Effects of Dietary Inclusion of Polyunsaturated Fatty Acids and Antioxidants on Semen Characteristics of Potchefstroom Koekoek

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***Abstract*—The aim of the study was to investigate the effect of dietary fish oil supplementation on the semen characteristics of the Potchefstroom Koekoek cockerels. Thirty cockerels were divided into five groups, kept in individual cages and received five different diets: Commercial layer diet (CL), commercial cockerel diet (CC), modified layer diet (ML), modified layer diet supplemented with 5% fish oil (MLP) and a modified layer diet supplemented with 5% fish oil, 200mg/kg vitamin E and 5mg/kg selenium. Semen was collected twice a week on Mondays and Fridays. Semen characteristics were evaluated. Supplementation of fish oil did not significantly improve semen volume and sperm concentration. However, supplementation with polyunsaturated fatty acids and antioxidants increased the percentage of live and normal, as well as total motility of spermatozoa. The percentage of spermatozoa with mid-piece abnormalities and static motility were reduced in cockerels fed the MLPA diet. The supplementation of polyunsaturated fatty acids and antioxidants was associated with improved semen characteristics. Similarly, the supplementation of antioxidants provided enhanced protection against mid-piece abnormalities**

***Index Terms*—fish oil, laboratory tests, CASA, vitamin E, selenium**

I. Introduction

Potchefstroom Koekoek (PK) is a South African breed bred in the 60s at the Potchefstroom Agricultural College. It is a dual purpose breed which is well known for fair egg production (210 eggs per year). Day-old chicks are easy to sex due to distinct sex markings. The PK is a preferred chicken over the high yielding strains for small scale farmers because of their high adaptability to harsh conditions. It is very hardy, need minimum vaccination care compared to other breeds and easy to keep. The meat from PK chickens is widely preferred because of pigmentation, taste and leanness. Production efficiency of poultry needs to be optimized, with the use of superior cockerels and high fertility rates [1].

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The impact of nutrition on semen characteristics has been reported [2], while a positive correlation between semen characteristics and fertility has been reported in several studies [3], [4]. Polyunsaturated fatty acids are components of spermatozoa and play a major role in the behavioural and function of spermatozoa [5]. Polyunsaturated fatty acids of n-3 and n-6 are essential since they cannot be synthesized and are acquired through feed intake. The composition of these polyunsaturated fatty acids is important to maintain the physical properties of spermatozoa [6]. It is not amazing that spermatozoa contain high levels of polyunsaturated fatty acids. The high levels of polyunsaturated fatty acids make the spermatozoa to be more susceptible to lipid peroxidation [1]-[3]. Lipid peroxidation is a major factor that causes infertility in males [7]. Antioxidant systems become necessary to counteract the effect of lipid peroxidation, therefore, protect spermatozoa from peroxidative damage [8]. Vitamin E is the main potent antioxidant. It protects the spermatozoa by breaking of the lipid peroxidation process, thereby maintaining the spermatozoa biophysical properties. Vitamin E is mainly located in the spermatozoa. Birds do not have the ability to synthesize Vitamin E, so dietary supplementation with this vitamin is necessary. Selenium-dependent glutathione is also a potent antioxidant. Glutathione is highly concentrated in the mid-piece of spermatozoa. It first functions as an antioxidant and during maturation in the *cauda epididymis* forms a structural part of spermatozoa [9].

This duality makes the glutathione to be important in male reproduction. It is not surprising therefore, that deficiency of selenium is associated with mid-piece abnormalities and reduced spermatozoa motility. The present study evaluates the effects of polyunsaturated fatty acid and antioxidants of the cockerel diet on semen characteristics.

II. Materials and Methods

A. Experimental Animals

The study was conducted between autumn and winter seasons (February and September 2013) at the

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Agricultural Research Council, Irene at the Poultry breeding Section. The area is located at 25°55' South latitude and 28°12' East longitude in Pretoria, South Africa. The area is situated on the Highveld at an altitude of 1525m above sea level. The weather conditions ranges from hot days and cool nights in summer (17.5℃ to 32℃) to moderate winter days with very cold nights (1℃ to 17℃) [10].

