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Original Research Article

ANTI-AGEING EFFECTS OF DIETARY BEE PRODUCTS AND CALORIE RESTRICTION ON SEMEN PRODUCTION AND OXIDATIVE DAMAGE IN OLDER BROILER BREEDER MALES

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Abstract: This study was conducted to investigate the effects of calorie restriction and dietary bee products (apilarnil plus royal jelly) supplementation on reproductive and oxidative responses and to determine the possibilities that these treatments may be used in retarding the reproductive ageing of broiler breeder males. At 52 weeks of age, broiler breeder males were assigned to four treatment groups. The control group was fed on restricted feed as recommended by the breeder company throughout the study; the *ad libitum* group was fed *ad libitum* for a four-week period; the bee products group was fed similar to the control group except that their diet was supplemented with apilarnil and royal jelly for a four-week period and in the last group calorie restriction (45 % of standard diet) was applied for a four-week period. After a four-week adaptation period, the experiment was continued for 18 weeks. The results obtained in the present study have demonstrated that the percentage of dead sperm was the most affected semen characteristic by reproductive ageing. Long-term moderate feed restriction could not prevent age-related declines in sperm production. Dietary bee products supplementation or calorie restriction for a four-week period positively affected the semen characteristics, and these beneficial effects could be maintained to some extend up until 72 weeks of age. Calorie restriction enhanced antioxidant defence for the first four-week period; however, this beneficial effect could not be sustained until the end of the experiment.

Key words: broiler breeder males; ageing; semen characteristics; oxidative stress; bee products; calorie restriction

Introduction

Although domestic roosters may live for more than 10 years, the fertility of commercial breeder strains declines during the last phase of their reproductive life, so they are kept for a much shorter period. Commercial broiler breeders have a relatively shorter reproductive life span, and their reproductive performance decreases, particularly after 45 weeks of age (1, 2). Therefore it is recommended to keep broiler breeder flocks in production until 61 to 64 weeks of age. The decline of fertility in ageing roosters was accompanied by a reduction in spermatozoa production and by a decrease in motility and viability of spermatozoa (3, 4, 5). In contrast, the demand for spermatozoa increases with ageing because of the changes in the oviduct conditions, especially in sperm storage tubules (6, 7). Therefore, one of the most important goals in the poultry industry is to extend the reproductive life span and retard signs of reproductive senescence, both in terms of animal welfare and production costs.

Calorie restriction (CR) has been shown to extend the life span and retard the onset of many age-related disorders in a variety of animal models (8, 9, 10). Short-term calorie or food restriction has been routinely used by the poultry industry for many years to extend the reproductive life span and delay the maturation of laying hens (11, 12).

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However, it has been suggested that severe CR caused a significant decrease both in testis weight and in plasma testosterone levels and an increase in plasma corticosterone levels (13, 14).

The cumulative oxidative damage to macromolecules caused by reactive oxygen species (ROS) is considered to be related to cellular senescence, life span, and fitness (15, 16). It is suggested that in avian cells the accumulation of ROS-induced damage would be slower due to lower ROS production, better resistance to oxidative stress, enhanced antioxidant capacity, and better DNA repair (16, 17, 18). If ageing is caused by ROS as described by the free radical theory or oxidative damage theory of ageing, supplementation of exogenous antioxidants may enhance the antioxidant defence capacity, slow down ageing, and prolong the reproductive life span.

Sperm membranes have a higher content of PUFAs (polyunsaturated fatty acids); therefore, they are more susceptible to oxidative damage. Oxidative stress may be reduced by antioxidant supplementation of broiler breeder diets (19).

Therefore, in this study, the natural bee products apilarnil and royal jelly have been used to retard the ageing process because of their antioxidant and antiaging activities.

Royal Jelly (RJ) is a functional food secreted by the hypopharyngeal and mandibular glands of worker bees. RJ has many properties, including antitumor, antibacterial, antioxidant, antiaging, hypotensive, growth-stimulating, and anti-inflammatory activities (20). It is believed that RJ can prolong life span because it prolongs the longevity of queens comparing to worker bees. In a mice model study, it is suggested that a 16-week RJ supplementation prolonged the average life span protecting the DNA and lowering oxidative stress (21).

