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Gender and Age Effects on the Expression of Genes Related to Lipid Metabolism in Broiler's Liver

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ABSTRACT

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Two experiments were conducted to assess gender (Experiment 1) and age (Experiment 2) effects on the expression of genes related to lipid metabolism in broiler chickens. The expression of fatty acid synthase (*FAS*), apolipoprotein A-I (*APOA-I*), apolipoprotein B (*APOB*), adiponectin (*ADIPOQ*), liver kinase B1 (*LKB1*), and AMP-activated protein kinase α-1 (*AMPKα-1*) genes was evaluated by qRT-PCR. In Experiment 1, we observed a gender effect on feed intake, as male broilers presented greater feed intake than females. Female broilers presented greater gene expression of *FAS*, and lower expression of *ADIPOQ* and *AMPKα-1*, than males. A gender effect was not observed for the gene expression of *APOA-I*, *APOB,* or *LKB1*. In Experiment 2, there was a significant age effect on feed intake and weight gain. Broilers 42 days of age presented greater feed intake and weight gain than 21-day-old birds. 21-day-old broilers showed greater expression of *APOA-I*, *ADIPOQ*, *LKB1*, and *AMPKα-1*, and lower *APOB* gene expression in the liver than 42-day-old broilers. Age had no effect on *FAS* gene expression. Our results show that the gender and age could act on the expression of genes related to lipid synthesis, such as *FAS* and *APOB*, and also on genes related to lipid oxidation, such as *ADIPOQ, LKB1,* and *AMPK*.

Keywords: apolipoprotein; lipogenesis; lipolysis

High body fat content in chickens has been the subject of numerous studies because it is related to reduced feed efficiency, reduced carcass yield, and lower customer acceptance of the final product (Wu et al. 2006). The accumulation of body fat is a result of absorption, synthesis, and oxidation of lipids (Smink et al. 2010) which are determined by the balance between lipogenesis, which occurs mostly in the liver of birds, and by lipolysis (β-oxidation) in mitochondria (Nelson and Cox 2011).

Lipid synthesis is performed by two enzyme complexes: acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). This metabolic process can be influenced by several factors, including the inhibition of ACC enzymatic activity by AMP-activated protein kinase (AMPK) action, which acts by phosphorylating the ACC enzyme and makes it inactive (Berg et al. 2012). Acetyl-CoA carboxylase inactivation is related to the predominance of β-oxidation, which provides energy to the body (Zhou et al. 2001).

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According to Saadoun and Leclercq (1987), the synthesis of lipids in adipose tissue is limited, and thus, lipid deposition in this tissue depends on the availability of lipid substrates derived from blood plasma. Lipids in the plasma originate from the diet and the hepatic lipogenesis process (Hermier 1997). Thus, lipid deposition and β-oxidation mechanisms depend on the transport of lipids. In the plasma, lipids are transported with lipoproteins, which contain apolipoproteins, such as apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB) (Ginsberg 2002).

Male and female broilers present some differences in the body fat deposition process, and females have a higher rate of fat deposition than males (Silva 2012). Studies suggest that this is possibly due to metabolic and hormonal differences (Tumova and Teimouri 2010). Similar to gender, the age of the chickens can also influence the fat synthesis rate, since greater fat content is observed in carcasses of older chickens (Zerehdaran et al. 2005).

Thus, the present study was developed under the hypothesis that the differences in fat deposition among chickens of different genders and ages may occur as a function of differences in the expression of genes related to lipid synthesis and oxidation. Therefore, our objective was to evaluate animal performance and the gene expression of fatty acid synthase (*FAS*), apolipoprotein A-I (*APOA-I*), apolipoprotein B (*APOB*), adiponectin (*ADIPOQ*), liver kinase B1 (*LKB1*), and AMP-activated protein kinase α-1 (*AMPKα-1)* in the liver of male and female 42-day-old broilers (Experiment 1), and in the liver of females 21 and 42 days of age (Experiment 2).

MATERIAL AND METHODS

This work was conducted in accordance with the specifications of the Ethics Committee of the State University of Maringá.

