Ciência Animal Brasileira
Brazilian Animal Science

DOI: 10.1590/1809-6891v25e-77462E

e-ISSN 1809-6891
Animal science | Research article

Reproductive efficiency of broiler breeder supplemented with canthaxanthin

Eficiência reprodutiva de matrizes de corte suplementadas com cantaxantina

Érica Crosara Ladir de Lucca[1], Patrícia Alves Teixeira[1], Matheus Vicente da Silva[1], Marcelo Emílio Beletti*[1]

[1] Universidade Federal de Uberlândia (UFU), Uberlândia, Minas Gerais, Brazil.
*Corresponding author: mebeletti@ufu.br

Abstract: The inclusion of substances with antioxidant properties in the diet of broiler breeders helps the enzymatic defense system in controlling the damage caused by free radicals in cells, for example, rooster spermatozoa. It is therefore hypothesized that supplementing broilers with canthaxanthin may have a positive effect on the reproductive system of broilers. The objective of this research was to study the action of the addition of canthaxanthin in the diet of broiler breeders on the fertilization and hatching rates of eggs and on the fertility of roosters. For the experiment, hens and roosters received, from 22 weeks of age, feed with and without supplementation of 6 ppm of canthaxanthin. Roosters between 30 and 50 weeks of age that received canthaxanthin in the diet showed less alterations in chromatin over the weeks of life. There were no differences between measurements of seminiferous tubules in different treatments. Canthaxanthin-supplemented broiler breeders had the highest rate of perforation of sperm in the perivitelline membrane, the best hatching rate and the highest fertility rate. It was concluded that the use of canthaxanthin as an antioxidant agent in the diet of broiler breeders improves reproductive efficiency.

Keywords: antioxidants; rooster; chromatin; fertility.

Resumo: A inclusão de substâncias com propriedades antioxidantes na dieta de matrizes auxilia o sistema de defesa enzimática no controle dos danos causados pelos radicais livres nas células, como exemplo, os espermatozoides dos galos. Propõe-se a hipótese de que a suplementação de matrizes de corte com cantaxantina possa ter um efeito positivo no sistema reprodutivo destes animais. O objetivo desta pesquisa foi estudar a ação da adição de cantaxantina na dieta de matrizes de corte sobre as taxas de fertilização e eclosão dos ovos e sobre a fertilidade de galos. Para o experimento, galinhas e galos receberam, a partir de 22 semanas de idade, ração com e sem suplementação de 6 ppm de cantaxantina. Galos entre 30 e 50 semanas de idade que receberam a cantaxantina na dieta apresentaram menos alterações na cromatina espermática ao longo das semanas de vida. Não houve diferença entre as mensurações dos túbulos seminíferos nos diferentes tratamentos. As

Received: October 06, 2023. Accepted: July 02, 2024. Published: September 30, 2024.

matrizes suplementadas com cantaxantina apresentaram a maior taxa de perfuração espermática na membrana perivitelina, melhor taxa de eclosão e maior taxa de fertilidade. Concluiu-se que o uso de cantaxantina como agente antioxidante na dieta de matrizes de corte melhora a eficiência reprodutiva.

Palavras-chave: antioxidantes; galo; cromatina; fertilidade.

1. Introduction

Free radicals are elements present in biological processes that are important in poultry reproduction and production, which can cause damage through the oxidation process in various biological systems. It is important to note that among the damage caused by free radicals, there are direct and indirect damages to chromatin, especially sperm chromatin, ranging from changes in chromatin compaction to spermatic DNA fragmentation [(1)]. The addition of substances with antioxidant properties in the broiler breeder diet helps the enzymatic defense system to control damage caused by free radicals in cells, such as spermatozoa [(2)].

Seminal plasma is naturally enriched with enzymatic and non-enzymatic antioxidants that protect sperm against oxidative stress, and a deficiency of these antioxidants is associated with male fertility problems [(3)]. In the case of the broiler breeder, after copulation, the spermatozoa remain stored in the sperm reservoirs of the oviduct and the hens assume the role of protection against the effects of oxidation on the sperm [(4)]. It has already been shown that the addition of canthaxanthin in the diet of broiler breeder males in the post-peak phase can be a tool to improve the fertility index of old breeders, as it improves the percentage of normal sperm, increases the dewlap weight and width, and increases testes weight and length [(5)].

In this perspective, the use of canthaxanthin added to the diet of roosters and female broiler breeders can exert its antioxidant role in three ways: 1) in the embryo – protecting embryonic tissues during incubation, 2) in the egg – protecting the yolk's nutrients during storage for the developing embryo and, 3) in aging broiler breeder– helping in the antioxidant mechanisms of the semen and oviduct and reducing the oxidative stress of sperm [(6)]. Based on this information that demonstrates the antioxidant role of canthaxanthin, and because it is a possible commercialization additive in industrial poultry farming, the objective of this work was to study the action of the addition of canthaxanthin in the diet of broiler breeders on the fertilization and hatching rates of eggs and on the fertility of roosters.

2. Material and methods

The methodology used was approved by the Ethics Committee on Use of Animals (CEUA) of the Federal University of Uberlândia, and the CEUA protocol certificate number is 012/15. The research was conducted in a conventional broiler breeders production shed located in the municipality of Uberlândia, Minas Gerais, Brzil. The collections took place between the months of January to July 2015. In this experiment, a total of 24,000 one-day-old chicks of

the Ross® AP95 strain were housed, 20,500 females and 3,500 males, which remained in the rearing period in two blackout sheds, with negative pressure, in separate specific pens for females and males.

Experimental rations were offered from the 22 weeks of life onwards. The treatments were defined according to the antioxidants added to the experimental diets, as follows: Treatment 1 - 10,500 females and 1,500 males, housed in a shed with supplementation of canthaxanthin (6ppm), from the 22nd week of life on; Treatment 2 - 10,500 females and 1,500 males, housed in a shed without canthaxanthin in the ration. The collections started in the 30th week of life of the flock and were repeated in weeks 40 and 50 of the animals' life.

