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Alpha-Lipoic Acid on Female Gamecocks Productive Parameters and Cardiac Mitochondrial Biogenesis

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Authors' contributions

This work was carried out in collaboration among all authors. Author BPM carried out the experimental trial. Author LG-D, carried out the experimental trial and contributed to the draft of the manuscript. Authors EPG, AV-E, and ASM contributed to draft the manuscript and author OMI designed the study, performed the statistical analysis, and wrote the manuscript. All authors read and approved this manuscript.

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ABSTRACT

Background: Alpha-lipoic acid (ALA) is an endogenous antioxidant responsible for the removal of free radicals in all cell types. In farm animals, its use improves weight gain, energy metabolism, and the response to oxidative stress. The purpose of this work was to measure the effect of ALA on productive variables and cardiac mitochondrial biogenesis in female fighting cocks (*Gallus gallus*).

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Methodology: Two groups of 38 4-week-old chicks received either 0 or 40 ppm of ALA in food for 9 weeks. Different parts of the carcass, additional to the liver and the heart, were weighed, the antioxidant capacity in serum was measured, and the expression of the mRNAs for five genes involved in mitochondrial biogenesis was quantified in the heart using real-time PCR.
Results: No differences were observed in growth performance between the two groups ($P > .1$). The carcass yield was higher in the birds that consumed ALA (69 vs. 65%; $P < .05$); however, no significant differences were observed in the breast, leg with thigh, wing, and liver yields ($P > .1$). Heart performance was higher (0.71%) in birds treated with ALA compared to the control group (0.67%; $P < .05$), and the total antioxidant capacity (599.71 vs. 554.64 UT, $P < .05$) was improved with ALA. The expression of the mRNAs for NRF1, NRF2, PGC1-α, MT-ND1, and SIRT1 was increased in the heart of the birds treated with ALA ($P < .05$), which may suggest that there was an increase in cardiac mitochondrial biogenesis.
Conclusion: The addition of ALA to the diet of fighting cocks could improve their strength during a fight; however, further testing is required to determine gender-specific effects of ALA supplementation.

Keywords: Gallus gallus; alpha-lipoic acid; productive parameters; cardiac hypertrophy; mitochondrial biogenesis; fighting cocks.

1. INTRODUCTION

Poultry is classified into three categories: meat production, egg production, and recreation or entertainment [1]. The latter include cockfighting, which has had an enormous influence on the domestication and spread of chickens in the world. Cockfighting has a long history that began over 2,500 years ago in China [2]. Cockfighting is important in the world, particularly in Mexico, due to the economic, social, and health impacts, regardless of some opposing groups, who consider it a controversial activity [2,1].

Additives are all those compounds that are added to food to improve, in some way, its appearance, shelf life, acceptance, ingestion, digestion, absorption, or metabolism, although strictly speaking, they are not essential for animal nutrition [3]. Some common food additives are binders, enzymes, flavorings, and antioxidants. Among the antioxidants, α-lipoic acid (ALA), a fatty acid derived from octanoic acid, is considered the ideal antioxidant because of its low redox potential and its capacity not only to scavenge reactive oxygen species but also to regenerate the activity of endogenous and exogenous antioxidants, such as glutathione and vitamins C and E, respectively [4]. Additionally, ALA participates as a cofactor for the pyruvate dehydrogenase and the α-ketoglutarate dehydrogenase complexes of the Krebs cycle, conferring it an important role in metabolism [5].

ALA has been used in the laboratory [6,7], and farm animals (broilers, quails, and swine), improving weight gain, reducing oxidative stress and ascites syndrome-related problems, and stimulating insulin sensitivity [8,9,10,11,12]. It is also known to regulate mitochondrial biogenesis through the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [13].

Several studies have suggested that ALA exerts cardio-protective effects against hypertension [14,15], diabetic cardiomyopathy [16], and ischemia-reperfusion injury [4].

It is known that the heart in birds is proportionally up to two times larger than that of a mammal of the same size and has an output volume capacity of up to seven times higher than that of a man or a dog [17].

With this background in mind and using the work by Díaz-Cruz et al. [9] as a reference, the objective of this work was to evaluate the effect of ALA in the diet of female fighting cocks on productive variables and cardiac mitochondrial biogenesis to assess the possibility of using this compound in the diet of such birds in the future.