Thereafter, cockerels were housed in individual cages of 47cm×32cm×61cm and 36cm×25cm×39cm respectively. Prior to use, the selected chickens were examined for external parasites, particularly poultry lice and treated accordingly. Feathers of cockerels around the vent area were clipped.

TABLE I. COMPOSITION OF THE EXPERIMENTAL DIETS

Composition %	MLD	ML+PD	ML+P+AD
Maize	69.27	69.27	69.27
Soyabean full fat	8.02	8.02	8.02
Soyabean 44	19.73	14.73	14.73
Fish oil	0.0	5.0	5.0
L-lysine	0.06	0.06	0.06
DL-methionine	0.03	0.03	0.03
Vit-min premix[1]	0.15		
Vit-min premix plus[2]		0.15	
Vit-min premix plus AO[3]			0.15
Limestone	0.9	0.9	0.9
Salt	0.2	0.2	0.2
Mono Ca phosphate	1.64	1.64	1.64

[1]Vitamin A 10,000,000IU, vitamin D3 3,000,000IU, vitamin E 20,000mg, vitamin K3 3,000mg, folic acid 500mg, niacin 30,000mg, D-calpan 10,000mg, vitamin B1 2,000mg, vitamin B2 5,000mg, vitamin B6 3,000mg, vitamin B12 20mg, biotin 60mg, Ethoxyquin 3,500mg, choline 300,000mg, iron 70,000mg, copper 8,000mg, zinc 100,000mg, cobalt 500mg, manganese 120,000mg, iodine 1,000mg, selenium 250mg, Ronozyme NP 90,000mg.

[2]Vitamin A 10,000,000IU, vitamin D3 3,000,000IU, vitamin E 20,000mg, vitamin K3 3,000mg, folic acid 500mg, niacin 30,000mg, D-calpan 10,000mg, vitamin B1 2,000mg, vitamin B2 5,000mg, vitamin B6 3,000mg, vitamin B12 20mg, biotin 60mg, Ethoxyquin 3,500mg, choline 300,000mg, iron 70,000mg, copper 8,000mg, zinc 100,000mg, cobalt 500mg, manganese 120,000mg, iodine 1,000mg, selenium 250mg, Ronozyme NP 90,000mg.

[3]Vitamin A 10,000,000IU, vitamin D3 3,000,000IU, vitamin E 200 000 mg, vitamin K3 3 000 mg, folic acid500 mg, niacin 30,000mg, D-calpan 10,000mg, vitamin B1 2,000mg, vitamin B2 5,000mg, vitamin B6 3,000mg, vitamin B12 20mg, biotin 60mg, Ethoxyquin 3,500mg, choline 300,000mg, iron 70,000mg, copper 8,000mg, zinc 100,000, cobalt 500mg, manganese 120,000mg, iodine 1,000mg, selenium 500mg, Ronozyme NP 90,000mg.

At 36 weeks of age, 60 cockerels were randomly allocated to five dietary treatments with 12 cockerels per dietary treatment. The three treatment diets are presented in Table I. The two control diets were commercial diets with unknown specifications due to commercial restrictions. The control diets consisted of a commercial layer diet (CL) (negative control); commercial cockerel layer breeder diet (CC) (positive control); modified layer diet (ML), modified layer diet +PUFA (MLP) modified layer diet+ PUFA+ antioxidants (MLPA). The dietary treatments were formulated to meet nutrient requirements of the cockerel breeder diet, as established by NRC [11]. Lighting regime was 16 hours light and 8 hour darkness. The experimental and control diets were presented in the form of mash and drinking water was supplied *ad libitum* by means of nipple drinkers.

B. Fatty Acid Analysis of the Dietary Treatments

Feed samples were collected from all five dietary treatments and separately packed in air-tight marked plastic bags. The feed samples were sent to the South African National Accreditation System (SANAS) accredited analytical laboratory at Irene ARC, for analysis of fatty acids [12].