Apilarnil is mainly a drone larvae extract that also contains small amounts of royal jelly, bee bread, honey, and propolis. Apilarnil has many pharmacological activities, such as anabolism stimulator, antiviral, immunomodulator, biostimulator (22, 23). Additionally, because it comes from a "male-like" structure, apilarnil is very rich in male-like hormones, so it stimulates the spermatogenesis (22, 24) and regulates the human endocrine system (25, 26, 27). It also has high levels of 10-HDA (10-hydroxy-2-decenoic acid), and many vitamins and minerals (27, 28) affecting antioxidant capacity. (29) reported that both the total antioxidant potential and activity of capturing the free radicals of RJ and drone larvae are sufficiently high, and their combination leads to extremely valuable products.

The present study was conducted to determine the possibilities that calorie restriction or dietary apilarnil and royal jelly supplementation may be used in retarding the reproductive ageing of broiler breeder males. We evaluated the effects of those dietary manipulations on the aging of roosters by quantifying lipid peroxidation, some antioxidant activities, and semen quality parameters. Moreover, these age-related parameters were compared with those of the *ad libitum* feeding group.

Materials and methods

The ethical committee approval of Ege University (2011-092) was granted in order to conduct this study.

Experimental design and diets

A total of 160 broiler breeder males (Ross 308) at 52 weeks of age were used in the present study. Males were identified with a leg tag and randomly assigned to 4 treatment groups with four replications. The treatments were 1) males were fed a restricted feed (135 g/day) as recommended by the breeder company (Control-CONT); 2) males were fed *ad libitum* for a four-week experimental period (*Ad libitum*-ADL), 3) males were fed similar to the CONT group except that diet was supplemented with bee products (apilarnil 5 g/d/male and royal jelly 200 mg/d/male) (Bee products-BP); 4) calorie restriction was applied at about 45 % of the standard diet for a four-week experimental period (Calorie restriction-CR). After a four-week adaptation period, the experiment was continued for 18 weeks. Males were placed in floor pens and fed a standard diet (Table 1) except for the four-week experimental period.

1: Allzyme SSF, *Aspergillus niger* (CBS 114.94) amylase, cellulase, phytase, xylanase, beta-glucanase, pectinase, protease.

2: 2 kg mineral mixture, antioxidant, 125.000 mg; copper, 10.000 mg; calcium D pantothenat, 15.000 mg; zinc, 100.000 mg; D-Biotin, 250 mg; iron, 60.000 mg; folic acid, 2.000 mg; iodine, 2.000 mg; cobalt, 500 mg; manganese, 80.000 mg; niacin, 55.000 mg; selenium, 250 mg.

3: 1 kg vitamin mixture, retinol-acetate, 13.000.000

IU; thiamine, 3.000 mg; cyanocobolamin, 40 mg; riboflavin, 12.000 mg; pyridoxine, 4.500 mg; cholecalciferol, 3.000.000 IU; α-tocopherol acetate, 100.000 mg; menadione, 5.000 mg.

4: Toxin binder, probiyotic, vitamin D_3

Table 1: Nutrient composition and analysis of standard and calorie restriction diets (g/kg)

Ingredients	**Standard diet (g/kg)**
Corn	748.49
Full fat soybean	40.00
Soybean cake	12.42
Sunflower cake	148.76
Acid oil	10.00
Limestone	23.53
MCP-22.7	4.28
Sodium sulphate	1.21
Salt	2.40
DL-Methionine	0.89
Vitamin C	0.20
L-Lysine sulphate	0.32
Colin chloride liquid	0.50
Enzyme mixture[1]	0.20
Trace Mineral mixture[2]	0.50
Vitamin mixture[3]	2.00
Other additives[4]	4.80
Analysed composition	**(g/kg)**
Dry matter	875.70
Crude protein	133.40
Crude fat	50.00
Crude fibre	50.70
Crude ash	56.60
ME (MJ kg^{-1})	12.01

Sample collection and analysis

Blood samples for biochemical analysis were collected from 10 males of each treatment group at the 4th, 8th, and 18th weeks of the trial. Lipid peroxidation (LPO) was ascertained by the formation of malondialdehyde (MDA), which was estimated using the thiobarbituric acid (TBARS) method (30). Superoxide dismutase (SOD) activity was determined using the commercially available enzyme kit (Ransod, RANDOX/SD-125). Glutathione peroxidase (GSH-Px) activity was determined using a Ransel kit (RANDOX/RS-504). The Randox Uric Acid (UA) Enzymatic Colorimetric method kit was used for uric acid analysis. Total antioxidant capacity (TAC) was measured using an Abbott Architect Analyzer commercial kit (Abbott Lab. Illinois, USA).