2. MATERIAL AND METHODS

2.1 Location

This experiment was performed in the facilities of the Juriquilla Campus of the National Autonomous University of Mexico (UNAM), located in the municipality of Querétaro, state of Querétaro in Mexico, with geographic coordinates 20°35'17" N, 100°23'17" W, and 1900 masl.

2.2 Animals and Treatments

A total of 76, 4-week-old female fighting cocks belonging to the Hatch, Sweater, Mclean,

Roundhead, and Grey strains, were used in this study. The birds were kindly donated by the "Melchor Ocampo" farm located in Tepotzotlán, State of Mexico.

On reception day, the birds were vaccinated against Newcastle (Newcastle La Sota, Merial Select, Gainesville, GA, USA) and the avian smallpox virus (Maver®, Mexico). On the sixth day after the reception, a coccidiostat (Baycox® 5%, Bayer, Mexico) was administered in the drinking water at a dose of 0.5 mL/L (mL L^{-1}) as a preventive treatment.

The birds were distributed following a completely randomized design into six pens containing 12 or 13 birds each. Each pen represented a repetition. Two diets were used (control and 40 ppm ALA) in a 9 weeks trial. Weight gain, food intake, and food conversion were registered. The animals were weighed every 14 days and at the time of sacrifice.

2.3 Diets

During the experiment, all the birds received a Purina™ feed, especially designed for gamecocks. Startina plus was used from 4 to 8 weeks of age and then Startina from 8 weeks to the end of the experiment. Both feeds were ground to facilitate mixing with the ALA. Table 1 shows the composition of both feeds.

2.4 Sacrifice

After the nine weeks of treatment (13 weeks of age), all birds were sacrificed at the animal facility of the UNAM Juriquilla Campus in full compliance with the Institutional Experimental Animal Care and Use Committee (CICUAE) standards. Blood samples were taken, the feet and skin were removed, and the animals were eviscerated to obtain the carcass weight, as well as the weights of liver and heart; finally, the carcasses were cut up and the right breast, right leg with thigh, and right-wing were weighed.

The hearts were sampled, immediately frozen in liquid nitrogen, and kept at -80°C until their analysis. Blood samples were centrifuged to obtain the serum for the antioxidant capacity test (QuantiChromTM Antioxidant Assay Kit, BioAssay Systems, Hayward, CA, USA).

A total of 50 birds, 25 controls, and 25 of the ALA treatment were used for the analysis of these data. For this purpose, 8 or 9 animals were taken randomly from each pen.

2.5 Heart Mitochondrial Biogenesis Gene Expression

A total of 50 hearts were taken to measure the changes in mitochondrial biogenesis by ALA feed; they were 4-5 of each pen. The synthesized cDNA was pooled into two samples of 4-5 birds each per pen, obtaining six samples for each treatment (n = 6).

Total RNA from the heart samples was purified using the TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and immediately frozen at -80°C. The RNA obtained was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) at 260 nm, and purity was assessed using the 260/280 nm ratios, which ranged from 1.8 to 2.0. The integrity of the RNA was visualized by electrophoresis in a 1% agarose gel.

After the evaluation of the concentration and purity of the RNA, cDNA was synthesized using 1 µg of total RNA, Oligo dT15, and SuperScript II Reverse Transcriptase (Invitrogen). The RNA samples were pre-treated with DNAse (Roche, Indianapolis, IN, USA) to eliminate genomic DNA contamination.

Specific primers for the nuclear respiratory factor 1 and 2 (NRF1, NRF2), sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), subunit 1 of NADH: ubiquinone oxidoreductase (ND1), peptidylprolyl isomerase A (PPIA), and ribosomal protein large P0 (RPLP0) were designed using the Oligo7 software (Table 2). The PCR products were sequenced in a 310 ABI Prism Sequencer with version 3 Big Dye (Applied Biosystems, Foster City, CA, USA) to verify their identity.

The expression of the mRNA for the genes described above was determined using a StepOne Real-Time PCR system (Applied Biosystems) and the LightCycler FastStart DNA Master Sybr Green I (Roche Applied Science, Mannheim, Germany).

The synthesized cDNA was pooled into two samples of 4-5 birds each per pen, obtaining six samples for each treatment (n = 6). The real-time PCR program used for NRF1, NRF2, PGC1α, ND1, RPLP0, and PPIA consisted of a 10-min

denaturation at 95°C, followed by 40 quantification cycles consisting of a 10-s denaturation at 95°C, 10-s annealing at 60°C, and a 15-s extension at 72°C; the melting curve started at 60°C for 1 min and ended with 15 s at 95°C. For SIRT1, the extension at 72°C was for 12 s and the melt curve analysis started at 55°C; the rest of the program was the same as for the other genes quantified.