C. Semen Collection

At 36 weeks of age, each cockerel was trained for semen collection using the massage technique for 14 days prior commencement of semen collection [13]. Cockerels characterized by positive response to massage technique and 75% motility using a Computer Assisted Sperm Analyser (CASA) (n=6) were selected from the base population (n=12) in each dietary treatment group. Individual males were considered to be the experimental unit of replication. Semen was collected twice weekly (Tuesdays and Fridays) from each cockerel throughout the experimental period. During semen collection, the tube was maintained at 40℃ in a thermo flask. To minimize stress and maximize the quality of semen, semen collection was done by the same persons, at the same time, under the same conditions. Special care was taken to avoid contamination of semen with faeces, urine and transparent fluid which lower semen quality.

D. Semen Volume and Sperm Concentration

Semen volume from each of the cockerel strains were determined by drawing the semen with a 1mL tuberlin syringe and reading directly to the nearest 0.01ml. Sperm concentration was determined using a spectrophotometer (Jenway 6310 spectrophotometer, Bibby Scientific, England).

The automated spermatozoa motility rate analyses, a Sperm Class Analyzer® [SCA] 5.0 (Microptic, Barcelona, Spain) at a magnification of 10×(Nikon, Japan) was used to analyze the spermatozoa motility. The settings of the CASA used to analyze the kinematic values of sperm are depicted in Table II. Semen samples were analyzed according to the manufacturer's recommendation. The sperm washing solution (Brackett and Olifant medium) was used. The wash solution was warmed to 40℃ on a warm plate before adding the semen sample. A total of 10µL semen was diluted with 500µL of medium in a 15ml tube (Falcon® 352099, USA) to perform the swim-up technique. The sperm solution was then incubated in the MCO-20 AIC Sanyo® CO_2 incubator (Sanyo, Japan) adjusted to 40℃ for five minutes. Following the incubation period, five µL of the semen solution was collected from the surface of the solution then pipetted onto a pre-warmed bevel-edged, frosted end microscope glass slide (76×26×1mm, Germany) and placed with a warmed cover slip (22×22mm, German) over a warm plate (Omron) adjusted at 40℃. For each sample, five randomly-selected fields containing between 200 and 400 motile tracks were examined. The generated data was

saved onto a Microsoft Excel® sheet for later statistical analysis.

TABLE II. SPERM CLASS ANALYZER® (V.4.0.0) SETTINGS USED TO ANALYZE THE SPERM CELL MOTILITY PARAMETERS

Parameters	Settings
Contrast	169
Brightness	470
Image/second	50
Optic	pH-
Chamber	Cover slide
Scale	10x
Particle area	5<190 µm2
Slow	<10 µm/s
Medium	<50 µm/s
Rapid	<100 µm/s

E. Statistical Analysis

The statistical program GenStat® [14] was used for the analysis of the data. Linear mixed model repeated measurements analysis, also known as REMl analysis [15] was applied to semen values. The means were separated using Fisher's unprotected t-test Least Significant Difference (LSD) and the data were tested at the 5 % level of significance.

III. RESULTS

The fatty acid compositions of the diets are depicted in Table III. The fatty acid composition of the diets clearly reflected the origin of the supplemented fish oil. As expected, the diets supplemented with fish oil contained a greater proportion of linolenic acid which was the major fatty acid of n-3. Supplementation of fish oil made the ratio of n-3 and n-6 to be as close as possible in the MLP and MLPA diets respectively. Because no n-3 was detected in LC diet, the ratio cannot be calculated.

The effects of various diets on the semen volume and concentration of cockerels are depicted in Table IV. Supplementation of polyunsaturated fatty acids had no significant difference on the treatments groups compared to other treatment groups. Cockerels fed diet supplemented with polyunsaturated fatty acids (MLP and MLPA) had similar semen volume and concentration compared to other treatment groups.

