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# Dietary glycerol for quail: association between productive performance and COX III mRNA expression

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**ABSTRACT.** This study was carry out to evaluate mRNA expression of mitochondrial cytochrome c oxidase III in the Pectoralis superficialis muscle of 28-day-old quails fed diets containing 0, 8, and 12% glycerol. Total RNA was extracted (N = 10) and cDNA was amplified using specifics primers for qRT-PCR. Feed efficiency and feed intake were evaluated. COX III mRNA expression in breast muscle was higher in the group fed with 12% glycerol (0.863 AU); no differences were observed in the expression of this gene between the muscle of animals fed diets without glycerol (0.357 AU) and 8% glycerol (0.415 AU).

 

Quails that showed greater COX III mRNA expression also showed the lowest feed efficiency. These results show that there is a difference in COX III mRNA expression in breast muscle of 28-day-old quail fed diets different concentrations of glycerol.

**Key words:** Electron transport chain; Gene expression; Oxidative phosphorylation; Energy metabolism

## INTRODUCTION

The use of by-products in animal diets may decrease production costs and therefore increase profitability. When financially profitable, glycerol, a byproduct of the biodiesel industry, can be used as a potential partial substitute for corn in animal feed (Cerrate et al., 2006) because of their similar energy values (Dozier et al., 2008). Glycerol is absorbed passively (Guyton, 1991), has a sweet taste and a low molecular weight (Rivaldi et al., 2007), and may influence feed intake (Pasquetti, 2011).

Inclusion of glycerol in the diet of quails and broiler chicken may affect their performance characteristics (Raber et al., 2009) as well as influencing mRNA expression of some mitochondrial genes related to the efficiency of ATP synthesis, such as *Cytochrome oxidase subunit III* (*COX III*) (Gasparino et al., 2012).

COX is located in complex IV and is the last protein of electron transport chain (Calhoun et al., 1994), where it receives electrons from cytochrome c, transfers them to a molecule of oxygen and reduces them to water, simultaneously pumping protons through the membrane, and transforming the energy generated in the redox reaction into proton motive force (Yoshikawa et al., 2006). COX activity can be modified in response to different treatments, (Scheffler, 1999; You et al., 2002), for this reason, COX has great importance in researches of mitochondrial energy efficiency. Changes in cellular efficiency may be due to lower expression of this gene (Kemp et al., 2003), resulting in animals being less efficient at converting food into body weight (Gasparino et al., 2012).

Therefore, there is a need to conduct studies on the effect of nutrients, and their levels, on the expression of genes related to the electron transport chain in order to better understand their influence on oxidative phosphorylation and its relationship with animal performance. Thus, this study has been developed to evaluate the effect of dietary inclusion of glycerol on the expression of *COX III* mRNA in breast muscle of quail, and on weight gain, food intake, and feed efficiency at 28 days of age.

## MATERIAL AND METHODS

This study was carried out in the poultry industry and the molecular genetics lab of the Department of Animal Science, Universidade Estadual de Maringá. The project was submitted and approved by the Ethics Committee under protocol No. 024/2011. For this study, 450 1-day old quails (*Coturnix coturnix*) were used. Animals were divided into three groups, each receiving different diet treatments: without the addition of glycerol, with the addition of 8% of glycerol, and containing 12% glycerol (Table 1). Diets were formulated based on corn and soybean meal according to the dietary recommendations of Rostagno et al. (2011) and the NRC (1994). Throughout the experimental period, the birds had free access to water and feed.

**Table 1.** Animal feed composition.

| Ingredients (kg) | Crude glycerin | | |
|---|---|---|---|
| | Without glycerol | 8% | 12% |
| Corn | 54.58 | 46.5 | 42.4 |
| Soybean meal 45% | 37.84 | 39.4 | 40.1 |
| Crude glycerin | - | 8 | 12 |
| Soybean oil | 2.88 | 1.44 | 0.73 |
| Dicalcium phosphate | 1.61 | 1.63 | 1.64 |
| L-Lysina HCL | 0.65 | 0.84 | 0.82 |
| DL-Methionine | 0.66 | 0.66 | 0.67 |
| L-Threonine | 0.44 | 0.43 | 0.43 |
| Salt | 0.4 | 0.4 | 0.4 |
| Vitamin supplement[1] | 0.4 | 0.4 | 0.4 |
| Limestone | 0.28 | 0.26 | 0.26 |
| Mineral supplement[1] | 0.07 | 0.07 | 0.07 |
| Antioxidant[2] | 0.01 | 0.01 | 0.01 |

[1]Vitamin/mineral supplementation (assurance levels per kg of product). Vitamin A, 4,500,000 IU; vitamin D3, 1,250,000 IU; vitamin E, 4000 mg; vitamin B1, 278 mg; vitamin B2, 2000 mg; vitamin B6, 525 mg; vitamin B12, 5000 µg; vitamin K3, 1007 mg; calcium pantothenate, 4000 mg; niacin, 10,000 mg; cholin, 140,000 mg; antioxidant, 5000 mg; zinc, 31,500 mg; iron, 24,500 mg; manganese, 38,750 mg; copper, 7656 mg; cobalt, 100 mg; iodine, 484 mg; selenium, 127 mg; [2]BHT (butyl hidroxy toluene).