Semen collection and evaluation

Semen samples were collected using the abdominal massage technique (at 2nd, 6th, 10th, and 16th weeks of trial) and evaluated in 20 minutes using Sperm Vision System (Minitüb Abfüll und Labortechnik Gmbh&Co.KG). Semen volume, motility (%), dead sperm (%), progressive motility (%) were determined in each semen sample.

Statistical analysis

The data were analysed using a one-way analysis of variance (ANOVA) with the General Linear Models (GLM) procedure of the SAS software (31). Significant differences between groups were determined by Student's t-test. Differences were considered to be significant at $P<0.05$ and the results were presented as the mean and standard error of the mean (SEM).

Results and discussion

The effects of dietary manipulations on semen quality parameters are presented in Table 2. As seen prior to treatments, there were no significant differences among groups for semen quality parameters. Dietary treatments caused dramatic changes in all semen parameters throughout the experimental period. At the 2nd week, BP supplementation positively affected semen quality. These males had the highest progressive sperm motility and semen volume. However, CR treatment adversely affected semen quality. Lower semen volume, lower sperm motility and higher dead sperm were observed in CR males compared to the other groups. After a four-week CR period, the detrimental effects of CR on semen production were gradually alleviated by increasing feed allocation.

Table 2: Semen quality characteristics in broiler breeder males during the experimental period (±SEM)

GROUP	Pretreatment				2 wk				6 wk				10 wk				16 wk				Pre vs Post-treatment			
	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume (ml)	Motility (%)	Progressive sperm motility (%)	Dead sperm (%)	Semen volume	Motility	Progressive sperm motility	Dead sperm
CONTROL	0.23 ± 0.02	98.06 ± 0.71	87.62 ± 0.75	2.13 ± 0.34	0.27 ± 0.01^{c}	95.21 ± 0.47^{b}	88.21 ± 0.52^{b}	9.56 ± 0.92^{a}	0.25 ± 0.01^{b}	88.39 ± 0.70^{b}	84.69 ± 0.92^{b}	11.27 ± 1.07^{a}	0.18 ± 0.02^{b}	85.36 ± 0.86^{b}	78.25 ± 0.97^{c}	16.32 ± 1.83^{b}	0.18 ± 0.01^{c}	84.26 ± 0.88^{b}	80.39 ± 0.93^{c}	16.92 ± 1.48^{a}	NS	***	**	***
ADL	0.31 ± 0.04	98.12 ± 0.55	84.97 ± 1.89	2.07 ± 0.68	0.39 ± 0.02^{b}	98.56 ± 0.53^{a}	87.21 ± 0.59^{b}	2.06 ± 0.62^{b}	0.21 ± 0.02^{bc}	82.11 ± 1.39^{c}	69.32 ± 1.03^{d}	8.66 ± 1.55^{a}	0.25 ± 0.04^{b}	78.21 ± 0.83^{c}	60.10 ± 1.06^{d}	24.34 ± 1.49^{a}	0.33 ± 0.04^{b}	80.95 ± 2.21^{b}	72.69 ± 2.03^{d}	20.48 ± 2.43^{a}	NS	***	**	***
BP	0.30 ± 0.07	97.51 ± 0.73	84.41 ± 2.31	1.73 ± 0.36	0.46 ± 0.03^{a}	95.31 ± 0.54^{b}	91.27 ± 1.31^{a}	2.33 ± 0.50^{b}	0.72 ± 0.10^{a}	98.96 ± 0.16^{a}	94.32 ± 0.49^{a}	0.41 ± 0.11^{b}	0.61 ± 0.02^{a}	99.26 ± 0.11^{a}	93.85 ± 0.30^{a}	0.79 ± 0.13^{d}	0.43 ± 0.01^{a}	97.89 ± 0.12^{a}	93.00 ± 0.35^{a}	1.51 ± 0.21^{b}	NS	NS	**	NS
CR	0.36 ± 0.05	97.64 ± 0.74	87.23 ± 2.32	2.45 ± 0.29	0.11 ± 0.01^{d}	88.21 ± 0.72^{c}	81.51 ± 0.73^{c}	7.89 ± 0.42^{a}	0.07 ± 0.01^{c}	81.58 ± 0.43^{c}	80.63 ± 1.31^{c}	9.23 ± 0.73^{a}	0.27 ± 0.02^{b}	87.15 ± 0.68^{b}	84.53 ± 0.45^{b}	5.51 ± 0.48^{c}	0.39 ± 0.01^{ab}	96.25 ± 0.66^{a}	89.33 ± 0.91^{b}	4.62 ± 0.94^{b}	NS	NS	NS	*
X±SEM	0.30 ± 0.02	97.83 ± 0.32	86.06 ± 0.94	2.09 ± 0.21	0.31 ± 0.03	94.32 ± 0.90	87.05 ± 0.89	5.46 ± 0.81	0.31 ± 0.06	87.76 ± 1.64	82.24 ± 2.10	7.39 ± 1.05	0.33 ± 0.04	87.50 ± 1.76	79.18 ± 2.85	11.74 ± 2.18	0.33 ± 0.02	89.84 ± 1.77	83.85 ± 1.89	10.88 ± 1.96				
Probability	NS	NS	NS	NS	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***				

