# Amelioration effects of n-3, n-6 sources of fatty acids and rosemary leaves powder on the semen parameters, reproductive hormones, and fatty acid analysis of sperm in aged Ross broiler breeder roosters

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**ABSTRACT** This study was conducted to evaluate the effects of dietary polyunsaturated fatty acids (PUFA) sources and rosemary leaves powder (RLP) on the semen quality, fatty acid analysis, and some reproductive hormones of senescent broiler breeder roosters. Thirty-five 45-wk-old Ross breeder roosters were randomly divided into 7 groups (5 birds/group), and received following treatments including control group (basal diet), fish oil (2%), corn oil (2%), an equal (50:50%) proportion of fish oil and corn oil (50:50%), fish oil (2%) with 5 g/kg capsulated RLP, corn oil (2%) with 5 g/kg capsulated RLP, and an equal (50:50) proportion of fish oil and corn oil (50:50%) with 5 g/kg capsulated RLP of diet for 60 D, during which time their seminal characteristics were evaluated every 20 D. At the end of the trial (on day 60), semen samples were tested for determination of sperm fatty acid analysis, lipid peroxidation, and some reproductive hormones. Results showed that feeding fish oil and fish/corn oil with RLP was associated with an increase in docosahexaenoic acid (C22:6n-3) and docosatetraenoic acid (C22:4n-6) in sperm. The fish oil diet increased the proportion of n-3 fatty acids in sperm, and as a consequence, the (n-6)/(n-3) ratio also decreased ($P < 0.05$). RLP (5 g/kg) to the fish and fish/corn-oil (50:50%)-based diet resulted in improvement in sperm concentration, total motility (%), sperm progressive motility (%), membrane integrity, and viability in terms 0 to 60 day trial ($P < 0.05$). Diets and age interacted to positively affect sperm concentration and sperm membrane integrity. Also this herbal antioxidant decreased the seminal content of malondialdehyde (MDA) significantly ($P < 0.05$). Testosterone and LH serum levels of reproductive hormones were significantly higher in fish and fish/corn-oil with RPL (50:50%)-based diet than other groups ($P < 0.05$). It can be concluded that RLP as an antioxidant could remarkably improve the effects of n-3 and n-3/n-6 PUFA on sperm characteristics, seminal MDA, and hormones levels in aged breeder roosters. The susceptibility of semen to lipid peroxidation was increased in chickens fed without RLP. Future studies are needed to disclose the causal mechanisms involved.

**Key words:** n-3: n-6 ratios, rosemary, rooster sperm, reproductive hormones, semen quality

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## INTRODUCTION

The genetic selection of broiler breeder lines (*Gallus gallus domesticus*) for the production of meat is primarily focused on producing broiler chickens that can obtain high slaughter weights with a reduced feed conversion ratio. However, these production successes of the final product (the broiler) sometimes are contrasted with the reproductive rates of the breeding stock as seen in a decline in fertility in the last weeks of life (Kirk et al., 1980; Vizcarra et al., 2010). Dietary manipulation by fatty acids (**FA**) has been suggested as a method of enhancing cockerel semen quality due to the strong relationship that exists between nutrition and overall flock fertility (Safari et al., 2018). Lipids are known to be constituents of avian semen and are also involved in the sperm biological activities (Cerolini et al., 2003). Dietary lipid or FA sources have then been thought to affect cockerel sperm composition and functionality in different ways (Bongalhardo et al., 2009), even when deposited proportionately in the sperm (Cerolini et al., 2003). The omega-6 (n-6) type of FAs (e.g., docosatetraenoic acid [**DTA**]) has been shown to be the most prevalent in cockerel sperm (Cerolini et al., 1997), as opposed to the omega-3 (n-3) type of FAs that are predominant in bull sperm (Kelso et al., 1997a). As already reported in men (Nissen and Kreysel, 1983), DTA is positively related with sperm motility in chicken also (Cerolini et al., 2002), even if present in low proportion. Dietary n-3 polyunsaturated fatty acids (**PUFA**) modifies spermatozoa FA profile (Castellano et al., 2010), and promotes

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the susceptibilities of membrane PUFAs to lipid peroxidation (Cerolini et al., 2000), resulting in changes of membrane integrity and function, and subsequently the changes of sperm motility and fertility (Yeste et al., 2011). These results imply that dietary n-3 may help to improve sperm functions, especially motility, by modifying membrane properties as well as decreasing spermatozoa lipid peroxidation.

In this regard, dietary manipulation of omega 3 FAs is an efficient strategy to change the FA profile and increase the concentration of n-3 PUFAs in the phospholipid bilayer of sperm (Blesbois et al., 1997b; Kelso et al., 1997b,c) and the turkey (Blesbois et al., 2004). The increased proportion of 22:5n-3 and 22:6n-3 in sperm lipid obtained by dietary linseed (Kelso et al., 1997c) and fish oils (Blesbois et al., 1997b) is associated with higher fertilizing ability of 39- and 47-wk-old cockerels. The same positive effects of n-3 sperm PUFAs and fertility have also been found in turkey breeders following fish oil supplementation from 48 to 58 wk of age (Blesbois et al., 2004). Safari et al. (2018) reported that n-3: n-6 FA ratios (0.16) supplemented with 200 mg vitamin E improved total sperm motility ($P < 0.05$) than the 0.09 and 0.23 ratios, both with and without vitE. Progressive motility, membrane functionality, and viability were significantly improved in the 0.16 and 0.23 ratios supplemented with vitE ($P < 0.05$). However, unsaturated fatty acids (**SFA**) readily undergo oxidation at the carbon atoms adjacent to the double bond to form hydroperoxidase (McDonald et al., 2011). Therefore, despite reports that long-chain (**LC**) n-3 FAs (e.g., docosahaexanoic acid [**DHA**]) incorporated in fish oil improve sperm progressive motility in cockerels (Cerolini et al., 2006), its susceptibility to peroxidation is a concern (Ollero and Alvarez, 2003). Therefore, it is necessary that when the FA sources are used as supplements of diet, an antioxidant compound is also applied with the FA sources. Rosemary (*Rosmarinus officinalis L.*) contains biologically active substances such as diterpenes, triterpenes, flavonoids, and polyphenols, as well as monoterpenes and sesquiterpenes (Hammerstedt, 1993). Its antioxidant capacity is mainly related to the presence of components like carnosol, rosmanol, isorosmanol, epirosmanol, carnosic, and rosmarinic acids (Wellwood and Cole, 2004). Carnosic and rosmarinic acids are 2 major phenolic constituents present in rosemary leaves and their antioxidant properties have been previously reported (Cuvelier et al., 1996). Further, the antioxidant potential of pure carnosic acid is several times greater than butylated hydroxytoluene and butylated hydroxyanisole (Richheimer et al., 1996). Carnosic acid and carnosol have been suggested to account for over 90% of the antioxidant properties of rosemary extract (Aruoma et al., 1992), although this has not yet been systematically verified. This high contribution of carnosic acid to antioxidative response of rosemary extract is also probably attributed to the great abundance of carnosic acid as compared to other rosemary phenolic diterpenes.