One of the evaluations to quantify the fertility of the rooster was the perivitelline membrane perforation count test. For the performance of the perivitelline membrane perforation count test, approximately 60 eggs were collected at each of the collection ages (30, 40, and 50 weeks of life of the broiler breeder). The sample number for this analysis was 10 eggs, per treatment (T1 – with canthaxanthin and T2 – without canthaxanthin) and by age. Eggs were randomly removed from the second egg collection of the day. After collection, they were sent to the laboratory where processing began.

The technique for counting the perforations used in the experiment was initially described by Bramwell and Howarth [(7)] and modified in some aspects by Donoghue [(8)]. Each egg had its yolk carefully separated from the white. A portion of approximately 1 cm² of the perivitelline membrane in the germinal disc region was removed and immersed in PBS to remove the remaining albumen and yolk. The piece of the membrane was placed and positioned stretched over a blade with the aid of tweezers and fixed with 20% formalin. Later, a few drops of Schiff's reagent were used as a dye. Coverslip was placed on the stained and fixed membrane and followed for analysis under a light microscope at 100x magnification. From each slide, the region where the sperm perforations had occurred was located and the number of perforations was counted.

For the analysis of testicular development, 10 roosters from each treatment were randomly collected, at the intermediate age of the test (40 weeks). Fragments of 0.5 cm^3 were obtained from the testis, from each rooster, and fixed in a 10% formalin solution for at least 24 hours. After this period, they were submitted to routine histological techniques (dehydration, diaphanization, and paraffin embedding), following the methodology proposed by Tolosa et al. [(9)]. Five-micrometer-thick sections were prepared on a microtome before being stained with hematoxylin-eosin. Subsequently, the prepared slides were observed under a light microscope, and microphotographed for analysis and measurements of the total diameter and height of the epithelium of the seminiferous tubules, using the HL Image® software. Twelve seminiferous tubules per animal were photographed and measured.

At the ages of 30, 40, and 50 weeks, approximately 20 roosters from each treatment were randomly selected. These cocks were separated from the females for 3 days before semen collection. Semen was collected as described by Burrows and Quinn [(10)], with the method of abdominal massage.

The 0.5mL rooster semen samples were processed for transmission electron microscopy; each sample was placed in tubes with 5% glutaraldehyde. After fixation, the semen was centrifuged 100 xg for 5 minutes, then washed in phosphate buffer three times and discarded the supernatant, post-fixed for four hours in 3% glutaraldehyde and one hour in 1% osmium tetroxide plus ferrocyanide 1.25% potassium. They were dehydrated in ascending series of acetone. Finally, the sediment was embedded in Epon resin (EMbed 812 ®, Electron Microscopy Sciences, Hatfield, PA, US) and subsequently cut in an ultramicrotome to obtain ultrathin cuts. The sections were contrasted with uranyl acetate lead citrate. All samples were analyzed in a transmission electron microscope Tecnai G2-12 Transmission Electron Microscope - SpiritBiotwin FEI - 120 kV (FEI company, Hillsboro, OR, US), when 80 heads of sperm from each sample were evaluated.

Heads that had homogeneously dark (electrodense) nuclei were considered to have chromatin with normal compaction. Heads that had a clear (electrolucent) or heterogeneous color nucleus were considered to have altered chromatin and were classified according to chromatin decompaction levels: mild, which has a small decompaction point in the chromatin structure; medium, where the chromatin presents between one and three decompaction points; intense, presence above three decompaction points or very heterogeneous presentation in more than one point of the sperm head.

The hatching data were obtained by the company Pole Alimentos, responsible for incubating the eggs, according to the hatching formula:

$$hatching = \frac{nº\ hatched\ chicks\ x\ 100}{nº\ total\ incubated\ eggs}$$

Eggs were incubated in multi-stage model machines, with an average storage period of seven days. On the 19th day of incubation, the eggs were transferred to hatchers, where they remained until they completed 21 days. For fertility analysis, eggs were submitted to breakage and visual analysis of the germinal disc and classified as fertile or non-fertile, on the second day of storage, after 50 weeks of age, weekly, 50 eggs per treatment, according to the company standard.

$$Fertility = \frac{nº\ fertile\ eggs\ x\ 100}{nº\ evalited\ eggs}$$

For statistical analysis, data were checked for the presence of outliers. Data referring to the spermatic perforation of the perivitelline membrane and hatching, tests were submitted to the normality and homoscedasticity test of variance and later to the Kruskal-Wallis test along with a Wilcoxon post hoc evaluation and Bonferroni correction was employed for comparison among the groups [11]. The level of significance was set at 0.05, in a bilateral test. To verify the existence or not of statistically significant differences between the fertility rates, after checking the normality of the data, the T-test was applied to the data in question.

The level of significance was set at 0.05, in a bilateral test. To verify the existence or not of statistically significant differences between testicular measurements, the t-test for two means was also applied after verifying the normality of the data. The level of significance was set at 0.05, in a bilateral test. For the chromatin compaction data, the binomial test for two proportions was applied, with a significance level set at 0.05. The Odds Ratio test was also applied to these data, which compares the relationship rate between two measures [12].

3. Result

According to the results, differences were found between the number of spermatic perforations, obtained at, 30, 40, and 50 weeks of age, and at the three ages observed, the highest rate of spermatic perforation in the perivitelline membrane (SPPM) occurred in the batch supplemented with canthaxanthin (Table 1). As a result of the present work, it was also observed a higher number of spermatic perforations in the perivitelline membrane of animals treated with the antioxidant additive at all tested ages (30, 40, and 50 weeks), also indicating possible improvements in fertility and hatching rates.

Table 1. Mean number of spermatic perforations in the perivitelline membrane of eggs from a batch supplemented with canthaxanthin (T1) and not supplemented with canthaxanthin (T2) at three different ages

Week	T1	T2	P-value
30	266 a	71 b	0.049*
40	344 a	114 b	0.041*
50	190 a	75 b	0.021*

Distinct letters indicate significant differences between values ($p \leq 0.05$).

The results of the statistical test to verify or not the difference between the number of SPPMs in relation to the age of the batches is represented in table 2. Regarding the analyzed lifetime, 30, 40, and 50 weeks, no differences were observed between the treatments. This means that in both treatments, the behavior of the average perforation in the perivitelline membrane of the eggs was similar, showing a proportional drop in the last week tested (Table 2). In Figure 1 there is an illustration of the sperm perforations in the perivitelline membrane. There were no differences between the measurements of total diameter and height of epithelium of the seminiferous tubules in the different treatments. (Table 3).