[abc]dMean values within the same column sharing a common superscript letter are not statistically different at P<0.05.*: P<0.05; **: P<0.01; ***: *: P<0.05; **: P<0.01; ***: P<0.0001; NS: Not Significant (P>0.05). ADL: *Ad libitum*; BP: Bee Products; CR: Calorie Restriction.

At the end of the experiment (16th week), all semen quality parameters of CR and BP males were significantly better than those of CONT and ADL. Semen volume was higher in ADL males than that of CONT, and there were significant differences in sperm motility and dead sperm between these groups.

The semen characteristics significantly changed throughout the experimental period (Table 2). The percentage dead sperm of ADL males markedly increased from 2.07 % at the beginning to 20.46 % on the 16th week of the experiment. Similarly, in CONT males, the percentage of dead sperm increased from 2.13 % to 16.92 % during the experimental period. At the end of the experiment, a 13-fold increase in percentage dead sperm of ADL males occurred in comparison with the BP males. It is noteworthy that dead sperm (%) was one of the semen characteristics most affected by age and dietary treatments.

At the end of the experiment, the ADL, BP, and CR males maintained semen volume similar to those observed at the beginning of the experiment while long-term restricted males (CONT) produced lower semen volume than the other groups did.

The results of the present study showed that sperm motility and progressive sperm motility significantly decreased while dead sperm dramatically increased in CONT males during the experimental period. These data imply that long-term feed restriction (135 g/d) recommended by the breeder company for males could not prevent age-related declines in semen production. Although during CR treatment period, all semen quality parameters were adversely affected, calorie restriction at 45 % of the standard diet for weeks retarded onset of age-related decline in semen quality. The effect of CR treatment observed in the present study was in agreement with previous studies reporting inadequate ME intake could be detrimental to semen production (4, 32, 33) and these detrimental effects could be revised by increasing male feed allocation (34, 35).

(36) suggested that there were no significant differences in sperm production, semen volume or sperm concentration between full-fed and feed restricted males. Moreover, they reported that while overfeeding might assist a male in semen production for the short-term, the long-term effects of being over-weight are negative ones. In agreement with these findings, we obtained no significant differences in sperm motility and dead sperm between ADL and CONT males, but semen volume was higher in ADL males.