Supplementing boar diet with rosemary oil extract containing 5.5% rosmarinic has resulted in an increase of sperm concentration, viability, and a significant reduction of return to estrus in sows inseminated with semen from treated males (Superchi et al., 2005). In addition, rosemary oil alleviated heat stress induced testicular lipid peroxidation and decreased number of spermatogenic cells in growing Japanese quail (Turk et al., 2016). In vitro inclusion of rosemary aqueous extract has also been successful in improving post-thawed sperm total and forward motility, viability, plasma membrane functionality, and malondialdehyde (**MDA**) concentration (Motlagh et al., 2014). It seems that supplementation of fish oil and rosemary leaves powder (**RLP**) in the diet of aged roosters might be beneficial for fertility. Therefore, the present study was designed to evaluate the semen parameters, sperm FA profile, and reproductive hormones in broiler breeder roosters fed different oil sources with RLP.

## MATERIALS AND METHODS

### Chemicals and Rosemary

All chemicals used in this study were obtained from Sigma Co. (St. Louis, MO) and Merck (Darmstadt, Germany) unless otherwise indicated. Approval for the present study was given by the Research Ethics Committees of Tarbiat Modares University. Fresh rosemary leaves were first collected, cleaned, sliced, and dried in the shade. Dried rosemary leaves were then milled (Moulinex/A320, Indonesia) and stored (22 to 25°C) in airtight plastic bags away from sunlight until use. The amount of carnosic acid as the main antioxidant compound of rosemary is 7% by weight of dried leaves (7% on weight basis of air-dried leaves).

### Experimental Birds, Housing, and Diet

Thirty-five 45-wk-old Ross 308 broiler breeder roosters were randomly divided into 7 groups (n = 5 birds/group), individually caged (60 × 50 × 75 cm), and kept under similar management conditions (a 15 L: 9D light schedule and 21°C ambient temperature). From 43 wk of age, all broiler breeder roosters were trained for semen collection according to the "massage" technique (Cole and Cupps, 1977). Body weight of broiler breeder roosters is shown in Table 2.

Treatment groups included control group (basal diet), fish oil (2%), corn oil (2%), an equal (50:50%) proportion of fish oil and corn oil (50:50%), fish oil(2%) with 5 g/kg capsulated RLP, corn oil (2%) with 5 g/kg capsulated RLP, an equal (50:50) proportion of fish oil and corn oil (50:50%) with 5 g/kg capsulated RLP of diet for 60 D, during which time their seminal characteristics were evaluated every 20 D. The basal diet in all experimental groups was a standard commercial mash for Ross broiler breeder roosters (Table 1). Components and consumption of the diet were based on instructions from the Ross 308 parent stock catalog, and

**Table 1.** Ingredients and the chemical composition of diets fed to broiler breeder roosters (DM basis).

| Ingredient (%) | Control diet (%) | Fish oil (2%) | Corn oil (2%) | Fish: corn oil (50%:50%) |
|---|---|---|---|---|
| Corn | 66.32 | 59.10 | 59.27 | 59.19 |
| Soybean meal (44%) | 8.50 | 7.67 | 7.72 | 7.69 |
| Wheat bran | 21.94 | 28.20 | 27.98 | 28.09 |
| Dicalcium phosphate | 1.00 | 0.92 | 0.92 | 0.92 |
| $CaCO_3$ | 1.11 | 1.14 | 1.14 | 1.14 |
| Sodium bicarbonate | 0.38 | 0.16 | 0.16 | 0.16 |
| Sodium chloride | 0.20 | 0.28 | 0.28 | 0.28 |
| Vitamin premix[1] | 0.25 | 0.25 | 0.25 | 0.25 |
| Trace-mineral premix | 0.25 | 0.25 | 0.25 | 0.25 |
| L-lysin HCl | 0.02 | 0 | 0 | 0 |
| Corn oil | 0 | 0 | 2 | 1 |
| Fish oil | 0 | 2 | 0 | 1 |
| DL-Met | 0.03 | 0.03 | 0.03 | 0.03 |
| Total | 100 | 100 | 100 | 100 |
| Composition | | | | |
| ME (kcal/kg) | 2,700 | 2,700 | 2,700 | 2,700 |
| CP (%) | 12 | 12 | 12 | 12 |
| Ca (%) | 0.7 | 0.7 | 0.7 | 0.7 |
| Avail.Phos (%) | 0.35 | 0.35 | 0.35 | 0.35 |
| Lysin (SID) (%) | 0.44 | 0.44 | 0.44 | 0.44 |
| Met+Cys (SID) (%) | 0.42 | 0.42 | 0.42 | 0.42 |
| Threonine (SID) (%) | 0.37 | 0.36 | 0.36 | 0.37 |

[1]The birds received diets containing 2% corn oil, 2% fish oil, 50:50% corn and fish oil, with 5 g/kg Rosemary powder leaves (RPL) for 60 D (45 to 53 wk of age). Supplied per kg diet: vitamin A, 12,000 IU; vitamin D3, 3,500 IU; niacin, 50 mg; vitamin E, 100 IU; vitamin K3, 5 mg; riboflavin, 12 mg; thiamin, 3.0 mg; D-pantothenic acid, 13 mg; folic acid, 2 mg; pyridoxine, 6 mg; vitamin B12, 0.03 mg, and biotin, 0.66 mg.c Supplied per kg diet: Fe ($FeSO_4 \cdot H_2O$), 50 mg; Mn ($MnSO_4 \cdot H_2O$), 120 mg; Zn (ZnO), 110 mg; Cu ($CuSO_4 \cdot 5H_2O$),10 mg; iodine (KI), 2 mg; and Se ($Na_2SeO_3$), 0.3 mg.

all diets were balanced to be isoenergetic and isonitrogenous. Ad libitum water was provided for roosters. Five grams of RLP per kg of diet was encapsulated to ensure that roosters received all 5 g, and the amount of rosemary powder was fed on a simple proportion and daily feed intake. Semen samples were collected from the roosters on days 0, 20, 40, and 60 of the experiment by the abdominal massage method for analysis (Burrows and Quinn, 1937). Hormonal analysis of the roosters was performed at the first and last days of the experiment (days 0 and 60). Lipid peroxidation and FA analysis of sperm were performed at the end of experiment (day 60). The FA compositions of the oils and diets used in this study are presented in Tables 3 and 4, respectively.