Quails were weighed at 28 days to determine body weight gain. The experimental diet and remains were weighed at 28 days to calculate feed intake. Feed efficiency was calculated as the ratio between the weight gain and feed intake. Mortality was considered for the feed conversion calculation. Ten quail from each treatment group were killed by cervical dislocation at 28 days. Breast muscle (Pectoralis superficialis) samples from these birds were collected, conditioned in RNA Holder® (BioAgency, Carlsbad CA, USA), and stored at -20°C until RNA extraction.

Total RNA was extracted using the Trizol® reagent (Invitrogen, Carlsbad, CA, USA) at a concentration of 1 mL for every 100 mg tissue. Muscle tissue was ground with an electric homogenizer until complete dissociation occurred. Subsequently, 200 µL chloroform was added and the sample was manually homogenized for 1 min. The homogenate was centrifuged for 15 min at 12,000 rpm at 4°C, and the liquid phase was collected and transferred to a test tube, where 500 µL isopropanol was added. Next, the material was homogenized, centrifuged for 15 min at 12,000 rpm and 4°C. The supernatant was discarded and the precipitate was washed with 1 mL 75% ethanol. The material was then centrifuged again at 12,000 rpm for 5 min, and the supernatant discarded. The precipitate was air-dried for 15 min and homogenized in distilled and deionized RNase-free water.

The total RNA concentration was determined with a spectrophotometer at 260 nm. RNA integrity was evaluated on a 1% agarose gel and visualized under ultraviolet light. RNA samples were treated with DNase (Invitrogen Corporation, Brazil) to remove residual DNA, according to the manufacturer protocol.

For preparation of cDNA, a SuperScript™ III First-StrandSynthesisSuper Mix kit (Invitrogen Corporation, Brazil) was used. Six-microliters of total RNA, 1 µL oligo (dT) (50 µM) and 1 µL annealing buffer were included. The reaction mixture was incubated for 5 min at 65°C and then placed on the gel for 1 min. Thereafter, 10 µL 2X First-Strand Reaction Mix solution and 2 µL solution containing the enzyme SuperScript III reverse transcriptase and RNase inhibitor were added. The solution was incubated for 50 min at 50°C for the synthesis

of cDNA. The reaction mixture was incubated again for 5 min at 85°C and then immediately placed on ice. Samples were stored at -20°C until analysis. Real-time PCR was performed using the SYBR GREEN fluorescent compound [SYBR® GREEN PCR Master Mix (Applied Biosystems, USA)].

Primers for *COXIII* (GenBank accession No. NP_006921) were obtained from Ojano-Dirain et al. (2007). Primers for β-actin, which acted as the endogenous control gene, were utilized. All analyses were performed in duplicate in a final volume of 25 μL. Data were analyzed by the SAS GLM procedure (SAS, 2000).The results of *COX III* gene expression analysis are presented as $2^{-\Delta Ct}$. UNIVARIATE analysis was adopted to verify the normality of the data. ANOVA was used with three treatments (without glycerol, 8 and 12% glycerol inclusion). Means were compared by the Tukey test ($P < 0.05$).

## RESULTS

The performance of quails who received different levels of dietary glycerol in the feed is shown in Table 2. No significant treatment effects on weight gain were observed, but there were significant increases in feed intake and in the efficiency of quail. Feed intake was significantly higher in the group of quails fed diets containing 12% glycerol than in the groups subjected to other treatments, and the consumption did not differ statistically between groups fed diets with 8% glycerol those fed diets without glycerol.

**Table 2.** Performance of quails fed different levels of glycerol.

| Characteristics[1] | Glycerol inclusion levels | | |
|---|---|---|---|
| | Without glycerol | 8% | 12% |
| WG (g) | 177.65 ± 15.34 | 179.51 ± 10.18 | 182.56 ± 21.31 |
| FI(g) | 345.32 ± 31.85[b] | 385.83 ± 24.42[b] | 437.73 ± 31.82[a] |
| FE | 0.502 ± 0.007[a] | 0.496 ± 0.017[a] | 0.414 ± 0.046[b] |

[a.b]Different letters on the same line represent different means ($P < 0.05$). [1]WG = weight gain; FI = feed intake; FE = feed efficiency.

In addition, birds fed diets containing 12% glycerol had the worst feed efficiency when compared with quails fed diets without glycerol or with 8% glycerol. This represented an average worsening of 8.5% in feed efficiency of the quails up to 28 days of age. Although no specific primers were found for the *COX III* gene, the *Gallus gallus* primers used in real-time PCR were shown to be suitable for the development of this study. Dissociation curve analysis revealed that there were no nonspecific products or primer dimer formation, confirming the reliability of the data. The expression of *COX III* mRNA in the breast muscle of 28-day-old quails is shown in Figure 1.