TABLE III. FATTY ACIDS COMPOSITION OF THE EXPERIMENTAL DIETS (% OF DIET)

Fatty acids	CL	CC	ML	MLP	MLPA
Saturated					
14:0	0.003	0.005	0.010	0.221	0.221
16:0	0.338	0.351	0.565	0.990	0.969
18:0	0.106	0.088	0.357	0.222	0.210
Total	0.447	0.444	0.932	1.433	1.400
Monounsaturated					
16:1n-7	0.004	0.011	0.012	0.288	0.283
18:1n-7	0.001	0.004	0.002	0.006	0.003
18:1n-9	0.020	0.992	1.702	1.598	1.446
20:1n-9	0.010	0.014	0.015	0.036	0.036
24:1n-9	nd	nd	0.001	0.036	nd
Total	0.035	1.021	1.732	1.964	1.768
PUFA n-3					
22:5n-3	nd	0.002	0.025	0.557	0.504
22:6n-3	nd	nd	0.009	0.340	0.313
Total		0.002	0.034	0.899	0.814
PUFA n-6					
18:2n-6	1.499	1.285	3.256	1.426	1.365
20:4n-6	nd	nd	0.011	0.018	0.004
22:4n-6	1.499	1.285	3.273	1.457	1.383
Total	2.998	2.570	6.541	2.901	2.752
Total PUFAs	2.998	2.572	6.575	3.800	3.566
Total n-6/n-3		1250	192	3.2	3.3
Total fatty acids	3.099	2.846	6.156	6.023	5.636

***nd=not detected**

TABLE IV. EFFECTS OF DIFFERENT DIETARY TREATMENTS ON SEMEN QUANTITY PARAMETERS OF COCKERELS

Parameters	Dietary treatments					p-value
	Layer diet	Commercial Cockerel	Modified layer	Modified layer + PUFA	Modified layer + PUFA+ Antioxidant	
Volume (ml)	0.49 ±0.03	0.50 ±0.04	0.44 ±0.04	0.38 ±0.03	0.43 ±0.04	0.479
Concentration ($\times 10^9$/ml)	5.2 ±0.03	5.0 ±0.03	5.1 ±0.03	5.1 ±0.03	4.8 ±0.03	0.816

Results of the effect of different dietary treatments on morphology are depicted in Table V. Overall, a significant large difference was observed between the cockerels fed five dietary treatments in live and normal spermatozoa. The extent to which spermatozoa displayed live and normal was significantly highest in the cockerels fed MLPA diet compared to other treatment groups. The spermatozoa quality was maintained in the MLPA group compared to other groups. Results revealed that the percentage of dead spermatozoa was not significantly different in any dietary treatment group. The cockerels fed MLPA diet had similar percentages of dead spermatozoa compared to other treatment groups. The percentage of spermatozoa with mid-piece abnormalities was significantly lower in the cockerels fed MLPA diet compared to other treatment groups. The integrity of the mitochondrial membrane potential was more preserved in the MLPA treatment group compared to other treatment groups.

Results of motility are summarized in Table VI. Following the spermatozoa analysis by CASA, a significant higher percentage of total motility was observed in the samples of cockerels fed MLPA diet compared to other treatment groups. Static motility is a parameter that depends on the outcome of total motility. When total motility increases, static motility decreases

and *vice versa*. Because the total motility was higher on the cockerels fed MLPA diet and lower on the cockerels fed other treatment groups; an opposite pattern of change was observed for the static motility values (Table I). Thus the changes in percentage of total motility had a negative effect on the proportions of static motility of the spermatozoa.

TABLE V. EFFECTS OF DIFFERENT DIETARY TREATMENTS ON SPERM MORPHOLOGY PARAMETERS OF COCKERELS

Parameters (%)	Dietary treatments				
	Layer diet	Commercial Cockerel	Modified layer	Modified layer + PUFA	Modified layer + PUFA+ Antioxidant
Live and normal	71.5±0.100d	76.1±0.101b	74.1±0.101c	76.4±0.101b	82.5±0.101a
Dead	7.8±0.1	7.3±0.1	7.4±0.1	7.4±0.1	6.7±0.1
Mid-piece	20.7±0.17a	16.6±0.17b	18.5±0.17ab	16.2±0.17b	10.8±0.17c

Values are expressed as mean ± SEM. Rows with different letters are significantly different ($p<0.05$).