**Table 3.** Fatty acids composition (g per 100 g of fatty acids) of fish oil and corn oil.

| Fatty acids (%) | | Fish oil | Corn oil |
|---|---|---|---|
| C14:0 | Myristic | 4.08 | – |
| C15:0 | Pentadecanoic | 4.75 | 2.84 |
| C16:0 | Palmitic | 18.70 | 14.06 |
| C16:1 *n-7* | Palmitoleic | 6.76 | 0.18 |
| C18:0 | Stearic | 4.15 | 2.22 |
| C18:1 *n-9* | Oleic | 33.24 | 28.85 |
| C18:2 *n-6* (LA) | Linoleic | 2.67 | 50.51 |
| C18:3 *n-3* (LNA) | Linolenic | 1.48 | 0.97 |
| C20:1 *n-9* | Eicosanoic | 2.38 | – |
| C20:0 | Arachidic | – | 0.37 |
| C20:4 *n-6* (ARA) | Arachidonic | 0.35 | – |
| C20:5 *n-3* | Eicosapentaenoic | 6.31 | – |
| C24:0 | Lignoceric | 0.44 | – |
| C22:5 *n-6* | Docosapentaenoic | 0.23 | – |
| C22:5 *n-3* | Docosapentaenoic | 0.88 | – |
| C22:6 *n-3 (DHA)* | Docosahexaenoic | 13.60 | – |
| Total | | 100 | 100 |
| *n-3* | | 22.7 | 0.97 |
| *n-6* | | 3.25 | 50.51 |
| *n-3/n-6* | | 6.86 | 0.02 |

**Table 4.** Fatty acid pattern of the experimental diets (% of total fatty acids).

| | Diets | | | |
|---|---|---|---|---|
| Fatty acid (% w/w) | Control diet (%) | Fish oil 2% | Corn oil 2% | Fish/corn oil (50%:50%) |
| C14:0 | 13.23 | 3.00 | 0.50 | 0.70 |
| C16:0 | 13.74 | 21.90 | 12.50 | 13.40 |
| C16:1 *n-7* | ND | 4.60 | 0.50 | 1.10 |
| C18:0 | 2.50 | 5.20 | 1.80 | 3.30 |
| C18:1 *n-9* | 27.71 | 23.10 | 25.00 | 29.70 |
| C18:2 *n-6* | 41.53 | 13.80 | 53.40 | 46.00 |
| C18:3 *n-3* | 1.29 | 2.20 | 2.60 | 4.00 |
| C20:4 *n-6* | ND[1] | 1.60 | 1.80 | 0.20 |
| C20:5 *n-3* | ND | 5.00 | 0.70 | 0.80 |
| C22:5 *n-3* | ND | 1.10 | 0.20 | 0.10 |
| C22:6 *n-3* | ND | 18.50 | 1.00 | 0.70 |
| Total n-3 fatty acids | 1.29 | 26.80 | 4.50 | 5.60 |
| PUFA[2] | 42.82 | 42.20 | 59.70 | 51.80 |
| Total n-6 fatty acids | 41.53 | 15.40 | 55.20 | 46.20 |
| Ratio *n–6 : n–3* | 32.19 | 0.57 | 12.27 | 8.25 |
| Total fat (%) | 8.40 | 8.43 | 8.44 | 8.43 |

[1]ND: no detection.
[2]PUFA is the sum of 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3.
Males received diets that contained either supplemental 2% fish oil (FO) or 2% corn oil (CO) from 45 to 53 wk of age.
All values are given as a percentage of total fatty acids.

**Table 2.** Effect of different treatments on body weight of aged broiler breeder roosters.

| | Diets | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Basal diet | Fish oil (2%) | Corn oil (2%) | Fish/corn oil (50:50%) | Fish oil+RLP* | Corn oil+RLP | Fish/corn oil (50:50%)+ RLP | SEM pooled | *P-value* |
| Body weight (in 0 D) | | | | | | | | |
| 4572.6 | 4616 | 4596.20 | 4585.40 | 4,568 | 4,622 | 4,589 | 86.58 | NS |
| Body weight (in 60 D) | | | | | | | | |
| 4816.80 | 4868.80 | 4833.40 | 4812.20 | 4,811 | 4,862 | 4849.4 | 86.82 | NS |
| Body weight (different 0 to 60 D) | | | | | | | | |
| 244.20 | 252.80 | 237.20 | 226.80 | 243 | 240 | 260.40 | 7.57 | NS |

### Experimental Design

The present study consisted of 7 treatments in a randomized complete design, with 5 replications of individual birds in each treatment. Thirty-five roosters were divided to 7 groups and in term 0 to 60 interaction between diet and time (diet × time) mentioned.

### Assessment of Semen Quality

Ejaculate from each rooster was individually evaluated. Semen volume was assessed in graduated collecting tubes. Sperm concentration was recorded after dilution of semen sample (1:200 with distilled water), and then a droplet of semen was placed on the Neubauer hemocytometer to determine the sperm concentration. Total and progressive motility of sperm were measured using Sperm Class Analysis software (**SCA**) (Shahverdi et al., 2015). Briefly, 8 $\mu$L sample of diluted semen with PBS (without calcium and magnesium, 1:10) was placed on the pre-warmed chamber slide (20 $\mu$m; Leja 4, Leja Products Luzernestraat B.V., Holland), and then total and progressive motility were measured. Eosin-nigrosin staining was used to determine the viability of sperm. For this purpose, smears were prepared on a warm slide and the stain was spread on a second slide. Viability was assessed by counting 200 sperms under phase-contrast at ×1,000 magnification. Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of the stain were counted as viable (Fattah et al., 2017). For evaluation of abnormal morphology, 3 drops (10 $\mu$L) of semen were pipetted into 1 mL Hancock's solution (Schäfer and Holzmann, 2000). Then, 10 $\mu$L of processed sperm was handled on a slide. The percentage of sperm abnormalities was recorded by counting a total of 300 sperm under a phase-contrast microscope (×1,000 magnification; immersion oil). Hypoosmotic swelling test was used to evaluate the plasma membrane functionality of the sperm (Revell and Mrode, 1994). This assay was carried out by mixing 5 $\mu$L of semen with a 50 $\mu$L hypoosmotic solution (100 mum/L, 57.6 mM fructose, and 19.2 mM sodium citrate). After 20-min incubation, sperm were checked under a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan) and 300 sperm with swollen and non-swollen tails were recorded.

