk
Corn	43.06
Soybean meal	17.50
Wheat	15.00
Full-fat soybean	12.00
Cottonseed meal	3.00
Fish meal	2.00
Limestone	5.40
Dicalcium phosphate	1.14
Salt	0.25
Vitamin premix[1]	0.25
Trace mineral premix[2]	0.10
L-Lys	0.19
DL-Met	0.11
Calculated composition	
ME (kcal/kg)	2,907
CP (%)	20.09
CF (%)	3.79
EE (%)	4.78
Ca (%)	2.52
Available P (%)	0.35
Met (%)	0.45
Met + cystine (%)	0.72
Lys (%)	1.00
L-carnitine (mg/kg)	8.68
Analyzed composition	
CP (%)	20.11
CF (%)	3.82
EE (%)	4.65

[1]Vitamin premix provided the following per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 2,000 IU; vitamin E, 35 mg; vitamin K_3, 4 mg; vitamin B_1, 3 mg; vitamin B_2, 7 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0.015 mg; niacin, 20 mg; Ca-D-pantothenate, 10 mg; folic acid, 1 mg; D-biotin, 0.045 mg; choline chloride, 125 mg; canthaxanthin, 2.5 mg; and apocarotenoic acid ester, 0.5 mg.
[2]Trace mineral premix provided the following per kilogram of diet: Mn, 80 mg; Fe, 60 mg; Zn, 60 mg; Cu, 5 mg; Co, 0.20 mg; I, 1 mg; and Se, 0.15 mg.

Data Collection

The growth performance of male Japanese quail breeders was evaluated by recording BW and feed intake. Individual BW of male quail breeders were recorded weekly from 5 to 20 wk of

Table 2. The effects of dietary L-carnitine supplementation on BW (g) of male Japanese quail breeders

	Levels of L-carnitine added (mg/kg)				
Age (wk)	0	250	500	SEM	*P*-value
5	199	190	205	4.07	0.320
6	200	192	210	4.29	0.234
7	202	192	209	4.30	0.284
8	206	195	211	4.22	0.279
9	208	196	213	4.15	0.214
10	211	197	214	4.02	0.190
11	209	198	209	4.02	0.479
12	209	196	212	4.00	0.225
13	203	193	213	3.85	0.096
14	207	196	214	4.03	0.172
15	211	195	212	4.17	0.163
16	202	194	210	3.60	0.224
17	200	194	211	3.78	0.167
18	203	193	209	3.79	0.210
19	195	193	211	3.76	0.090
20	194	189	200	3.58	0.478

age. However, feed intake of male quail breeders were recorded at 20 wk of age. Individual plastic feed troughs were utilized to measure feed intake per quail.

For sperm fertilizing ability tests, a total of 45 females and 45 males at 5 wk of age were divided into 3 groups, each of which included 15 female and 15 male Japanese quails, and were kept in individual cages throughout the experiment. Females were naturally inseminated. They were separated at 19 wk of age, after which, 1 male and 1 female were housed together per pen until 19 wk of age. Eggs were collected daily for 7 d at 10, 13, 16, and 19 wk of age to determine fertility and hatchability as fertilizing ability of semen. Eggs were stored at 10 to 15°C and 75% RH, set weekly in multistage incubators [38], and incubated at 37.5°C and 65 to 70% RH. All eggs were transferred into hatching baskets at 14 d of incubation and incubated up to hatch at 37.1°C and 80% RH to determine the hatchability results. After hatching, remaining eggs were opened, and the numbers of those containing dead and no embryos were recorded. Only those containing no embryos were classified as infertile.

Pooled semen of 7 male Japanese quail from each group was routinely collected twice a week by male stimulation by female method and by the dorso-abdominal massage technique when males were 20 wk of age. Female quails used for male stimulation were sexually matured (in a laying phase), characterized by quiet temperament, and displayed the tolerance reflex enabling a male to ascent on a female quickly. Care was taken to avoid any contamination of semen with cloacal products. Yellow semen samples and semen contaminated with blood, urine, or feces were also systematically discarded to avoid the deleterious semen products described by Hess and Thurston [39].

For sperm viability, expressed as percentage of dead sperm, number of viable spermatozoa in total sperm, was determined using the eosin B staining method as described by Ozkoca [40]. The staining solution was prepared by adding 2 g of eosin B stain and 3 g of sodium citrate into distilled water. The solution was filtered with a paper filter before being used. The staining was performed with 1 drop of fresh semen into 2 drops of staining solution on a microscope slide. Using another slide, a smear was made and allowed to dry. Unstained (intact) and red-colored (with damaged membranes) spermatozoa were counted as a counterstain. Dead spermatozoa retained more stain and appeared dark, whereas the viable ones appeared clear. Sperm viability was defined as the percentage of intact cells at 20 wk of age.