TABLE VI. EFFECTS OF DIFFERENT DIETARY TREATMENTS ON SPERMATOZOA MOTILITY OF COCKERELS

Parameters (%)	Dietary treatments				
	Layer diet	Commercial Cockerel	Modified layer	Modified layer + PUFA	Modified layer + PUFA+ Antioxidant
Total motility	93.17±0.85b	94.92±0.95ab	93.86±0.89ab	95.18±0.79ab	96.00±0.85a
Static	6.87±0.85a	5.07±0.96ab	6.13±0.90ab	4.82±0.79ab	3.99±0.85b

Values are expressed as mean ± SEM. Rows with different letters are significantly different ($p<0.05$).

IV. DISCUSSION

The results obtained in this study confirm the previous findings with turkeys [16] and show that supplementation with polyunsaturated fatty acid does not improve semen quantity, such as semen volume and sperm concentration. These results suggest that supplementation with polyunsaturated fatty acids cannot improve the quantitative characteristics of cockerel semen. These results are in line with the findings of Edens and Sefton [17], that supplementation of polyunsaturated fatty acids does not improve semen quality.

Supplementation of polyunsaturated fatty acids and antioxidants significantly increased the percentage of live and normal spermatozoa of the cockerels fed MLPA diet. The high percentage of live and normal spermatozoa can be ascribed to the protective effect of antioxidants against lipid peroxidation [3]. The protective effect of antioxidants supplementation of spermatozoa has been evidently confirmed. The increase in the activity of antioxidants attained in the cockerels fed the diet supplemented with polyunsaturated fatty acids and antioxidants was related with superior protection against lipid peroxidation compared to the other treatment groups. As vitamin E and selenium-dependent glutathione in spermatozoon are considered to be the main antioxidants that bath lipid peroxides and protect spermatozoa against damage caused be reactive oxygen species, the products of lipid peroxidation *in vivo* [18], its protective effect is related with this function. The outcomes in this study show the impact of vitamin E and selenium on the protection of spermatozoa against lipid peroxidation.

It is apparent that the number of dead spermatozoa is affected by male effect, collection method and technician experience. Considering the non-significance among dietary treatment groups in dead spermatozoa, individual variation in the dietary treatment groups were considered. It is a common practice that breeding cockerels are selected on the traditional method of phenotypical characteristics. In this study, the male effect was eliminated by pre-selecting cockerels with 75 percent and above motility using CASA analysis. The semen samples were deliberately not pooled to increase replicates which is essential to increase power of statistical analysis. irrespective of supplementing polyunsaturated fatty acid and antioxidants, the present study indicates that the dead spermatozoa were similar in all dietary treatment groups, emphasizing the consistency in semen collection by the technician. In contrast, Golzar-Adabi *et al.* [19] found a significant difference between treatment groups in dead spermatozoa when supplementing polyunsaturated fatty acid and antioxidants in Japanese quail. The discrepancies among studies could be the above mentioned authors used the traditional method to select the Japanese quail males used in the study.

Morphologically anomalous spermatozoa are more vulnerable to oxidative stress. The production of reactive oxygen species by spermatozoa with mid-piece abnormalities can induce oxidative stress, resulting in cellular dysfunction by a process called lipid peroxidation [18]. Lipid peroxidation is associated with mid-piece abnormalities [7]. Considering this results, the high percentage of mid-piece abnormalities observed in the other treatment groups (CL, CC and ML) may be due

to high production of reactive oxygen species. The situation was prevented in this study by supplementing antioxidants in the diet in the cockerels fed MLPA diet compared to other treatment groups.

Previous investigations have associated spermatozoa with increased percentage of mid-piece abnormalities to impaired motility [17], [19]. A similar connection was found in this study, as a significant high mid-piece abnormalities resulted in low motilities. In cockerels fed the MLPA diet, supplemented with polyunsaturated fatty acid and antioxidants, the percentage of total motility was the highest compared to other treatment groups, while the percentage of spermatozoa with mid-piece abnormalities was correspondingly decreased respectively.

V. Conclusion

The present study indicates that dietary polyunsaturated fatty acids of n-3 can improve the sperm quality but not semen quantity characteristics in cockerels and also emphasize the importance of antioxidants in preventing mid-piece abnormalities. This study showed that polyunsaturated fatty acids supplementation with antioxidants may have beneficial effect on semen quality in Potchefstroom cockerels.

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