The expression of *COX III* mRNA in the Pectoralis superficialis muscle of quails was higher in the animals fed diets containing 12% glycerol, and this level of supplementation increased the expression of *COX III* mRNA by 91% (compared to the expression without glycerol treatment) and 64% (compared to the expression under 8% glycerol treatment). No difference in *COX III* expression was observed between the group supplemented with 8% glycerol and those not receiving glycerol treatments. The relationship between *COX III* and feed efficiency was inversely proportional (Figure 2).

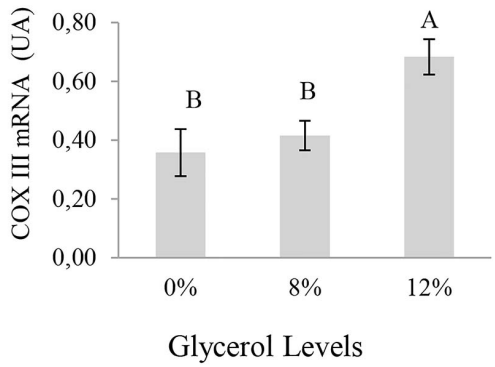


**Figure 1.** *COX III* mRNA expression. Letters on the bars represent the comparison of mRNAs expression means. Different letters represent a statistical difference ($P < 0.05$) as determined by the Tukey test.

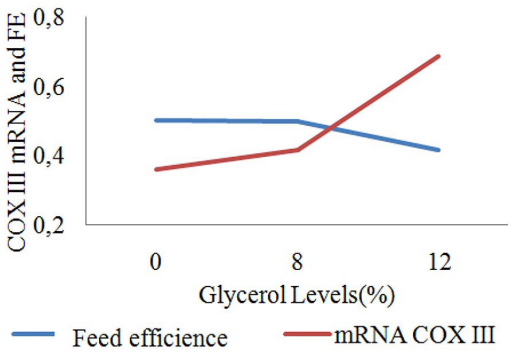


**Figure 2.** Inverse relationship between *COX III* expression and feed efficiency (FE) in response to increased dietary glycerol.

## DISCUSSION

The level of dietary glycerol inclusion in this study differentially influenced the expression of *COX III*. These changes in gene expression may partly explain the results obtained for quail performance. Feed intake showed a raising, being the higher consumption observed in animals fed diet containing 12% glycerol. This may have occurred due to the increased palatability of diets containing glycerol, which has a sweet taste and may influence feed intake (Pasquetti, 2011). There was no increase in weight gain, thus promoting a worse feed conversion in animals receiving 12% glycerol. According to the literature, the maximum recommended inclusion of glycerol in the diets of quails up to 14 days of age is 10% (Pasquetti, 2011). Therefore, inclusion of 12% glycerol may have exceeded the metabolic capacity of the animal, thus limiting its absorption (Min et al., 2010). Moreover, the inclusion of glycerol at 8 and 12% promoted an increase in apparent litter humidity, suggesting an increased rate of digesta passage and compromising nutrient utilization as suggested by Gianfelici (2009).

In broilers, a relationship between mitochondrial function and feed efficiency has

been reported (Parker et al., 2008). During oxidative phosphorylation, there may be errors in the movement of protons from the inter membrane space to the mitochondrial matrix (Brand et al., 1995) due to the uncoupling of proton transport by specific proteins that function as membrane uncouplers, which may cause mitochondrial inefficiency (Rolfe and Brand, 1997).

The relationship between COX III and feed efficiency was inversely proportional (Figure 2). Birds receiving feed supplemented with 12% glycerol showed higher *COX III* expression and worse feed efficiency than birds receiving no glycerol and 8% glycerol. Similar to this finding, other studies have found increased expression of *COX III* in birds with poor feed efficiency (Iqbal et al., 2004; Iqbal et al., 2005; Lassiter et al., 2006; Tinsley et al., 2010), suggesting that there is a relationship between poor feed efficiency and increased *COX III* expression.

According to Iqbal et al. (2004), although the exact mechanism of action of COX III has not yet been fully determined, the high expression of *COX III* in low feed efficiency birds could represent a compensatory response against possible oxidative stress caused by enhanced protein oxidation. The increase in *COX III* expression in animals fed a diet containing 12% glycerol is a strong indication of the likely increase in reactive oxygen species caused by diet, since the function of COX III is more closely linked to stress oxidative responses and activities relating to changes in proton position (You et al., 2002).

The results of this study show that even when diets are isocaloric, the source of energy and nutrient levels used influence the gene expression of COX III, which consequently influences the performance of quail at 28 days of age. This indicates that molecular studies that evaluate gene expression assist in our understanding of mechanisms that may improve or worsen the performance of birds for different nutrients, and their levels used in feed. Dietary inclusion of 8% glycerol led to similar quail performance as was observed for those fed diets without glycerol, suggesting that its inclusion up to this level would not cause adverse effects.

In conclusion, the inclusion of glycerol in diets affects the mRNA expression of *COX III* in the breast muscle of quails as well as feed intake and feed efficiency of birds up to 28 days old.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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