The expression of the mRNA for NRF1, NRF2, PGC1-α, ND1, and SIRT1 was normalized relative to the geometric mean [18] of PPIA and RPLP0, which were evaluated as the best housekeeping genes by the Excel add-in Normfinder (MDL, Aarhus, Denmark) using the $2^{-\Delta Ct}$ method [19,20].

2.6 Statistical Analysis

A split-plot in time design was used for the analysis of the live weight, voluntary intake, and daily weight gain variables. The weights of the breast, leg-thigh, wing, liver, and heart were expressed relative to the weight of the carcass and were analyzed as the arcsine of the square root of this value in a completely randomized design. The antioxidant capacity and the expression of the mRNA for NRF1, NRF2, PGC1α, ND1, and SIRT1 were analyzed in a completely randomized design as well.

In all cases, the general linear model procedure (GLM) of the statistical analysis software SAS [21] was used with the significance level set at $P < .05$. Least square means (LSM) ± standard error of the mean (SEM) were used to analyze the differences within each data block.

3. RESULTS AND DISCUSSION

No differences between treatments ($P > .1$) were found in the productive variables (Table 3). It has been observed that ALA administration in healthy and obese diabetic rats reduces body weight [6,22,23]; however, it has also been reported that ALA does not affect body weight in rats [24]. In broiler chickens, the addition of 40 ppm ALA to the diet improved the final body weight and the food conversion in a seven-week trial [9], which was not observed in this experiment. Our results suggest that in female gamecocks, ALA administration should be for a longer period (> 9 weeks) to observe effects on weight gain. Díaz-Cruz et al. [9] observed that treatment with ALA in broiler chickens had an increase of 124 g gain relative to the control animals ($P < .05$) from the third up to the seventh week of the production period.

The carcass yield was improved with ALA (65% vs. 69%; $P < .05$; Table 4). In mice, treatment with ALA causes carcass weight and body fat loss due to a reduction in food intake and an increase in fatty acid oxidation, respectively [6]. Kim et al. [25] observed that ALA decreased the activity of AMP-activated protein kinase (AMPK) in the hypothalamus of obese rodents, causing weight loss as a consequence of the decrease in food intake and the increase in energy expenditure. These reports are contrary to what was observed in this work, in which no differences in the breast, leg with thigh, or wing yields concerning the carcass yield were observed between treatments ($P > .1$). Even when the carcass yield of the treated birds was greater ($P < .05$), no increase in their muscle mass (breast, leg with thigh, and wing) was observed. This difference could be represented in strength for the fight as a result of the ability of ALA to improve insulin sensitivity in skeletal muscle [26].

In the liver, no differences between control and treated birds ($P > 0.1$) were observed. Cremer et al. [27] observed that the livers of female rats treated with 180 mg ALA kg^{-1} body weight during 24 months showed a decrease in their weights as compared to those of the controls. Furthermore, Ide et al. [28] showed that ALA reduces fatty acid synthesis in the liver. Those results are different from our work; however, the administration of ALA was for a shorter time 22 days (vs. 9 weeks in this work) and ALA concentrations administered were between 2 and 20 times higher than the used in the present report.

The hearts of the treated birds were bigger than those of the control animals ($P < .05$; Table 4). Valdecantos et al. [29] observed a decrease in the absolute weight of the hearts from male rats treated with ALA in a dose approximately 200 times higher than the one received by the fighting chicks used in this experiment.

In humans, it is known that several hereditary or familial factors are affecting the size of the heart [30]. In animals, for example, racehorses, heart hypertrophy due to hereditary factors and the normal physiological maturity is unclear compared with the one caused by exercise; however, Sleeper et al. [31], found that heart size, determined by echocardiographic measurements, in Arabian endurance horses is related with performance in elite vs. non-elite animals, which is similar to the findings for English thoroughbred horses.