### DNA Fragmentation

The SCSA procedure was used to determine DNA damage in spermatozoa using flow cytometry analysis (Evenson and Wixon, 2005). An aliquot of washed spermatozoa in PBS was diluted to a concentration of 3 × 106 spermatozoa/mL. The cell suspension was treated with an acid detergent solution contained 0.1% Triton X-100, 0.15 mol/l NaCl, and 0.08 N HCl for 30 s, and then stained with 6 mg/L purified acridine orange (**AO**) in a phosphate-citrate buffer. The AO binds to single-stranded DNA and emits a red fluorescence that detected using a 670 bandpass filter (Fl-3). The percentage of DNA fragmentation in spermatozoa was calculated by DNA fragmentation index obtained from the ratio of red cells to the total of red green cells (Hosseinifar et al., 2015).

### Abnormal Morphology

For the evaluation of abnormal morphology, 3 drops (10 $\mu$L) of semen samples were pipetted into 1 mL of Hancock's solution (Schaäfer and Holzmann, 2000) consisted of 62.5 mL formalin (37% formaldehyde), 150 mL sodium saline solution, 150 mL PBS buffer solution, and 500 mL double-distilled water. To detect the different features of abnormality in acrosome, head, and tail, 10 mL of processed semen samples were handled on a slide. The percentage of abnormality was determined by counting about 300 spermatozoa under a phase-contrast microscope (×1,000 magnification; oil immersion).

### MDA Assay

Malondialdehyde concentrations, as indicator of lipid peroxidation (**LPO**) in semen, were measured using the thiobarbituric acid reaction (Esterbauer and Cheeseman, 1991). Quantification of thiobarbituric acid reactive substances was performed by comparing the absorption with the standard curve of MDA equivalents generated by the acid catalyzed hydrolysis of 1,1,3,3-tetra-methoxypropane.

### Detection of the Reproduction Hormones

Blood samples were drawn from the brachial vein at days 0 and 60 of the experiment. Then, they were centrifuged at 3,000 rpm for 10 min at 4°C to separate the plasma and stored at –20°C for further assessment of reproductive hormones. Plasma concentrations of luteinizing hormone (**LH**) and follicle-stimulating hormone (**FSH**) were measured using a chicken ELISA kit (Crystal Day, Shanghai, China), according to the manufacturer's instruction. All assays were performed in 96-well plates, and absorbance was measured at 450 nm. For evaluation of testosterone, an ELISA kit (Monobind Inc., Costa Mesa, CA) was used. Intra-assay coefficients of variation and sensitivity of the testosterone assay were 6.08% and 0.0576 ng/mL, respectively.

### Assessment of Sperm FA Analysis

Fatty acid analysis of sperm at the end of experiment (day 60) was measured according to the method of Ommati et al. (2013) with a slight modification. Sperm suspension (500 $\mu$L) was centrifuged at 3,200 rpm for 10 min and washed in PBS. For extraction of lipid, chloroform methanol (2:1 vol/vol) was dissolved in a NaOHMeOH solution (2%), and methylated by

**Table 5.** Seminal attributes in aged Ross 308 breeder roosters fed fish/corn oil with rosemary.

| | Diets | | | | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Basal diet | Fish oil (2%) | Corn oil (2%) | Fish/corn oil (50:50%) | Fish oil (2%)+ RLP | Corn oil (2%)+ RLP | Fish/corn oil (50:50%)+ RLP[7] | SEM pooled | Diet | Time | Diet × Time |
| Semen volume (mL) | 0.36 | 0.44 | 0.34 | 0.45 | 0.47 | 0.43 | 0.50 | 0.09 | NS | NS | NS |
| Concentratio (n) | $3.58^{b}$ | $3.66^{b}$ | $3.64^{b}$ | $3.76^{b}$ | $4.40^{a}$ | $3.41^{b}$ | $4.67^{a}$ | 0.05 | <0.0001 | <0.0001 | <0.0001 |
| Total motility% | $80.92^{b}$ | $81.04^{b}$ | $80.28^{b}$ | $80.61^{b}$ | $86.31^{a}$ | $79.85^{b}$ | $86.77^{a}$ | 0.06 | <0.0001 | <0.0001 | <0.0001 |
| Progressive motility% | $40.29^{b}$ | $39.47^{b}$ | $39.11^{b}$ | $39.32^{b}$ | $46.51^{a}$ | $37.90^{b}$ | $45.83^{a}$ | 0.55 | <0.0001 | <0.0001 | <0.0001 |
| VCL[1] | 162.10 | 164.04 | 162.37 | 166.40 | 165.50 | 160.60 | 165.85 | 1.96 | NS | 0.007 | 0.01 |
| VSL[2] | 68.26 | 70.75 | 66.77 | 67.32 | 68.20 | 68.40 | 68.67 | 0.67 | 0.05 | 0.0003 | 0.03 |
| VAP[3] | $40.27^{a\text{-}c}$ | $38.18^{b,c}$ | $40.66^{a\text{-}c}$ | $40.67^{a\text{-}c}$ | $44.67^{a,b}$ | $37.34^{c}$ | $46.37^{a}$ | 0.93 | NS | <0.0001 | <0.0001 |
| LIN[4] | 42.18 | 43.16 | 41.31 | 40.52 | 41.32 | 45.14 | 41.50 | 1.05 | NS | 0.004 | 0.01 |
| STR[5] | $55.75^{b,c}$ | $54.25^{c}$ | $61.56^{a\text{-}c}$ | $60.79^{a\text{-}c}$ | $65.67^{a,b}$ | $54.67^{c}$ | $67.59^{a}$ | 1.11 | 0.003 | <0.0001 | <0.0001 |
| ALH[6] | $7.81^{b,c}$ | $7.25^{c}$ | $7.65^{b,c}$ | $8.90^{b,c}$ | $10.01^{a,b}$ | $8.76^{b,c}$ | $11.42^{a}$ | 0.33 | 0.007 | <0.0001 | <0.0001 |
| Morphology% | 16.48 | 16.76 | 15.97 | 15.85 | 15.54 | 16.38 | 13.27 | 0.50 | NS | <0.0001 | 0.003 |
| Membrane Integrity% | $77.40^{b,c}$ | $75.67^{c}$ | $76.88^{b,c}$ | $76.57^{c}$ | $84.12^{a}$ | $73.90^{c}$ | $81.44^{a,b}$ | 0.58 | <0.0001 | <0.0001 | <0.0001 |
| Viability% | $80.67^{a,b}$ | $76.17^{b}$ | $77.59^{a,b}$ | $78.94^{a,b}$ | $80.62^{a,b}$ | $76.98^{b}$ | $82.50^{a}$ | 0.58 | NS | <0.0001 | <0.0001 |
| DNA $Fr^{+}$ | 8.10 | 9.12 | 9.03 | 8.59 | 8.31 | 8.08 | 8.54 | 0.37 | 0.2 | <0.0001 | NS |
| DNA $FR^{-}$ | 91.90 | 90.87 | 90.97 | 91.41 | 91.69 | 89.42 | 91.46 | 0.51 | NS | <0.0001 | <0.001 |
| MDA (nmol/mL) | $3.19^{a,b}$ | $3.25^{a}$ | $3.57^{a}$ | $3.31^{a}$ | $2.66^{b,c}$ | $2.59^{c}$ | $2.64^{b,c}$ | 0.06 | 0.002 | <0.0001 | <0.0001 |