Table 2. Probability test for the variables analysis time – 30, 40 and 50 weeks - and treatment, with canthaxanthin (T1) and without canthaxanthin (T2), for the perivitelline membrane perforation test

Treatment	weeks		P-value
T1	30 a	50 a	0.42
T2	30 a	50 a	0.96

Distinct letters indicate significant differences between values ($p \leq 0.05$).

Table 3. Results of testicular measurement, total diameter and height of epithelium of the seminiferous tubules of roosters supplemented (T1) and not supplemented (T2) with canthaxanthin, at 40 weeks of life

Measure	T1	T2	P-value
diameter (µm)	232.58 a	207.49 a	0.06
Epithelium (µm)	67.83 a	61.54 a	0.22

Distinct letters indicate significant differences between values ($p \leq 0.05$).

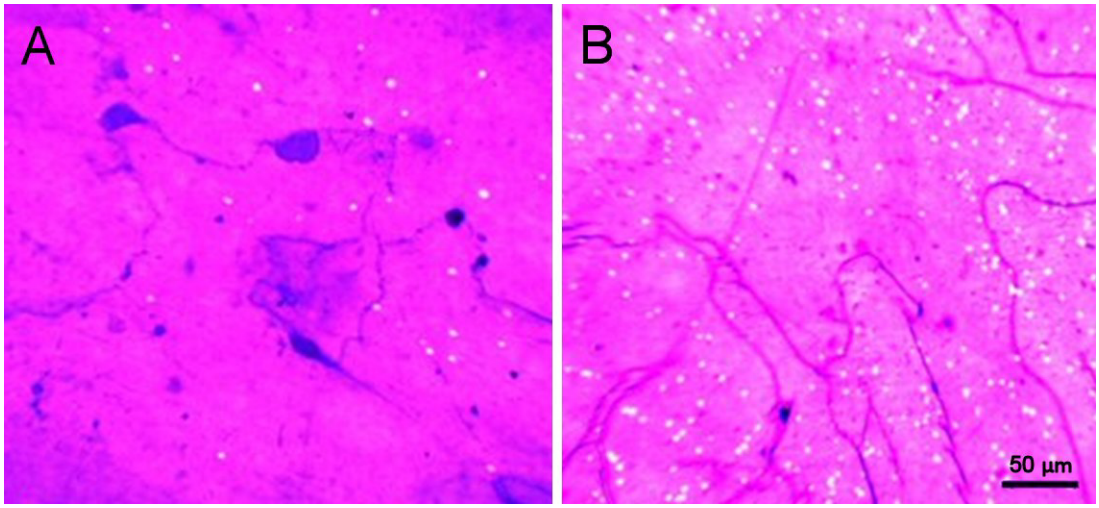

Figure 1. Perivitelline membrane with a low number of spermatic perforations, B- with a large number of perforations in the spermatic membrane. Barr = 50 µm

There was a difference between treatments at week 30 of life, and roosters that received canthaxanthin had a greater amount of normal sperm. As for the degree of decompaction, at this age, it was possible to observe differences in terms of the weak classification level, that is, non-supplemented roosters had a greater amount of sperm with only one decompaction point, in the entire extension of their head. (Table 4). At week 50, it was also possible to observe a greater amount of normal sperm in the roosters that received canthaxanthin. In all degrees of chromatin decompaction, there were differences indicating that roosters that did not receive the antioxidant in the diet had a greater amount of sperm with partial or total chromatin decompaction (Table 5).

Table 4. Degree of chromatin decompaction observed in sperm from roosters supplemented (T1) and not supplemented (T2) with canthaxanthin, at 30 weeks of life

	Degree of decompaction in sperm			
	Normal	Mild	Medium	Intense
T1	79.45 a	3.08 a	5.82 a	11.64 a
T2	67.96 b	15.49 b	5.99 a	10.56 a
P-value	0.00*	0.00*	0.99	0.98

Distinct letters indicate significant differences between values ($p \leq 0.05$).

Table 5. Degree of chromatin decompaction observed in sperm from roosters supplemented (T1) and not supplemented (T2) with canthaxanthin, at 50 weeks of life

	Degree of decompaction in sperm			
	Normal	**Mild**	**Medium**	**Intense**
T1	89.31 a	0.85 a	5.98 a	3.85 a
T2	59.11 b	7.73 b	17.68 b	15.47 b
P-value	0.00*	0.00*	0.00*	0.00*

Distinct letters indicate significant differences between values (p ≤ 0.05).

We observed that at T2 the amount of sperm with some degree of chromatin decompaction increased in relation to the lifetime of the roosters, that is, older roosters had higher amounts of partial or total chromatin decompaction. At T1, the degree of decompaction was lower, that is, the roosters that received the antioxidant in the diet presented a smaller amount of chromatin change over the weeks of life. (Table 6). Figure 2 illustrates the different degrees of chromatin decompaction of rooster sperm.

Table 6. Number of sperm from roosters supplemented (T1) and not supplemented (T2) with canthaxanthin, which showed abnormal chromatin compaction, at 30 and 50 weeks of life

Week	T1	T2	P-value
30	20.54 a	32.04 b	0.01*
50	10.68 a	40.88 b	0.00*

Distinct letters indicate significant differences between values (p ≤ 0.05).