At 20 wk of age, the semen samples of 5 male Japanese quails in each experimental group were pooled at 2100 h by the abdominal massage technique to determine lipid peroxidation of the semen; consequently, 3 replications were obtained per experimental group [41]. The collection vial was sealed to minimize possible contamination and evaporation. Care was taken to avoid any contamination of semen with cloacal products and water. Lipid peroxidation of the semen was determined by measuring malonaldehyde (**MAL**), which is the primary stable by-product of lipid peroxidation using the procedure of Neuman et al. [20]. The pooled semen samples were diluted (1:2) by using a 0.90% NaCl (pH 6.5, 284 mOsm) solution according to a modified procedure of Cecil and Bakst [12], and 50-μL aliquots of this mixture were pipetted into separate 20-mL-capacity glass vials, along with 125 μL each of 1-m*M* sodium L-ascorbate and 0.2 m*M* ferrous sulfate. Sodium L-ascorbate and ferrous sulfate were added to each sample to promote

Table 3. The effects of dietary L-carnitine supplementation on BW, feed intake, testes weight, dead sperm, and multinucleated giant cells per testes of male Japanese quail breeders

	Levels of L-carnitine added (mg/kg)				
Parameters	0	250	500	SEM	*P*-value
Live BW[1] (g)	194	189	200	3.58	0.478
Feed intake[1] (g/quail per d)	20	20	20	0.32	0.927
Absolute testes weight[2] (g)	6	5	6	0.22	0.454
Relative testes weight[2] (g/g)	0.030	0.028	0.030	0.0009	0.734
Dead sperm[3] (%)	18[a]	13[b]	9[b]	1.22	0.002
Multinucleated giant cells per testes[2]	4[a]	2[b]	1[c]	0.21	0.000

[a–c]Means within the same row with no common superscripts differ significantly ($P < 0.05$).
[1]Values represent the means of 15 observations (15 birds/dietary treatment) at 20 wk.
[2]Values represent the means of 15 birds per dietary treatment.
[3]Values represent the means of 30 observations at 20 wk of feeding the diets (15 birds/dietary treatment × twice per wk).

peroxidation [14]. Each vial was loosely capped to prevent evaporation but also to further circulate O_2. Samples were incubated at 37°C on a shaker at 150 rpm for 4 h. After incubation, samples were immediately placed on ice, and 150 μL of 40% trichloroacetic acid; 150 μL of PBS, pH 7.0; and 500 μL of 1% 1,1,3,3 TBA [2-TBA (4,6-dihydropyrimidine-2-thiol)] [42] diluted in PBS were added to each sample. Samples were mixed, capped, and incubated at 80°C for 20 min. Following the second incubation, 1 mL of deionized water was added to each vial. Samples were transferred to 2.0-mL microcentrifuge tubes and centrifuged at 1,500 × *g* for 15 min at 25°C. The resulting supernatant was measured with a spectrophotometer at 520 nm to determine MAL production. The MAL level of the sample was calculated by comparing it with the optical density produced by MAL standard [43].

At 20 wk of age, all 15 male Japanese quails from each experimental group were euthanized, and testes were excised for histological and mor-

Table 4. The effects of dietary L-carnitine supplementation on fertility and hatchability rate of set and fertile eggs

	Levels of L-carnitine added (mg/kg)				
Reproduction parameters[1]	0	250	500	SEM	*P*-value
10 wk					
Fertility (%)	93	96	98	2.09	0.610
Hatchability (%)					
Set eggs	84	85	91	3.39	0.643
Fertile eggs	82	91	95	3.18	0.256
13 wk					
Fertility (%)	81	92	94	3.03	0.166
Hatchability (%)					
Set eggs	80	88	90	2.97	0.316
Fertile eggs	96	96	98	1.03	0.680
16 wk					
Fertility (%)	86	89	90	2.78	0.896
Hatchability (%)					
Set eggs	76	81	86	3.41	0.511
Fertile eggs	88	89	96	2.29	0.287
19 wk					
Fertility (%)	75	83	88	3.69	0.364
Hatchability (%)					
Set eggs	69	72	76	4.23	0.810
Fertile eggs	80	91	92	2.92	0.167

[1]Values represent the means of 60 observations of wk 10, 13, 16, and 19 of feeding the diets (15 female birds/dietary treatment × 4 wk).