Table 1. Diets

Type of feed	Age, weeks	Feed (Purina™)	Moisture, % (Max)	Protein, % (Min)	Fat, % (Min)	Fiber, % (Max)	Nitrogen free extract, %	Ash, % (Max)	Ca, % (Min)	P, % (Min)
Starter	4-8	Startina Plus	12	28	2	8	42	8	0.9	0.6
Grower	8-end	Iniciarina gold	12	21	2	9	46	10	0.0	0.5

Table 2. Primers used for the amplification of specific DNA sequences by qPCR

Gene	Reference sequence (accession number)	Primer position (5' → 3')	Fragment size	Annealing temperature (°C)
Chicken NRF1	NM_001030646.1 *Gallus gallus* nuclear respiratory factor 1 (NRF1), mRNA	Forward: TCTACGCATTTGAGGATCAGC Reverse: GCCACTGCAGAATAATTGACT	220 bp	60
Chicken NRF2	NM_001007858.1 *Gallus gallus* GA binding protein transcription factor alpha subunit (NRF2), mRNA	Forward: TGCACATTATTCCAGCATCCG Reverse: GAAATACAGTCCCGAGCGTCT	190 bp	60
Chicken PGC1α	XM_040699025.1 *Gallus gallus* PPARG coactivator 1 alpha (PGC1A), mRNA	Forward: ATCCAGATCACCGTACAGTCG Reverse: TCCCTCAGATCTTTTCGGGTT	216 bp	60
Chicken ND1	AB753756.1 *Gallus gallus* mitochondrial NADH dehydrogenase subunit 1 (ND1)	Forward: ATTTCTCCTAGCCATATCAAGC Reverse: TTAAGGTGTAATTGCCGCTCA	180 bp	60
Chicken Sirtuin 1 (SIRT1)	NM_001004767.1 *Gallus gallus* sirtuin 1 (SIRT1), mRNA	Forward: TGCTCCCAGAAACGATCCCT Reverse: CCGGCTCCTGTCAAGACCA	175 bp	60
Chicken PPIA	NM_001166326.1 *Gallus gallus* peptidylprolyl	Forward: AGCACTGGGGAGAAAGGATT	248 bp	60

Gene	Reference sequence (accession number)	Primer position (5' → 3')	Fragment size	Annealing temperature (°C)
	isomerase A (PPIA), mRNA	Reverse: AGCCACTCAGTCTTGGCAGT		
Chicken RPLP0	NM_204987.1 *Gallus gallus* ribosomal protein, large, P0 (RPLP0), mRNA	Forward: AGACTGGAGACAAGGTGGGA Reverse: ACACCCTCCAGAAAGCGAGA	172 bp	60

Table 3. Effect of the addition of ALA to the diet on productive variables (body weight, daily weight gain, accumulated food intake, and food conversion) in female fighting cocks

Treatment	BW_i (g)		BW_f (g)		DWG (g d^{-1})		AFI (kg)		FC	
	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM
Control	284.1	7.2	1043.1	17.2	12.0	10.6	149.4	4.2	3.8	0.7
ALA	287.5	5.5	1021.5	14.0	11.7	9.5	146.7	4.4	4.1	0.5

ALA= alpha-lipoic acid; BW_i= initial body weight; BW_f= final body weight; DWG= daily weight gain; AFI= accumulated food intake; FC= food conversion; $\bar{x}$/LSM= mean value/least square mean; SEM= standard error of the mean. P > 0.1, n = 3

Table 4. Effect of the addition of ALA to the diet on carcass weight and breast, leg-thigh, wing, liver, and heart yields

Treatment	Carcass weight (g)		Carcass yield (%) †		Breast (%)		Leg-thigh (%)		Wing (%)		Liver (%)		Heart (%)	
	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM
Control	673.2	14.9	65[a]	2.2	30.7	2.7	13.5	2.7	6.5	3.2	3.2	0.3	0.67[a]	0.002
ALA	694.2	14.9	69[b]	2.1	29.9	2.7	13.3	2.7	6.3	3.3	3.2	0.3	0.71[b]	0.002

† *The values for breast, leg-thigh, wing, liver, and heart are express relative to the carcass, as %. Different letters within columns indicate statistical differences (P < .05, n = 3). $\bar{x}$= mean; SEM= standard error of the mean*

In this work, the increase in heart weight of the treated birds could indicate an added effect of ALA and the cardiac capacity of these birds, since it is likely that they were selected in great part due to their musculoskeletal capacity [32].
In the Thoroughbred industry, it has long been believed that large hearts were associated with racing success [33]. Recent data have demonstrated a significant linear relationship between British Horseracing Board Official rating or Timeform rating and heart size measured by echocardiography in 200 horses engaged in National Hunt racing (30,33). If VO_2, max, and heart size are the more important predictors of performance for equine athletes, the effect of ALA on heart size observed in this experiment could be beneficial for gamecocks

The total antioxidant capacity in serum of the treated birds was higher than in control birds (P = .014; Table 5). Chen et al. [34] observed an increase in the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in serum, liver, and breast muscle of broilers treated with ALA but no increase in the total antioxidant capacity. Jia et al. [35], on the contrary, observed a dose-dependent increase of the total antioxidant capacity in the serum of broilers treated with ALA. In racehorses, administration of ALA in doses from 2 to 10 g d^{-1} decreases oxidative stress produced during training and intense exercise [36,37]. Therefore, it is possible to deduce that ALA in fighting cocks could improve their response capacity to the oxidative stress induced by their physical activity during training or a fight.