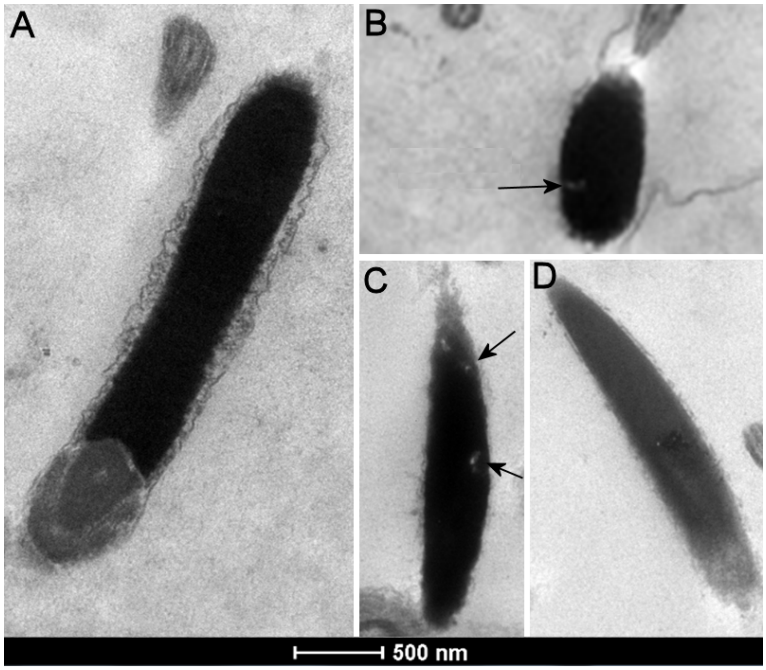

Figure 2. Electron micrograph of rooster sperm heads. A: Sperm head with normal chromatin compaction. B: Mild degree of sperm chromatin decompaction characterized by a small lighter area (arrow); C: Medium degree of chromatin decompaction characterized by several small lighter areas (arrows); D: Sperm head with intense chromatin decompaction and characterized by a lighter region that affects almost the entire head. Barr = 500 nm

According to the Odds Ratio, between the number of changes in the chromatin compaction of treatments in week 30 of the rooster's life, the probability of observing alterations in the sperm chromatin compaction of roosters not supplemented with canthaxanthin (T2) is greater than in supplemented roosters. At week 50, this probability increases, in this case, the greater the chance of observing changes in the chromatin compaction of sperm from roosters that did not receive the antioxidant in the diet (Table 7). This suggests that the negative effect of age on the reproductive efficiency of the female broiler breeder was minimized by the addition of canthaxanthin to the diet. We observed differences in the hatch rate in the period corresponding to weeks 35 to 45 weeks, that is, for these ages, the weekly hatch rates showed improvement in the flocks that received the antioxidant in the diet (Table 8). The average fertility was analyzed in the period of 50-60 weeks, it was possible to observe differences between fertility rates, and the lot that was fed with the addition of canthaxanthin showed higher fertility rates (table 9).

Table 7. Odds Ratio, between number of changes in chromatin compaction of treatments 1 and 2, at ages 30 and 50 weeks

Week	T1:T2	P-value
30	1.82	0.00*
50	5.78	0.00*

Distinct letters indicate significant differences between values ($p \leq 0.05$).

Table 8. Average hatching analyzed in three periods, of eggs from broiler breeders supplemented (T1) and not supplemented (T2) with canthaxanthin

Period (weeks)	T1	T2	P-value
29 - 35	83.12 a	79.37 a	0.14
35 – 45	86.16 a	82.66 b	0.00*
45 – 55	77.85 a	73.86 a	0.17

Distinct letters indicate significant differences between values ($p \leq 0.05$).

Table 9. Average fertility analyzed over a period of eggs from broiler breeders supplemented (T1) and not supplemented (T2) with canthaxanthin

Period (weeks)	T1	T2	P-value
50 – 60	91.54 a	88.36 b	0.02*

Distinct letters indicate significant differences between values ($p \leq 0.05$).

4. Discussion

The broiler breeders that received canthaxanthin in their diet showed better improvement in reproductive efficiency, with better hatching rates at the age of 35 to 45 weeks of life, and higher fertility rate after 50 weeks of life, a period in which the fertility analysis was possible to be performed in a field. The improvement in the reproductive indices of the batch that contains canthaxanthin can be explained by the antioxidant effect that the additive promotes, reducing the oxidative stress of sperm.

The correlation between fertility and hatching and the number of perforations in the perivitelline membrane was found by Christensen et al. [13], where, in their study, strains selected for rapid growth had lower rates of sperm perforation in the perivitelline membrane, when compared to strains selected for egg production. Consequently, egg fertility and embryo viability were also negatively affected.

This correlation between hatching, fertility, and perivitelline membrane perforations can be explained, as according to Robertson et al. [14], in order for sperm to perforate, a complex interaction of hormonal and metabolic regulatory parameters is necessary, together with factors such as sperm motility, the connection between the oocyte and sperm, induction of the acrosomal reaction and the hydrolysis of the perivitelline membrane. According to the author, the technique of counting spermatic perforations in the perivitelline membrane evaluates the result of the sum of all the events mentioned above, which gives the test a wide capacity for evaluating avian fertilization and hatchability rates.

Triques et al. [5], verified in their research that the increase in the number of perforations is directly linked to the number of sperm that manage to reach the oocyte in the infundibulum, thus indirectly indicating the reproductive capacity of roosters. He concluded that the results suggested that adding antioxidants to the ration of breeding roosters resulted in an increase in the number of sperm produced by males or that cells deposited in the vagina had better survival conditions in the oviduct and were able to reach the infundibulum soon after ovulation.

Regarding the lifespan of the birds, we observed that the average number of SPPMs was lower at 50 weeks when compared to the results of 30 and 40 weeks. Still, it was found that the treatment that received the antioxidant in the diet showed smaller changes in chromatin compaction in the 50 weeks of life, when compared to week 30 of the birds' life. This suggests that the addition of the antioxidant was important in protecting the effects of age on chromatin compaction, although drilling results showed a decline.

Regarding the testicular development results, where in this research, no differences were observed between the measurements of total diameter and height of epithelium of the seminiferous tubules in the different treatments, also in their research Triques et al. [5], where the effect of dietary antioxidant supplementation on reproductive characteristics of male broiler breeders in the post-peak production phase was evaluated, the authors also found no differences in testicular development.

Mcgary et al. [15] stated that there is a positive correlation between testicle weight and spermatic production and that this may be one of the factors that differentiate the fertility index of the lot. Likewise, as described by Keel and Abney [16], altered testicular volumes may mean inhibition of testicular development and all of its exocrine and endocrine functions. In addition, Heinlein and Chang [17] correlated that a germinal epithelium, responsible for the production of luminal fluids, is an essential prerequisite for spermatogenesis. However, Yama et al. [18] described that it is not entirely possible to describe the relationship of tubular geometric changes with sperm production, since morphometric data are only a part of

the complete image, and that other factors are clearly important such as the number of spermatogenic cells, by Sertoli, and that these data should serve as preliminary models for a study of the fertilizing capacity as a whole.