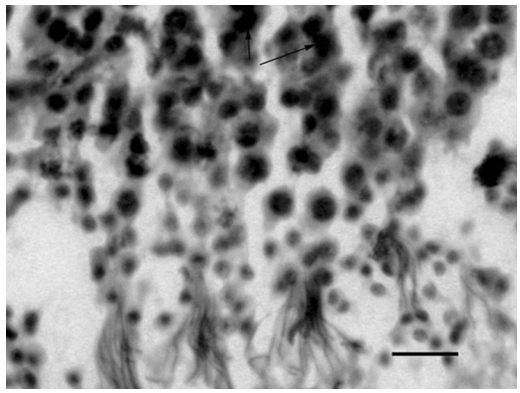

Figure 1. Testis from a Japanese quail breeder fed a control diet. Magnification is 50×. Bar scale is 15 μm. Arrows indicate multinucleated giant cells.

phological analyses. The right and left testes of each quail were weighed. Testes weights were expressed relative to BW. The left testis of each quail was cut into serial cross-sections 5 mm in thickness, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for routine histological and morphological examination [44, 45]. Histological examination of 5 preparations of the left testis of each quail was conducted blindly under light microscope.

Statistical Analysis

Semen traits, feed intake, BW, fertility rate, hatchability rate of set and fertile eggs, and the quantitative detection of multinucleated giant cells and testes weights were analyzed by using SPSSWIN [46] statistical program with a 1-way ANOVA. Significant differences among treatment means were determined with Duncan's multiple range test [47]. Threshold for significance was $P < 0.05$.

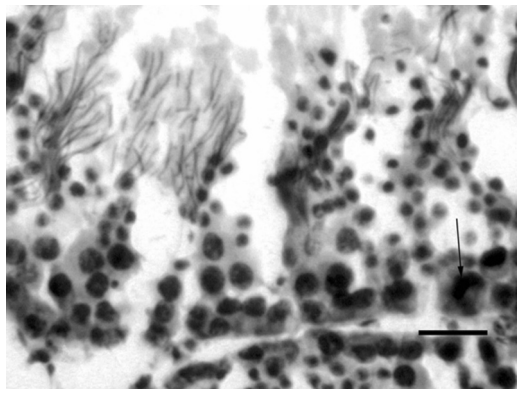

Figure 2. Testis from a Japanese quail breeder fed a diet supplemented with 250 ppm L-carnitine. Magnification is 50×. Bar scale is 15 μm. Arrows indicate multinucleated giant cells.

RESULTS AND DISCUSSION

The supplementation of dietary L-carnitine at levels of 250 or 500 mg/kg to a basal diet did not influence ($P > 0.05$) BW in mature male Japanese quails (Table 2). Supplementation of dietary L-carnitine at levels of 0, 250, or 500 mg/kg of diet did not affect ($P > 0.05$) BW, feed intake, or testes weight of mature male Japanese quail (Table 3). These results are in agreement with those reported by Neuman et al. [20], in which dietary supplemental carnitine (500 mg/kg) did not influence BW, feed consumption, or testes weight of mature White Leghorns. However, the supplementation of dietary L-carnitine at levels of 250 or 500 mg/kg to a basal diet significantly increased sperm viability (% dead sperm) in mature male Japanese quails.

Lipids are a basic component of semen, contributing to the membrane structure of spermatozoa, the metabolism of the sperm cells, and their ability to fertilize the female gamete. There is considerable evidence to indicate that the lipid composition of the sperm membrane is a major determinant of the cold sensitivity, motility, and overall viability of spermatozoa [3]. The presence of the high concentrations of long chain PUFA of the n-6 series in avian spermatozoa increases their susceptibility (vulnerability) to lipid peroxidation and limits the viability of chicken and turkey spermatozoa. Lipid peroxidation plays a key role in the aging of spermatozoa by shorting its lifetime in vivo as well as during the in vitro conservation of sperm for artificial insemination. The peroxidation process comes with extensive structural alterations, especially in the acrosomal section of the spermatozoa, fast and irreversible loss of motility, extensive metabolic changes, and high rate of leakage of intracellular spermatic constituents. Semen or spermatozoa is normally equipped with diverse enzymatic (glutathione peroxidase, SOD) protection against lipid peroxidation in birds. However, an increase in lipid peroxidation with aging may be correlated with