In the BP group, progressive motile sperm significantly increased while there were no negative changes in the other semen characteristics at the end of the experiment compared to the pretreatment period, suggesting that age-related declines in semen production could be alleviated with dietary BP supplementation. Congruent with these results, it was reported that RJ administration caused an increase in sperm production, sperm motility, and higher testosterone levels in lab animals (37, 38, 39). (40) suggested that the androgenic effect of apilarnil on chickens was higher than its anabolic effect. Supporting this report, (41) obtained that apilarnil administration at an early age increased testicular weights and testosterone production and stimulated comb growth in broiler males. (42) also reported that RJ administration has a positive effect on libido, semen quality, sperm output, testosterone level, and fertility of heat-stressed male rabbits. Controversially, (43) suggested that high dose RJ (800 mg/kg) administration for four weeks adversely affected the reproductive system of pubescent male rats, but these detrimental effects are alleviated to some extend by the cessation of RJ.

The effects of dietary manipulation on some antioxidant activities and MDA levels are presented in Table 3. On the 4th week, dietary treatment significantly affected TAC and UA levels, but no significant effects were observed in MDA, SOD, and GSH-Px levels. The TAC and UA levels were significantly higher in the CR group than in others. At the end of the experiment, there were no significant differences in antioxidant capacity and MDA levels among the treatment groups.

Table 3: Some biochemical parameters of broiler breeder males during the experimental period (±SEM)

GROUP	UA (mg/dL)	TAC (mmol/L)	SOD (U/L)	GSH-Px (U/L)	MDA (umol/L)
			4 wk		
CONTROL	10.18±1.09[b]	0.76±0.00[b]	1.27±0.26	22983.17±1710.69	1.92±0.10
ADL	7.22±1.02[b]	0.78±0.14[b]	1.59±0.18	23553.67±2851.07	1.87±0.19
BP	10.28±1.24[b]	1.04±0.14[b]	1.62±0.24	28788.20±3675.19	1.72±0.24
CR	25.38±1.58[a]	1.62±0.07[a]	1.42±0.20	20808.80±3371.93	2.02±0.37
Probability	***	**	NS	NS	NS
			8 wk		
CONTROL	9.80±0.99	0.78±0.05	1.70±0.08	24845.67±2707.96ab	2.58±0.83
ADL	7.94±1.12	0.96±0.10	1.57±0.16	30443.83±2994.62[a]	2.62±0.49
BP	8.56±1.18	1.08±0.26	1.25±0.17	19736.00±910.62[b]	1.58±0.19
CR	8.98±0.99	0.97±0.12	1.41±0.21	18596.00±1869.43[b]	1.22±0.16
Probability	NS	NS	NS	**	NS
			18 wk		
CONTROL	7.30±0.50	0.74±0.07	1.16±0.38	28829.17±4625.01	1.88±0.17
ADL	8.14±0.84	0.89±0.11	1.64±0.21	28227.33±1525.50	2.37±0.65
BP	8.10±0.87	0.77±0.00	1.54±0.17	24122.00±2406.23	3.40±1.21
CR	8.33±1.08	0.81±0.08	1.66±0.15	29366.00±6130.38	1.14±0.21
Probability	NS	NS	NS	NS	NS

[ab]Meansvalues within the same column sharing a common superscript letter are not statistically different at P<0.05.
*: P<0.05; **: P<0.01; ***: P<0.0001; NS: Not Significant (P>0.05). ADL: *Ad libitum*; BP: Bee Products; CR: Calorie Restriction. UA: Uric Acid; TAC: Total Antioxidant Capacity; SOD: Superoxide Dismutase; GSH-Px: Glutathione Peroxidase; MDA: Malondialdehyde.

Overexpression of antioxidant enzymes has been considered to be a protective response to oxidative stress (44, 45, 46). Therefore, both increased antioxidant activity and no significant increase in MDA level of CR males may be interpreted as a protective response to LPO damage. Supporting our results, previous studies showed that in calorie-restricted animals, free radical generation and LPO decreased (47, 48) antioxidant defence capacity was enhanced (49), DNA damage decreased (50), and the rate of the ageing process was modulated by retarding many age-related physiological declines (51). (52) suggested that a-10 day fasting treatment can be used to decrease oxidative stress-mediated injury in aged hens without affecting the welfare of hens from previous fasting experiments.

After a four-week treatment period, it was observed no significant difference among treatment groups in MDA levels and antioxidant capacity except GSH-Px activity on the 8th week, suggesting that all groups had similar LPO responses and that there were no significant differences in possible age-related oxidative stress.