González-Morán et al. [19] stated that until the 12 weeks of life of the rooster, the seminiferous tubules of the testes are formed only by a simple layer of Sertoli cells and spermatogonia, evolving, as the animal matures, to a stratified seminiferous epithelium and notorious reduction of interstitial tissue in the sexually mature rooster. Triques et al. [5] concluded that antioxidant supplementation from the early stage of the rooster's life, and not just in the post-peak phase as occurred in their experiment, could have some effect on tubular morphometry. In the present study, roosters began to receive the antioxidant additive in their diet at 22 weeks of age, which may also have contributed to this result. Although there is no statistical difference in the histomorphometry performed in the present study, the height of the epithelium tended to be higher in the treated animals (p=0.06).

In addition to morphological changes, chromatin decompaction also has negative consequences for fertility. During the spermatogenesis phase of mammals, most histones are replaced by protamine, this exchange is of great importance for inducing sperm chromatin condensation, but a small percentage of histones remain retained in the DNA, and defects in the conversion of histones into protamine result in defective chromatin compaction [20, 21]. It is known that in mammals the existence of histones is normal in sperm, but there may be a permanence greater than the maximum 15% proportion of histones in the chromatin structure [22], which would also lead to looser chromatin, interfering with male fertility.

In this study, the results of sperm chromatin compaction indicated a greater degree of partial or total decompaction of roosters not supplemented with canthaxanthin. In addition, the batch not supplemented with canthaxanthin had a lower hatching rate between 35 and 45 weeks of life, and a worse fertility rate.

Johnson et al. [23] and Carrel [24] showed that sperm chromatin alterations are not necessarily marked by DNA damage but may be in histones found in small non-protamine regions and that would be important carriers of epigenetic information, necessary for the early embryonic development. In situations where sperm chromatin disturbances are severe, the fertilization rate is low and embryonic development is impaired [21]. Thus, this could also be another explanation for the worse hatching rates, and a greater degree of chromatin decompaction of unsupplemented sperm, in this research.

According to Opuwari and Henkel [1], free radicals can damage chromatin, ranging from changes in compaction to serious changes in DNA. Therefore, the antioxidant effect of canthaxanthin probably also collaborated in the protection of chromatin and not only the sperm membrane. According to Souza et al. [25], canthaxanthin plays an important role in biological processes, such as maintaining membrane fluidity through its antioxidant action.

Rodrigues et al. [26] observed a greater number of chromatin alterations in older animals, with less homogeneous chromatin and less compaction compared to normal heads, which could be negatively influencing fertility. Soares and Beletti [27] also observed that old roosters

(60 weeks) had more alterations in chromatin compaction than young roosters. Rocha Júnior and Baião [28] evaluated semen from young (35 weeks) and old (68 weeks) roosters and found no significant difference in sperm physical characteristics (motility, vigor, and turbulence), showing that the fall in fertility in old roosters would be caused by other factors, such as loss of protection against oxidative stress in females, for example.

Rosa et al. [29] fed hens and roosters with 6ppm of canthaxanthin, from 46 to 66 weeks of age, and found an improvement in fertility and a decrease in embryonic mortality. Contributing to these data already described in the literature, this research demonstrated that the use of canthaxanthin in the diet improves the sperm chromatin compaction indices, suggesting that the additive minimizes the harmful effects of free radicals on sperm from the ejaculate while they remain stocked in the sperm storage tubules (SST) of females, which contributes to better sperm perforation rates and better hatching and fertility rates in the supplemented batch.

Although it is found in the literature that in semen from fertile roosters there is a small amount of sperm with low chromatin compaction and morphological alterations [30], it is also known that morphological defects in sperm can have serious implications for the hydrodynamic properties of these cells, including negative effects on normal rectilinear movements and uneven progressions, decreasing fertility rates [31].

Soares and Beletti [27] comment that according to studies in mammals, chromatin alterations could not alter the sperm capacity to fertilize the oocyte, that is, the fertility rate could be altered or not, but would interfere in the embryo's evolution, preventing the formation of blastocysts or even leading to death in pre-hatch stages, thus having a direct impact on the hatching rate.

Other studies in mammals that conclude in the same direction as our findings are Ellington et al. [32] and Twigg et al. [33], who concluded that some sperm with chromatin abnormalities can fertilize oocytes in vivo and in vitro, but DNA damage may remain throughout the embryonic period to induce apoptosis and further fragmentation of the embryo, which can lead to miscarriage. Twigg et al. [33] comment that under normal circumstances, damage to the DNA-protein complex could be repaired by the oocyte, with no major consequences.

The likely contribution of hens to improving fertility and hatching rates should be considered, as in this study, females also received the antioxidant in their diet. According to Bonagurio et al. [34], canthaxanthin and 25-hydroxycholecalciferol supplementation increased the expression of antioxidant enzymes (GPx-7, SOD1, and catalase) in the vaginal mucosa of quail, proposes a positive effect on fertility, through the protection of sperm present in the host glands against oxidative damage, resulting in an improvement in reproductive performance.

The action of dietary carotenoids possesses an important role in protecting the sperm plasma membrane against oxidative stress [35]. Another important fact is the pro-vitamin A action of canthaxanthin. Vitamin A deficiency affects the productive performance of males, affecting spermatogenesis, through the inability to protect cells in the reproductive tissue

and gonads against oxidative stress [36]. According to Najafi et al. [37], canthaxanthin reduces membrane lipid peroxidation in sperm, improves sperm motility, reduces agents of oxidative stress, and maintains fluidity, membrane integrity, suppressing caspase-3 activity and increasing superoxide dismutase activity. Supplementation of frozen human sperm with canthaxanthin at concentrations of 10 and 25 μM obtained increase progressive motility, improved acrosome integrity, sperm morphology, and chromatin compaction after thawing when compared to the control group [37].