[1]VCL: average velocity measured over the actual point to point track followed by the cell.
[2]VSL: average velocity measured in a straight line from the beginning to the end of track.
[3]VAP: path velocity of the smoothed cell path.
[4]LIN: average value of the ratio VSL/VCL; measured the departure of the cell track from a straight line.
[5]STR: average value of the ratio VSL/VAP; measured the departure of the cell path from a straight line.
[6]ALH: amplitude of lateral head displacement corresponding to the mean width of the head oscillation as the sperm swam.
[7]Rosemary leaves powder.

boron trifluoride. Methylated FAs were separated by n-hexane and saturated NaCl solutions, and were then analyzed by gas chromatography (Unicam 4600, Cambridge, UK), using a BPX-70 column (0.25 mm i.d., 30 m, 0.25 $\mu$m film thickness; J & W Scientific, Folsom, CA) and a flame ionization detector (Unicam 4600, Cambridge, UK).

### Statistical Analyses

All data are presented as least squares means ± SEM. Each individual rooster was considered as an experimental unit in all statistical analyses. Before analysis, the data were tested for normality using the Shapiro–Wilk test with a univariate procedure of SAS 9.1 (SAS Institute Inc., Cary, NC). Single and repeated measurement data were analyzed by PROC GLM and PROC MIXED, respectively. When significant treatment differences were detected, LSMEANS was used to compare individual least-squares means with Turkey's test. In all statistical analyses, the significance level was set at $P < 0.05$.

## RESULTS

### Semen Parameters

Dietary oil sources with or without RLP had no significant effects on semen volume, VCL, LIN, DNA $Fr^{+}$, DNA $Fr^{-}$, and morphology on period (0 to 60) they were measured ($P > 0.05$, Table 5). The body weight of roosters on day 0 and at the end of the experimental period was not significantly different (Table 2). At days 0 to 60, diets supplemented with fish and fish/corn oil and RPL resulted in a significantly higher percentage of total motility, semen concentration, and progressive motility ($P < 0.0001$) than diets supplemented with corn oil and without rosemary. Also in this period, diet supplemented with fish oil and RPL significantly increased the percentage of membrane integrity compared to other groups except fish/corn oil with RPL ($P < 0.0001$). The percentage of VAP, STR, and ALH was affected by diets at throughout the length of the experiment ($P < 0.05$). A 12 to 19% improvement in sperm progressive motility was noted in roosters fed fish and fish/corn oil with rosemary of diet when compared to control and corn oil groups ($P < 0.05$). RLP feeding (5 g/kg) in combination with n-3 (2% fish oil) and n-3: n-6 (50:50) resulted in a significant increase in semen concentration, which ranged from $4.67 \times 10^{9}$ spermatozoa/mL in n-3: n-6 (50:50) group to $4.40 \times 10^{9}$ spermatozoa/mL in n-3 (2% fish oil) group. Recorded semen concentration values ranged from 3.63 (day 0) to $3.07 \times 10^{9}$ spermatozoa/mL (day 60) for control group that was not significant with other group except n-3 (2% fish oil) and n-3: n-6 (50:50) combined with RLP. The corresponding values in those fed n-3: n-6 (50:50) combined with RLP ranged from 4.05 to 5.37 ($\times 10^{9}$ spermatozoa/mL) at days 7 and 60, respectively. Roosters in n-3 (2% fish oil) combined with RLP group recorded a maximum value of $5 \times 10^{9}$ spermatozoa/mL at days 40, slightly decreased and reached to $4.40 \times 10^{9}$ at the end of trial. Total sperm production was significantly affected by feeding of PUFA sources and RLP. The highest value was observed in n-3: n-6 (50:50) combined with RLP ($2.33 \times 10^{9}$ spermatozoa/ejaculate)

**Table 6.** The main effect of time on sperm quality in broiler breeder roosters.

| item | Semen volume (mL) | Semen concentration ($10^9$ spermatozoa/mL) | Total motility% | Progressive motility (%) | VCL (%) | VSL (%) | VAP (%) | LIN (%) | STR (%) |
|---|---|---|---|---|---|---|---|---|---|
| Day(s) | | | | | | | | | |
| 0 | 0.49 | 3.73[a,b] | 81.88[b] | 39.93[b] | 165.81 | 68.01[a,b] | 40.43[b,c] | 41.07[a,b] | 59.81[a,b] |
| 20 | 0.42 | 3.99[a,b] | 84.95[a] | 40.07[b] | 159.82 | 68.26[a,b] | 42.31[a,b] | 44.26[a] | 60.24[a,b] |
| 40 | 0.40 | 4.09[a] | 85.49[a] | 43.33[a] | 161.21 | 70.69[a] | 45.66[a] | 43.82[a] | 65.23[a] |
| 60 | 0.40 | 3.68[b] | 76.70[c] | 41.49[a,b] | 168.50 | 66.39[b] | 36.28[c] | 39.48[b] | 54.88[b] |
| SEM pooled | 0.04 | 0.08 | 0.49 | 0.54 | 1.92 | 0.84 | 0.91 | 0.84 | 1.47 |
| *P-value* | 0.11 | <0.0001 | <0.0001 | <0.0001 | 0.01 | 0.001 | <0.0001 | 0.008 | <0.0001 |

[a–c]Within rows, least squares (means ± S.E) with different superscripts significantly ($P \leq 0.05$).