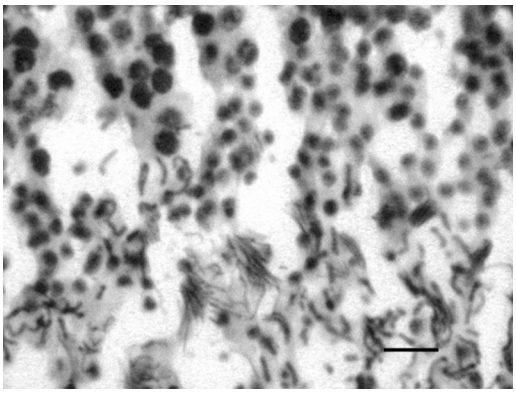

Figure 3. Testis from a Japanese quail breeder fed a diet supplemented with 500 ppm L-carnitine. Magnification is 50×. Bar scale is 15 μm. Note the absence of multinucleated giant cells.

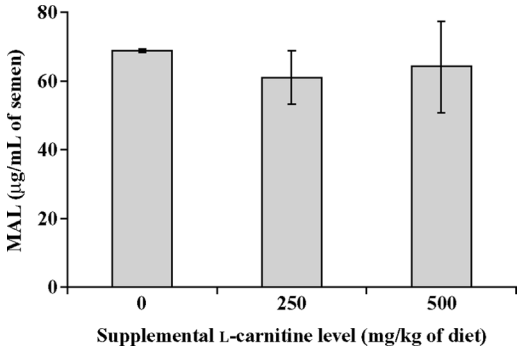

Figure 4. The effect of feeding the diet supplemented with 0, 250, or 500 mg of L-carnitine/kg on sperm malonaldehyde (MAL) production when MAL was expressed per milliliter of semen.

decreased antioxidant enzyme status and activities. L-Carnitine supplementation increased overall antioxidant enzyme activities as a function of the duration of treatment, thus decreasing the levels of free radicals available for lipid peroxidation. As a result of the mode of action of L-carnitine, dietary supplementation of 250 or 500 mg/kg of L-carnitine may increase the viability of the mature male Japanese quail breeder spermatozoa. As indicated in Table 3, quails fed carnitine had significantly ($P < 0.05$) fewer multinucleated giant cells per testes than quail fed the control diet (Figure 1, 2, and 3). The presence of the multinucleated giant cells is considered to be a sign of abnormalities in the divisional mechanism of primary spermatocytes. Testicular multinucleated giant cells are described as a degenerative syndrome resulting presumably from the inability of tetraploid primary spermatocytes to complete meiotic division; thus, maturation arrests at the spermatid stage of development [20, 48]. Multinucleated giant cells consist primarily of aggregates of degenerated spermatocytes and spermatids and are often sloughed into the lumen of seminiferous tubules [20, 45].

Supplemental dietary carnitine did not significantly affect the fertility and hatchability rate of set and fertile eggs (Table 4). In the present study, the fertility rate showed numerically a negative correlation with the age of the male Japanese quail breeders but was positively correlated with dietary L-carnitine supplementation.

When the level of lipid peroxidation was expressed as micrograms of MAL per milliliter of semen, no differences among dietary treatments were observed (Figure 4). Sperm counts were not done in the current study, so the MAL assay was not adjusted for sperm numbers. If the data were expressed as micrograms of MAL per billion sperm cells, birds fed diets supplemented with L-carnitine may have produced significantly lower amounts of MAL as compared with birds fed the control diet as reported by Neuman et al. [20], who indicated that no differences were observed between birds fed a diet supplemented with 500 mg of L-carnitine/kg of diet or a control diet when the data were expressed as micrograms of MAL/mL of semen. Additionally, samples were not adjusted for sperm numbers.

CONCLUSIONS AND APPLICATIONS

1. Dietary L-carnitine supplementation at 0, 250, or 500 mg/kg did not significantly affect BW, feed intake, testes weight, fertility rate, hatchability rate of set and fertile eggs, and MAL production (μg/mL of semen) of male Japanese quail breeders. However, quails fed diets supplemented with 250 or 500 mg of L-carnitine/kg of diet had significantly fewer multinucleated giant cells per testes and percentage of dead sperm than quails that received a control diet.

2. To identify the key role of L-carnitine as an antioxidant, especially in regard to effects on semen traits and reproduction parameters of Japanese quail breeders, the responses to supplemental dietary L-carnitine of Japanese quail fed diets with different dietary fat source rich in C18:2n-6 or C22:6n-3 could be further investigated.
3. Additional studies are required to explore the antioxidant role that L-carnitine has in Japanese quail breeders.

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