Rocha et al. [6] observed an increase in vitamin A in egg yolks from batches treated with canthaxanthin and related the improvement in the fertility of the broiler breeders to two factors: an increase in vitamin A and antioxidant protection of sperm. Rosa et al. [38] show us that high levels of carotenoids in the diet of breeders act on resistance to lipid peroxidation, and therefore, the presence of these carotenoids in the egg would have the action to aid development by protecting against lipid peroxidation. Araujo et al. [39] believe that the increase in the fertility of eggs from supplemented broiler breeders is due to the ability of canthaxanthin to prevent the oxidation of lipids present in the yolk.

The decline in reproductive efficiency with the increasing age of the broiler breeders is already known in the literature. Rocha et al. [6], a history that regardless of diet, fertility reduced by 0.75% per week of aging in broiler breeders between 50 and 60 weeks of age. The negative effect of age on fertility could be explained by the greater susceptibility of sperm from old roosters to oxidative damage, which, in this research, was compensated by the addition of canthaxanthin to the diet, due to the better results obtained by those breeders who received the antioxidant. 54-week-old broiler breeders supplemented with canthaxanthin had an increased hatch rate when compared to non-supplemented breeders [38].

On the ability to neutralize free radicals in relation to age, Weir and Robaire [40] compared the antioxidative enzymatic activity of sperm and the production of free radicals in the maturation of sperm in old and young rats and observed a decrease in the antioxidant capacity associated with the increase in the production of free radicals with the aging of animals. The authors concluded that the drop in spermatic quality of older animals is associated with increased susceptibility of sperm to oxidative damage. As for the reduction in fertility due to factors related to broiler breeders, there may have been a reduction in the efficiency of the antioxidant mechanism of action of SST on sperm stored in the oviduct, with advancing age [41], which would have been compensated by the addition of canthaxanthin in the diet of females.

The “natural” decline in the duration of fertility with age, observed in this research, may also be associated with an easier release of sperm in the SST of hens, reducing the number of sperm able to carry out oocyte fertilization [42]. As for fertility, with regard to factors related to chickens, the addition of canthaxanthin also for females may have contributed to the improvement in the efficiency of the antioxidant mechanisms of action of SST on sperm stored in the oviduct, with advancing age [4,41]. Broiler breeders supplemented with canthaxanthin and 25-hydroxycholecalciferol in their diet had higher results for egg production, fertility, total

hatchability, and fertile egg hatchability, and had lower results for early embryo mortality when compared to breeders without supplementation [39].

Therefore, in addition to males, it would also be important to supplement females during the reproductive phase, due to their role in the conduction and preparation of sperm to the fertilization site. According to Gumulka and Kapkowska [43], the duration of fertility is more related to the female, due to a possible loss in the storage capacity of the seminal storage tubules. They observed that fertility rates decreased with the increasing age of females, even when they were inseminated with semen from younger roosters.

Several authors propose that for sperm to survive within the SST of birds and maintain their fertilization capacity, they need a contribution from this female structure, which has, suggested by their studies, the primary functions of being a source of substrates for the metabolism of resident sperm; being a source of macromolecules that alter the fertilization function and providing protection against oxidative stress [44].

The effects of the use of canthaxanthin as an antioxidant additive in diets have already been described in the literature by other authors, demonstrating the great interest of the broiler breeder market for this additive, as it is commercially available. Triques et al. [5] found that antioxidant supplementation (canthaxanthin and vitamin D) can positively influence the fertility rate in the broiler breeders' batch. And they concluded that eggs from sheds where the roosters were supplemented with a blend of antioxidants showed a greater amount of sperm perforations in the perivitelline membrane, indicating the possibility of gains in fertility using these additives, resulting in greater hatchability.

Bisht et al. [3] and Bansal and Bilaspuri [35] highlighted dietary antioxidants in reducing the oxidative stress of sperm, consisting of vitamins C, E, beta-carotenes, carotenoids, and flavonoids. Rocha et al. [6], in their study, found that canthaxanthin is included in the group of carotenoids and, added to the diet of broiler breeders, can play its antioxidant role in three ways: 1) in the embryo – protecting embryonic tissues during incubation, 2) in the egg – protecting the yolk's nutrients during storage for the developing embryo and, 3) in the heavy broiler breeders – supporting the antioxidant mechanisms of the semen and oviduct and reducing the oxidative stress of sperm.

According to Rosa et al. [38], the high levels of canthaxanthin present in the diet and absorbed by the breeders are transferred to the egg yolk, which leads to increases in carotenoid levels in the developing embryo, and the high levels of carotenoids remain present in chicks throughout of the first week after hatching. Moreover, The increase in the concentration of canthaxanthin being present in the egg yolk is correlated with increased resistance to oxidative stress. According to Duarte et al. [2], the inclusion of Canthaxanthin and 25-hydroxycholecalciferol in the diet reduced embryo mortality and increased the hatching percentage and the number of viable chicks produced by birds.

5. Conclusion

Including canthaxanthin in the diet of broiler breeders enhances the fertilization and hatching rates of eggs and the fertility of roosters.

Conflicts of Interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization: Lucca, E. C. L and Beletti, M. E. *Formal Analysis*: Lucca, E. C. L and Beletti, M. E. *Funding acquisition:* Beletti, M. E. *Investigation:* Lucca, E. C. L and Teixeira, P. A. *Methodology:* Lucca, E. C. L and Beletti, M. E. *Resources:* Beletti, M. E. *Validation:* Lucca, E. C. L; Teixeira, P. A; Beletti, M. E and Silva, M. V. *Visualization:* Lucca, E. C. L. *Writing (original draft):* Lucca, E. C. L; Teixeira, P. A; Beletti, M. E and Silva, M. V. *Writing (review & editing):* Lucca, E. C. L; Teixeira, P. A; Beletti, M. E and Silva, M. V.

Acknowledgments

The authors would like to thank the funding for the realization of this study provided by the Brazilian agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior -Brasil), Finance Code 001, and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico -Brasil)

References

1. Opuwari CS, Henkel RR. An Update on Oxidative Damage to Spermatozoa and Oocytes. BioMed Research International, v.2016, p.1-11, 2016. Available from: https://doi.org/10.1155/2016/9540142.

2. Duarte V, Minafra CS, Santos FR, Perim FS. Inclusion of canthaxanthin and 25-hydroxycholecalciferol in the diet of broiler breeders on performance and incubation parameters. Ciência Rural. 2015; 45(11):2050-2055. Available from: https://doi.org/10.1590/0103-8478cr20140564 .

3. Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility. Nature Reviews Urology. 2017; 14(8):470-485. Available from: https://doi.org/10.1038/nrurol.2017.69

4. Bakst MR, Bauchan G. Apical blebs on sperm storage tubule epithelial cell microvilli: Their release and interaction with resident sperm in the turkey hen oviduct. Theriogenology. 2015; 83(9):1438–1444. Available from: https://doi.org/10.1016/j.theriogenology.2015.01.016.

5. Triques GE, Schmidt JM, Oro CS, Bordignon HF, Donin DG, Fernandes JIM. Effect of dietary antioxidant supplementation on reproductive characteristics of male broiler breeders during the post-peak production phase. Semina: Ciências Agrárias. 2016; 37(4):2557-2566. Available from: https://doi.org/10.5433/1679-0359.2016v37n4Supl1p2557

6. Rocha JSR, Barbosa VM, Lara LJC, Baião NC, Cançado SV, Lana AMQ, Pompeu MA, Vasconcelos RJC, Machado ALC, Miranda DJA, Fernandes MNS, Mendes PMM. Efeito do armazenamento e da cantaxantina dietética sobre a qualidade do ovo fértil e o desenvolvimento embrionário. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2013; 65(3):792-800. Available from: https://doi.org/10.1590/S0102-09352013000300027 .

7. Bramwell RK, Howarth BJr. Preferential attachment of cock spermatozoa to the perivitelline layer directly over the germinal disc of the hen's ovum. Biology Reproduction. 1992; 47(6):1113-1117. Available from: https://doi.org/10.1095/biolreprod47.6.1113 .

8. Donoghue AM. The effect of twenty-four hour in vitro storage on sperm hydrolysis through the perivitelline layer of ovipositioned turkey eggs. Poultry Science. 1996; 75(8):1035-1038. Available from: https://doi.org/10.3382/ps.0751035 .

9. Tolosa EMC, Rodrigues CJ, Behmer OA, Neto AGF. Manual de técnicas para histologia normal e patológica. 1st ed. São Paulo: Editora Manole, 2003. 341p. Portuguese.

10. Burrows WH, Quinn JP. The collection of spermatozoa from the domestic fowl and turkey. Poultry Science. 1937; 16(1):19-24. Available from: https://doi.org/10.3382/ps.0160019.

11. Pantos K, Sfakianoudis K, Maziotis E, Rapani A, Karantzali E, Gounari-Papaioannou A, Vaxevanoglou T, Koutsilieris M, Simopoulou M. Abnormal fertilization in ICSI and its association with abnormal semen parameters: a retrospective observational study on 1855 cases. Asian Journal of Andrology. 2021; 23(4):376-385. Available from: https://doi.org/10.4103/aja.aja_84_20.

12. Rumel D. "Odds ratio": algumas considerações. Revista Saúde Pública. 1986; 20(3):253-258. Available from: https://doi.org/10.1590/S0034-89101986000300011.

13. Christensen VL, Fairchild BD, Ort DT,Nestor KE. Dam and sire effects on sperm penetration of the perivitelline layer and resulting fecundity of different lines of turkeys. Journal Applied of Poultry Research. 2005; 14(3):483-491. Available from: https://doi.org/10.1093/japr/14.3.483 .

14. Robertson L, Brown HL, Staines HJ, Wishart GJ. Characterization and application of an avian in vitro spermatozoa-egg interaction assay using the inner perivitelline layer from laid chicken eggs. Journal of Reproduction and Fertility. 1997; 110(2):205-211, 1997. Available from: https://doi.org/10.1530/jrf.0.1100205.

15. McGary S, Estevez I, Bakst MR, Pollock DL. Phenotypic traits as reliable indicators of fertility in male broiler breeders. Poultry Science. 2002; 81(1):102-111, 2002. Available from: https://doi.org/10.1093/ps/81.1.102.

16. Keel BA, Abney TO. Influence of bilateral cryptorchidism in the mature rat: Alterations in testicular function and serum hormone levels. Endocrinology. 1980; 107(4):1226-1233. Available from: https://doi.org/10.1210/endo-107-4-1226.

17. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: an overview. Endocrine Reviews. 2002; 23(2):175-200. Available from: https://doi.org/10.1210/edrv.23.2.0460.

18. Yama OE, Duru FI, Oremosu AA, Noronha CC, Abayomi O. Stereological evaluation of the effects of Momordica charantia, antioxidants and testosterone on seminiferous tubules of rat. International Journal of Morphology. 2011; 29(3):1062-1068. Available from: http://dx.doi.org/10.4067/S0717-95022011000300068.

19. González-Morán MG, Guerra-Araiza C, Campos MG, Camacho-Arroyo I. Histological and sex steroid hormone receptor changes in testes of immature, mature and aged chickens. Domestic Animal Endocrinology. 2008; 35(4):371-379. Available from: https://doi.org/10.1016/j.domaniend.2008.08.001.

20. Yoshida K, Muratani M, Araki H, Miura F, Suzuki T, Dohmae N, Katou Y, Shirahige K, Ito T, Ishii S. Mapping of hitone-binding site in histone replacement-completed spermatozoa. Nature Communications. 2018; 9(1):3885. Available from: https://doi.org/10.1038/s41467-018-06243-9.

21. Colaco S, Sakkas D. Paternal factors contributing to embryo quality. Journal of Assisted Reproduction and Genetics.2018;.35(11):1953-1968. Available from: https://doi.org/10.1007/s10815-018-1304-4 .

22. Beletti ME. Cromatina espermática: quebrando paradigmas. Revista Brasileira de Reprodução Animal. 2013; 37(2):92-96. Available from: http://cbra.org.br/pages/publicacoes/rbra/v37n2/pag92-96%20(RB465).pdf .

23. Johnson GD, Lalancette C, Linnemann AK, Leduc F, Boissonneault G, Krawetz SA. The sperm nucleus: chromatin, RNA, and the nuclear matrix. Reproduction. 2011; 141(1):21-36. Available from: https://doi.org/10.1530/REP-10-0322.