**Table 7.** The main effect of time on sperm quality in broiler breeder roosters.

| item | ALH (%) | Morphology | Membranes integrity | Viability (%) | DNA $Fr^+$ | DNA $Fr^-$ | MDA (nmol/mL) |
|---|---|---|---|---|---|---|---|
| Day(s) | | | | | | | |
| 0 | 10.59[a] | 14.12[b] | 80.49[a] | 81.04[a] | 7.91[b] | 92.09[a,b] | 3.08[a,b] |
| 20 | 7.35[b] | 14.01[b] | 76.75[b] | 77.66[b] | 6.07[b] | 93.93[a] | 2.74[b] |
| 40 | 9.68[a] | 14.58[b] | 79.70[a] | 83.19[a] | 8.33[b] | 90.24[b,c] | 2.90[b] |
| 60 | 7.68[b] | 20.49[a] | 75.04[b] | 74.38[c] | 11.85[a] | 88.15[c] | 3.40[a] |
| SEM pooled | 0.33 | 0.59 | 0.61 | 0.67 | 0.53 | 0.71 | 0.36 |
| *P-value* | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

[a–c]Within rows, least squares (means ± S.E) with different superscripts significantly ($P \leq 0.05$).

compared to other groups. For total sperm, there was a tendency ($P = <0.0001$) for an effect of treatment × time. Other semen characteristics were affected ($P < 0.03$) by treatment or treatment × time except semen volume (Table 5). Subsequent analysis revealed an effect ($P \leq 0.0001$) of treatment × period for sperm concentration cells; treatments with omega-3 and n-3: n-6 (50:50%) FAs combined with 5 g/kg RPL increased ($P \leq 0.0001$) the sperm concentration from $3.86 \times 10^9$, $4.05 \times 10^9$ (mean + S.E.M.) during day 0 to $4.40 \times 10^9$ and $4.67 \times 10^9$ during days 0 to 60, respectively (S.E.M = 0.05 treatment × period, $P = 0.0001$). Semen volume for days 0 to 60 was 0.36 mL for control roosters and 0.47 and 0.50 mL, respectively, for roosters fed the diet supplemented with omega-3 FAs and n-3: n-6 (50:50%) FAs combined with 5 g/kg RPL (*S.E.M.* = 0.09; treatment × period, $P = 0.65$). Sperm viability percentage was significantly improved in n-3 and n-3: n-6 combined with 5 g/kg RLP fed birds (Table 5). In addition, birds received of n-3: n-6 combined with 5 g/kg RLP diet recorded the highest value (82.50%) among treated groups. Spermatozoa from birds in n-3 and n-3: n-6 combined with 5 g/kg RLP groups had significantly higher plasma membrane integrity functionality than other groups (84.12 and 81.44 respectively), although there was no significant difference between others.

In spite of the fact that oil sources increase oxidation and MDA, rosemary was able to decrease MDA levels in oil treatments. This useful effect was significant between groups ($P < 0.05$). The main effect of time on sperm quality in Ross 308 male breeder roosters shows that in day 40 semen collection impression of treatment was clear; in fact, in this time improve semen quality is more tangible (Tables 6 and 7).

## Fatty Acid Composition of Sperm

The FA composition of sperm according to the diet supplied to the breeders is presented in Table 8. Dietary supplementation with fish oil (FO group also with RLP) resulted in a partial remodeling of the phospholipid FA profile of sperm. The proportion of C22:6n-3 in the sperm phospholipid fraction was 2.5-fold higher in the FO group compared to the corn oil group, and the proportion of C22:5n-3 also significantly increased ($P < 0.05$). As a consequence, the n-6/n-3 ratio was significantly decreased to 2.91 (with RLP) in the FO group compared to the control and corn oil group. The proportion of C18:2n-6, essential precursor of the n-6 series, was significantly decreased in the fish and fish oil with RLP groups compared to the control group ($P = 0.001$); however, the proportion of n -6 FAs in the sperm was not significant, but the highest proportion is seen in corn oil groups. The proportion of C22:3n-9 was not significantly modified by the diet. The proportions of SFA and total LC-PUFA were not affected by the dietary treatment. The combination of fish and corn oil (50:50%) was successful to transfer n-3 LC-PUFA to sperm ($P < 0.05$).

## Hormonal Parameters

Hormonal concentrations of roosters were not significantly different at day 0 ($P > 0.05$, Table 9). At day 60, a significantly higher concentration of testosterone was

**Table 8.** Fatty acid composition of sperm phospholipid from broiler breeder rooster fed different oil sources with rosemary.

| | Diets | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Basal diet | Fish oil (2%) | Corn oil (2%) | Fish/corn oil (50:50%) | Fish oil+RLP | Corn oil +RLP | Fish/corn oil (50:50%)+ RLP[3] | *SEM pooled* | *P-value* |
| Fatty acids% | | | | | | | | | |
| C16:0 | 14.90 | 15.60 | 14.51 | 15.22 | 15.60 | 14.51 | 15.23 | 0.69 | NS |
| C18:0 | 23.90 | 23.67 | 22.50 | 23.09 | 23.67 | 22.50 | 23.08 | 0.70 | NS |
| C18:1 *n*-9 | 12.40 | 11.60 | 12.30 | 11.95 | 10.50 | 12.30 | 11.95 | 0.38 | NS |
| C18:1 *n*-7 | 2.30 | 2.17 | 2.20 | 2.19 | 2.17 | 2.20 | 2.16 | 0.13 | NS |
| C20:1 *n*-9 | 3.70[a] | 2.94[a,b] | 2.40[b] | 2.50[b] | 2.92[a,b] | 2.37[b] | 2.46[b] | 0.20 | 0.003 |
| C20:0 | 0.90 | 1.00 | 0.80 | 0.90 | 0.96 | 0.81 | 0.90 | 0.12 | NS |
| C18:2 *n-6* | 4.90[a] | 3.33[b] | 4.91[a] | 4.20[a,b] | 3.22[b] | 4.88[a] | 4.20[a,b] | 0.24 | 0.001 |
| C20:3 *n-6* | 2.10[a] | 1.70[a,b] | 1.49[b] | 1.62[a,b] | 1.70[a,b] | 1.52[b] | 1.62[a,b] | 0.11 | 0.03 |
| C20:4 *n-6* | 9.70 | 7.64 | 10.37 | 9.03 | 7.63 | 10.33 | 8.97 | 0.67 | NS |
| C22:3 *n-9* | 3.50 | 3.20 | 2.91 | 3.07 | 3.24 | 2.87 | 3.03 | 0.14 | NS |
| C22:4 *n-6* | 19.20[a,b] | 17.00[b] | 20.10[a] | 18.80[a,b] | 17.07[b] | 21.09[a] | 18.76[a,b] | 0.78 | 0.001 |
| C22:5 *n-3* | 0.80[c] | 1.65[a] | 1.04[b,c] | 1.35[a,b] | 1.67[a] | 1.04[b,c] | 1.38[a,b] | 0.10 | <0.0001 |
| C22:6 *n-3* | 1.70[d] | 8.50[a] | 3.41[c] | 6.10[b] | 8.57[a] | 3.43[c] | 6.23[b] | 0.46 | <0.0001 |
| SFA[1] | 39.70 | 40.27 | 37.81 | 39.20 | 40.23 | 37.82 | 39.21 | 1.45 | NS |
| PUFA[2] | 41.90 | 43.02 | 45.14 | 44.16 | 43.18 | 44.22 | 44.20 | 2.47 | NS |
| n-6 | 35.90 | 29.67 | 37.78 | 33.65 | 29.71 | 37.82 | 33.55 | 1.79 | 0.20 |
| n-3 | 2.50[c] | 10.15[a] | 4.45[c] | 7.45[b] | 10.23[a] | 4.47[c] | 7.61[a,b] | 0.55 | <0.0001 |
| *n-6/n-3* | 14.51[a] | 2.93[c] | 8.59[b] | 4.54[c] | 2.91[c] | 8.58[b] | 4.43[c] | 0.39 | <0.0001 |