It is reported that RJ has high antioxidant activity and scavenging ability against free radical (20, 29, 39). However, in the present study, BP supplementation (royal jelly plus apilarnil) for a four-week period could not enhance the antioxidant capacity, indicating that the antioxidant properties of these products were probably not adequate to affect the oxidative status of males. Supporting this result, (53) reported that RJ and its bioactive component 10-HDA, did not scavenge any ROS; dietary RJ might have protective effects against tissue damage through other mechanisms other than ROS scavenging. There was no report focusing on the antioxidant effect of apilarnil in poultry; therefore, the present results could not be compared with other studies in the literature.

As a result, it was obtained that the ageing effect was most pronounced in the percentage of dead sperm, especially in males fed ad libitum and restricted as recommended by the breeder companies. Royal jelly plus apilarnil supplementation or calorie restriction for a

four-week period positively affected the semen characteristics of broiler breeder males at 56 weeks of age, and these beneficial effects could be maintained to some extent until 72 weeks of age. Supporting these results, (54) determined that antioxidant diet supplementation resulted in a higher percentage of normal sperm cells in male broiler breeders older than 50 weeks.

Calorie restriction has been enhanced antioxidant defence for the first four-week period, indicating a protective mechanism against oxidative stress. However, after the calorie restriction period, this positive effect could not be sustained. A four-week calorie restriction period may not be long enough to induce a long-lasting oxidative response; a longer time may be required to obtain long term antiaging and antioxidative effects.

In conclusion, it seems to be possible that dietary bee products supplementation or calorie restriction can be used to slow down the rate of the ageing process and extended reproductive life span of broiler breeder males by retarding an age-related decline in semen production. However, the long-term moderate feed restriction recommended by the breeder companies could not prevent age-related decline in semen quality parameters.

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The authors declare that they have no conflict of interest.

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VPLIV ČEBELJIH PRIDELKOV IN OMEJEVANJA KALORIJ NA PROIZVODNJO SEMENA IN OKSIDATIVNI STRES PRI STAREJŠIH SAMCIH PLEMENSKIH BROJLERJEV

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Povzetek: Študija je bila izvedena z namenom raziskovanja učinkov omejevanja kalorij in dodajanja prehranskih čebeljih pridelkov (apilarnil in matični mleček) na reprodukcijske in oksidativne odzive ter ugotoviti možnosti uporabe prehranskih dodatkov za zaviranje reproduktivnega staranja samcev plemenskih brojlerjev. Pri starosti 52 tednov so bili samci plemenskih brojlerjev razporejeni v štiri skupine. Kontrolna skupina je bila ves čas študije krmljena z restrikcijsko krmo po priporočilih podjetja, ki se ukvarja z gojenjem plemenskih broilerjev; skupina *ad libitum* je bila štiri tedne hranjena *ad libitum*; skupina, pri kateri so bili dodani čebelji pridelki je bila krmljena podobno kot kontrolna skupina, le da je bila njihova prehrana štiri tedne dopolnjevana z apilarnilom in matičnim mlečkom, zadnja skupina pa je štiri tedne dobivala kalorično omejeno hrano (45 % običajne prehrane). Po štiritedenskem prilagoditvenem obdobju se je poskus nadaljeval še 18 tednov. Rezultati, pridobljeni v tej študiji, so pokazali, da je bila najbolj prizadeta značilnost staranja povišan odstotek mrtvih semenčic v ejakulatu. Dolgoročna zmerna omejitev krme ni preprečila starostnega zmanjšanja proizvodnje smenčic. Dodatek prehranskih čebeljih pridelkov ali omejevanje kalorij v obdobju štirih tednov je pozitivno vplival na značilnosti semena. Ti blagodejni učinki so se ohranili vse do starosti do 72 tednov. Omejitev kalorij je okrepila tudi antioksidativno obrambo v prvih štirih tednih raziskave; vendar pa se je ta ugodni učinek kasneje izgubil.

Ključne besede: samci plemenskih brojlerjev; staranje; značilnosti semena; oksidativni stres; čebelji proizvodi; omejitev kalorij