24. Carrel DT. Epigenetics of the male gamete. Fertility and Sterility. 2012; 97(2):267-274. Available from: https://doi.org/10.1016/j.fertnstert.2011.12.036.

25. Souza HM, Arruda LCP, Monteiro MM, Nery IHAV, Araújo Silva RAJ, Batista AM, Guerra MMP. The effect of canthaxanthin on the quality of frozen ram spermatozoa. Biopreservation and Biobanking. 2017; 15(3):220–227. Available from: https://doi.org/10.1089/bio.2016.0049.

26. Rodrigues ACN, Rocha JV, Beletti ME. Análise computacional da compactação da cromatina de espermatozoides de galo. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2009; 61(6):1302-1307. Available from: https://doi.org/10.1590/S0102-09352009000600008 .

27. Soares JM, Beletti ME. Avaliação da integridade cromatínica de espermatozóides de galos (Gallus gallus, Linnaeus, 1758) de linhagem pesada de duas idades. Brazilian Journal of Veterinary Research and Animal Science. 2006a; 42(4):543-553. Available from: https://doi.org/10.11606/issn.1678-4456.bjvras.2006.26471.

28. Rocha Júnior JM, Baião NC. Características físicas do sêmen de galos de matriz pesada com 35 e 68 semanas de idade. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2001; 53(6):683-685. Available from: https://doi.org/10.1590/S0102-09352001000600012.

29. Rosa AP, Scher A, Sorbara JO, Boemo LS, Forgiarini J, Londero A. Effects of canthaxanthin on the productive and productive and reproductive performance of broiler breeders. Poultry Science. 2012; 91(3):660-666. Available from: https://doi.org/10.3382/ps.2011-01582.

30. Soares JM, Beletti ME. Avaliação da morfologia e da compactação cromatínica em espermatozóides de galo (Gallus gallus, Linnaeus, 1758) através de microscopia electrônica de transmissão. Brazilian Journal of Veterinary Research and Animal Science. 2006b; 43(4):554-560. Available from: https://doi.org/10.11606/issn.1678-4456.bjvras.2006.26472.

31. Beletti ME, Costa LF, Guardieiro MM. Morphometric features and chromatin condensation abnormalities evaluated by toluidine blue staining in bull spermatozoa. Brazilian Journal Morphology Science. 2005; 22(2):85-90. Available from: http://www.jms.periodikos.com.br/article/587cb4587f8c9d0d058b460a/pdf/jms-22-2-587cb4587f8c9d0d058b460a.pdf

32. Ellington JE, Evenson DP, Fleming JE, Brisbois RS, Hiss GA, Broder SJ, Wright RW Jr. Coculture of human sperm with bovine oviduct epithelial cells decreases sperm chromatin structural changes seen during culture in media alone. Fertility and Sterility. 1998; 69(4):643- 649. Available from: https://doi.org/10.1016/S0015-0282(98)00023-5.

33. Twigg JP, Irvine DS, Aitken RJ. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Human Reproduction. 1998; 13(7):1864-1871. Available from: https://doi.org/10.1093/humrep/13.7.1864

34. Bonagurio LP, Cruz FK, Kaneko IN, Matumoto-Pinto PT, Murakami AE, Santos TC. Dietary supplementation with canthaxanthin and 25-hydroxycholecalciferol has beneficial effects on bone and oxidative metabolism in European quail breeders. Poultry Science. 2020; 99(10):4874-4883. Available from: https://doi.org/10.1016/j.psj.2020.06.021 .

35. Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International. 2010; 2011:686137. Available from: https://doi.org/10.4061/2011/686137.

36. Vanderhout SM, Rastegar Panah M, Garcia-Bailo B, Grace-Farfaglia P, Samsel K, Dockray J, Jarvi K, El-Sohemy A. Nutrition, genetic variation and male fertility. Translational Andrology and Urology. 2021; 10(3):1410-1431. Available from: https://doi.org/10.21037/tau-20-592.

37. Najafi L, Halvaei I, Movahedin M. Canthaxanthin protects human sperm parameters during cryopreservation. Andrologia. 2019; 51(10):e13389. Available from: https://doi.org/10.1111/and.13389.

38. Rosa AP, Bonilla CE, Londero A, Giacomini CB, Orso C, Fernandes MO, Moura JS, Hermes R. Effect of broiler breeders fed with corn or sorghum and canthaxanthin on lipid peroxidation, fatty acid profile of hatching eggs, and offspring performance. Poultry Science. 2017; 96(3):647–658. Available from: https://doi.org/10.3382/ps/pew294.

39. Araujo LF, Araujo CSS, Pereira RJG, Bittencourt LC, Silva CC, Cisneros F, Hermes RG, Sartore YGA, Dias MT. The dietary supplementation of canthaxanthin in combination with 25OHD3 results in reproductive, performance, and progeny quality gains in broiler breeders. Poultry Science. 2019; 98(11):5801-5808. Available from: https://doi.org/10.3382/ps/pez377.

40. Weir CP, Robaire B. Spermatozoa have decreased antioxidant enzymatic capacity and increased reactive oygen species production during aging in the brown Norway rat. Journal of Andrology. 2007; 28(2):229-240. Available from: https://doi.org/10.2164/jandrol.106.001362.

41. Rutz F, Anciuti MA, Pan EA Fisiologia e manejo reprodutivo de aves. In: Macari M, Mendes AA. 1st ed. Manejo de matrizes de corte, Campinas: FACTA, 2005. pp. 76-143.Portuguese.

42. Rutz F, Anciuti MA, Xavier EG, Roll VFB, Rossi P. Avanços na fisiologia e desempenho reprodutivo de aves domésticas. Revista Brasileira de Reprodução Animal. 2007; 31(3):307-317. Available from: http://cbra.org.br/pages/publicacoes/rbra/download/307.pdf

43. Gumułka M, Kapkowska E. Age effect of broiler breeders on fertility and sperm penetration of the perivitelline layer of the ovum. Animal Reproduction Science. 2005; 90(1-2):135-148. Available from: https://doi.org/10.1016/j.anireprosci.2005.01.018.

44. Bakst, M.R. Role of the oviduct in maintaining sustaines fertility in hens. Journal Animal Science. 2011; 89(5):1323-1329. Available from: https://doi.org/10.2527/jas.2010-3663.