[a–c]Within rows, least squares (means ± S.E) with different superscripts significantly ($P \leq 0.05$).
Only fatty acids representing more than 1% are reported.
[1]Total saturate fatty acids.
[2]Total polyunsaturated fatty acids.
[3]Rosemary leaves powder.

**Table 9.** Effect of different treatments on plasma hormone of aged broiler breeder roosters.

| | Diets | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Basal diet | Fish oil (2%) | Corn oil (2%) | Fish/corn oil (50:50%) | Fish oil+RLP[1] | Corn oil+RLP | Fish/corn oil (50:50%)+ RLP | *SEM pooled* | *P-value* |
| Hormones (in 0 D) | | | | | | | | | |
| LH | 4.44 | 4.71 | 4.68 | 4.11 | 4.55 | 4.77 | 4.37 | 0.23 | NS |
| FSH | 5.69 | 5.80 | 6.23 | 5.95 | 5.79 | 5.75 | 5.75 | 0.25 | NS |
| Testosterone | 3.08 | 3.62 | 3.11 | 2.79 | 2.91 | 3.97 | 3.15 | 0.23 | NS |
| Hormones (in 60 D) | | | | | | | | | |
| LH | 3.87[c,d] | 3.73[c,d] | 2.97[d] | 5.44[b] | 4.24[c] | 4.25[c] | 6.56[a] | 0.21 | <0.0001 |
| FSH | 6.51 | 6.81 | 6.40 | 5.81 | 6.49 | 6.62 | 6.77 | 0.24 | NS |
| Testosterone | 2.26[b] | 2.85[b] | 2.41[b] | 3.75[a,b] | 3.07[b] | 2.63[b] | 4.66[a] | 0.34 | 0.0003 |

[a–d]Within rows, least squares (means ± S.E) with different superscripts significantly ($P \leq 0.05$).
[1]Rosemary leaves powder.
FSH, follicle-stimulating hormone; LH, luteinizing hormone.

observed in roosters fed the fish/corn oil with 5 g rosemary ($P = 0.0003$) compared to other groups. There was no significant difference in testosterone concentration among other treatments except fish/corn. The concentration of FSH was not significantly affected by the different diets at day 60 ($P > 0.05$). The LH concentrations on day 60 showed similar trends as for testosterone concentration ($P < 0.05$).

## DISCUSSION

This study was conducted from 45 to 53 D, age during which the semen quality of roosters has a tendency to decline. Therefore, such method as adjusting the PUFA sources with a natural antioxidant to improve semen quality will be valuable. This study reported the effects of dietary n-6: n-3 ratio and RLP on semen quality, FA composition, and lipid peroxidation in aged roosters. The effectiveness of dietary rosemary supplementation with PUFA in improvement of sperm quality, sperm FA composition, and some hormones of roosters was assessed in this experiment.

This study clearly indicated that dietary fish: corn oil (50:50%) supplementation with RLP resulted in improving all semen quality traits included in this experiment, followed by the results of fish oil. On the other hand, the worst results of semen quality traits were recorded by corn oil treatment. Blesbois et al. (1997a) concluded that transfer of essential FAs from the diet to the semen is effective, and this transfer may have biological effects on semen quality and fertilizing

ability of semen. However, those researchers found that there was clear influence of dietary lipids on spermatozoa FA profile; the proportion of n-3 FAs in spermatozoa from males fed fish oil compared to corn oil was higher (9.6% vs. 4.3%) and that of n-6 FAs was lower (22.4% vs 33.3%). Furthermore, the total n-6: n-3 of spermatozoa were 7.6 and 2.3 and seminal plasma was 154 and 2.8 for corn oil and fish oil, respectively. Hudson and Wilson (2003) reported that providing fish oil to broiler breeder males throughout their life may be simple means to maintain fertilizing ability of spermatozoa of these birds. Kelso et al. (1997b,c) suggested that a small increase in the proportion of n-3 FAs in semen phospholipids induced by enriching the diet with alpha-linolenic acid is associated with a significant improvement in semen quality of cockerel. Cerolini et al. (2000) reported that supplying the roosters with fish oil as a source of omega-3 FAs resulted in improvement in semen quality as compared with soybean or evening primrose oils (as a sources of omega-6 FAs).

Bongalhardo et al. (2009) indicated that diets containing lipids from different sources would differentially modify the lipid contents of membranes from sperm heads and bodies; they also found high correlations between these changes in lipids content and sperm concentration. Safarinejad et al. (2010) reported the fertile men were found to have higher blood and sperm levels of all 3 omega-3 FAs, while infertile men had significantly higher blood rations of omega-6 to omega-3 FAs. The number of spermatozoa per ejaculate decreased by 50% between 26 and 60 wk of age in birds fed the maize oil diet; this age-related decreased in the number of spermatozoa was almost completely prevented by feeding the birds with the oils enriched in either C22:6n-3 or C20:4n-6 (Surai et al., 2001). However, testis mass at 60 wk of age was approximately 1.5 times greater in birds given the tuna orbital and arasco oil diets compared with those given the maize oil diet (Surai et al., 2001). Estienne et al. (2008) reported that the number of sperm was increased and some of characteristics of sexual behavior were altered in boars fed a diet supplemented with omega-3 FAs.