Table 5. Effect of the addition of ALA to the diet on the antioxidant capacity in serum (obtained) from female fighting cocks (after sacrifice)

Treatment	Trolox units	
	LSM †	SEM
Control	554.6 [a]	12.75
ALA	599.7 [b]	12.75

† Different letters within columns indicate statistical differences (P = .014, n = 3). $\bar{x}$/LSM= mean value/least square mean; SEM= standard error of the mean

Fig. 1 shows that ALA treatment increased the expression of the mRNA for NRF1, NRF2, SIRT1, PGC-1α, and ND1 (P < .05); these genes participate in mitochondrial biogenesis. This is a complex process that requires the coordinate expression and assembly of several proteins coded by both the nuclear and mitochondrial genomes [38]. Pershadsingh et al. [39] demonstrated that ALA is a peroxisome proliferator-activated receptor γ and α (PPARγ and PPARα) agonist and their coactivator PGC-1α can activate SIRT1, regulating mitochondrial biogenesis [40]. This family of coactivators plays an important role in this process through interactions with transcription factors, such as NRF1, which controls the transcription of both the nuclear and mitochondrial genomes and directly activates the mitochondrial transcription factor A (TFAM), a basic component of the mitochondrial transcription machinery [38].

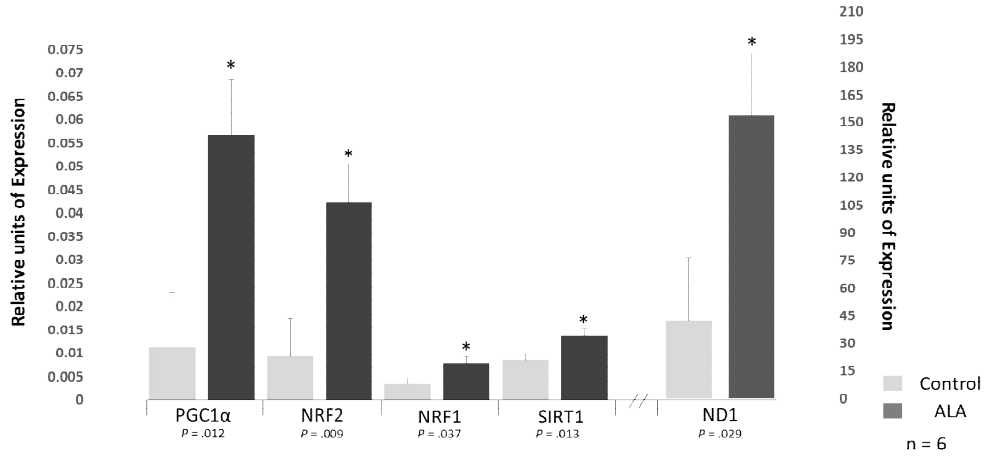

Fig. 1. Effect of ALA on the relative expression of NRF1 and SIRT1 in heart of female fighting cocks. Different literals represent statistical differences
**P < .05; n =6*

Training is important in elite sports and during physical activities and the wide variety of responses observed is dependent on the individual's genotype [41]. Based on the results obtained in this work, it can be concluded that the addition of 40 ppm ALA to the diet of fighting cocks increases the size and the mitochondrial biogenesis of their hearts, improving their physical and metabolic responses for the purpose these animals are being produced for.

4. CONCLUSION

Alpha-lipoic acid improved the weight of the heart concerning the carcass, which could represent an advantage in fighting cocks because of a possible greater cardiovascular capacity. The expression of both genes evaluated was increased, indicating an increase in mitochondrial biogenesis in the heart. ALA also improved the total antioxidant capacity, which could enhance their oxidative stress response. More research on the effects of ALA at the cellular and molecular levels is still necessary, mainly in the liver, skeletal muscle, and heart, as well as in male fighting cocks.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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