Our data showed an increase in seminal volume of birds receiving fish oil and fish/corn oil (50:50%) with 5 g RLP/kg diet, but this increase was not significant. The cause for this effect is unknown; however, it might be inferred that an increased secretary and/or a decreased absorptive activity in the rete testis or efferent ducts might have contributed to enhancing the seminal volume (Ommati et al., 2013). In the present study, the greatest progressive sperm motility was observed in the roosters consuming fish oil and fish/corn oil (n-6: n-3 50:50%) with rosemary diets. This is consistent with the research conducted in cockerels, in which diets with n-6: n-3 ratios ranging from 6:1 to 9:1 improved the fertilizing ability of spermatozoa (Zanini et al., 2003).

An improvement in sperm motility in aged roosters consuming fish/corn oil (n-6: n-3 50:50%) with rosemary diets in the present study is in agreement with the results in a previous study using rosters (Surama et al., 2003). Those effects were dependent on fish oil quantity and sources and the duration of supplementation.

Useful effects of RLP on sperm quality have been reported but separately in combined basal diet (Borghei-Rad et al., 2017). In this experiment, RLP used to decrease negative effects of PUFA oxidation. Age-related reduction of antioxidant system along with naturally higher PUFA contents in plasma membrane of rooster spermatozoa makes them vulnerable to lipid peroxidation (Kelso et al., 1996). Being feed supplement in poultry industry, natural antioxidants have been included in the diet to improve sperm quality, performance, and reproductive outcomes. A growing body of evidence has documented the positive effects of herbal antioxidants including dried ginger rhizome (Akhalghi et al., 2014) and RLP (Borghei-Rad et al., 2017) on rooster sperm quality, with regard to having active antioxidative substances such as phenolic diterpenes, flavonoids, phenolic acids and volatile oils (Ho et al., 2000).

When the cockerels were fed the fish/corn-oil-based diet, the MDA level of the semen increased due to the fish and corn oil. However, in this present work, with an increasing supply of RLP in the fish/corn oil-based diet, an increase in semen quality was observed. It has been reported that sperm motility negatively correlated with the content of MDA in spermatozoa (Suleiman et al., 1996). On the other hand, a positive correlation has been documented between total PUFA, DHA, and n-3 PUFA with sperm motility, viability, normal morphology, and normal plasma membrane (Am-in et al., 2011). The association between increased lipid peroxidation and decreased sperm motility, which has been reported by Surai et al. (2001), was certified in our work. Reactive oxygen species induced a rapid loss of intracellular adenosine tri-phosphate that results in axonemal damage and subsequently a decrease in sperm motility and viability (Agarwal et al., 2014) In addition, sperm in vitro senescence and loss of motility are biochemical causes of LPO (Surai et al., 2001). Fluidity and permeability of plasma membrane play a substantial role in sperm motility and viability, which is in concert with our results showing the lower oxidative stress results in higher protection of plasma membrane FAs and subsequently higher sperm motility and viability.

The results of this experiment allow certain inferences regarding the necessity for a balanced ratio of n-6: n-3 in the diet. For instance, the reproductive performance of the cockerels fed on corn oil was inferior to that with other group. Probably, this effect was related to the higher ratio of n-6: n-3 FAs in the diet, which resulted in a higher n-6 FA deposition in the spermatozoa, and may subsequently influenced cell membrane flexibility. Kelso et al. (1997a) suggested that an increase in the availability of n-6 FAs inhibited a possible incorporation of FAs of the n-3 series in the spermatozoa. Although supplementing rosemary to corn oil increases n-6: n-3 and causes imbalance, in reality this herb protects n-6 PUFA and might have a higher ratio of n-6:

n-3. Hazim et al. (2010) reported the worst results for semen quality when diets of quail males supplemented with corn oil, whereas those supplemented with fish oil improved reproductive performance of Japanese quail males. The 6.6 dietary ratio (n-6: n-3) contributed to a greater progressive sperm motility ($P < 0.05$) than the 14.4 and 2.2 dietary ratio, and this ratio also enhanced the total antioxidant capacity ($P < 0.05$) in seminal plasma boars more significantly (Liu et al., 2017).

However, the appropriate ratio of n-3/n-6 PUFAs for sperm quality improvement and reproductive hormones in senescent broiler breeder roosters was not known. Both n-6 and n-3 PUFAs affect reproduction. Many studies have shown the effects of n-3/n-6 PUFA ratio on male reproduction. GnRH, which is released by the hypothalamus, stimulates release of FSH and LH from the pituitary gland (De kretser, 1979). Some study indicated that spermatogenesis and steroidogenesis in the avian testis are dependent on FSH, LH, and testosterone (Vizcarra et al., 2010). FSH and LH regulate spermatogenesis via cyclic adenosine 3, 5' '-monophosphate (cAMP) (Huang et al., 2003). LH binds to receptors in the membranes of Leydig cells, and stimulates the secretion of testosterone. Testosterone levels determine the testicular development and behavior of roosters (Yan et al., 2013). Also, this hormone may act on Sertoli and peritubular cells of the seminiferous tubules and stimulate spermatogenesis. Feng et al. (2015) showed that the concentrations of GnRH, FSH, LH, and testosterone were positively related to the quality and morphology of sperm. Similarly, Yan et al. (2013) reported that the concentrations of GnRH, FSH, LH, and testosterone increased with increasing n-3/n-6 PUFA ratio, and that lower and higher n-3/n-6 ratios have opposite effects on reproduction. These results indicate that an appropriate n-3/n-6 PUFA ratio is important for the development of spermatogonia in roosters.

PUFAs act via cell surface and intracellular receptors/sensors that control cell signaling and gene expression patterns (Calder, 2012). Some effects of n-3 PUFAs appear to be mediated by, or at least associated with, changes in the FA composition of cell membranes. GnRHR, FSHR, and LHR have important roles in the regulation of male reproduction (Millar et al., 2004). Yan et al. (2013) reported that relative GnRHR, FSHR, and LHR mRNA levels at 21 and 35 D differed significantly among ratios of n-3/n-6 PUFAs. This evidence suggests that PUFAs may affect cellular responses through changes in membrane fluidity, receptor binding characteristics, or their downstream activation.

## CONCLUSION

The results of the present study show that adding RLP to fish and fish/corn oil (50:50%) diets broiler breeder roosters resulted in significant improvement in semen quality traits, FA composition, and some sexual hormones in comparison with corn oil alone. Therefore, this herb could be used as a beneficial tool (as a natural antioxidant) in combined fish and fish/corn oil for improving reproductive performance of aged breeder roosters based on inclusion of these oils in their diets. Dietary treatment of roosters with an appropriate n-3/n-6 PUFA ratio (fish/corn oil+RLP treatments) increased hormone secretion, thereby improving sperm quality. These findings provide a sound basis for synergism n-3/n-6 PUFA in combined RLP being beneficial to aged broiler breeder roosters reproduction.

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