Experimental design

Experiment 1. This study was conducted to evaluate the effect of broiler chicken (Cobb 500, *Gallus gallus*) gender on performance and on the expression of genes related to lipid metabolism. For the experiment, 60 females and 60 males (21-day-old) were used. The animals were separated in collective cages (at a density of 10 birds/m^2); each cage with 10 birds was considered as one experimental unit for performance parameters (n = 6). The birds were raised in an acclimatized room at comfortable temperature according to Cobb-Vantress (2016): temperature was maintained at 25°C with 60% relative humidity and then decreased gradually to 18°C and 60% relative humidity until day 42. Throughout the experimental period, the animals had free access to water and a balanced diet to meet their nutritional demands (Rostagno et al. 2011), consisting of a corn and soy-based feed with 19.7% crude protein and 3170 kcal/kg. A 24-h light schedule was used.

Weight gain was calculated as: (broiler weight at 42 days of age – weight at 21 days of age). Feed intake was calculated as the difference between the amount of feed offered in the experimental period and the feed residue at day 42. The feed intake and weight gain were corrected for mortality.

Experiment 2. This study was conducted to evaluate the effect of age of female broilers on performance and on the expression of genes related to lipid metabolism. For the experiment, one hundred one-day-old female broilers were used. The animals were separated in collective cages (at a density of 10 birds/m^2), and each cage with 10 birds was considered as one experimental unit for performance parameters (n = 10). The birds were raised in an acclimatized room at thermoneutral according to Cobb-Vantress (2016) as described above. Throughout the experimental period, the animals had free access to water and a balanced diet to meet their nutritional demands (Rostagno et al. 2011), consisting of a corn and soy-based feed with 21.6% crude protein and 3052 kcal/kg in the starter period (1–21 days), and 19.7% crude protein and 3170 kcal/kg in the grower period (22–42 days). A 24-h light schedule was used.

Feed intake was calculated as the difference between the amount of feed offered through the experimental period and the feed residue at day 21 for the first experimental period (days 1–21), and between the amount of feed offered through the experimental period and the feed residue at day 42 for the second experimental period (days 22–42). To calculate the weight gain, all birds were weighed at days 1, 21, and 42. The feed intake and weight gain were corrected for mortality.

At the end of each experiment, birds were slaughtered by cervical dislocation. The liver was collected and placed in liquid nitrogen and subsequently stored in a freezer at –80°C until the time of RNA extraction.

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Gene expression. Gene expression in Experiments 1 and 2 was evaluated in the liver. For gene expression analysis birds were considered as experimental units (n = 6). Total RNA was individually extracted from six birds of each treatment using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions (1 ml/100 mg tissue). All of the materials used had been previously treated with the RNase inhibitor RNase AWAY® (Invitrogen). The total RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. The RNA integrity was analyzed using 1% agarose gel stained with 10% ethidium bromide and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen) according to the manufacturer's instructions to remove potential genomic DNA contamination. A SuperScript™ III First-Strand Synthesis Super Mix kit (Invitrogen) was used for cDNA synthesis from 1 µg of DNase-treated total RNA, according to the manufacturer's instructions. The cDNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. The cDNA samples were diluted to 40 ng/µl and stored at –20°C until further use as a template in the amplification reaction.

Real-time PCR was performed using the fluorescent dye SYBR Green (SYBR® Green PCR Master Mix, Applied Biosystems, USA). The amplification reaction consisted of 5 µl of diluted cDNA, 0.5 µl of each primer (forward and reverse) at 10 µM (final concentration in the reaction of 200 nM), 12.5 µl of SYBR® Green PCR Master Mix, and water up to a total volume of 25 µl. To measure each gene reaction efficiency, a series of 25 µl reactions was performed similar to that above using as a template 5 µl of the cDNA pool derived from a serial dilution. Thermal cycling parameters for all genes were the following: hot-start, 95°C for 10 min, followed by 40 cycles of denaturation and annealing/extension, 95°C for 15 s, 60°C for 1 min, and then, melting curve, 65–95°C.

The primers used for gene amplification reactions were designed according to Lei and Lixian (2012) and Jiang et al. (2014) (Table 1). Two endogenous controls, *β-actin* and *GAPDH*, were tested, and *β-actin* (Accession No. L08165) was selected because its amplification efficiency was higher and similar to the amplification efficiency of the target genes. All of the analyses were performed in duplicate, each in a volume of 25 µl.

Gallus gallus specific primers were used in the amplification of all genes. The amplification efficiencies were similar for the genes of interest, with 90–110% efficiency. The analyses of the dissociation curves did not reveal any non-specific PCR products, such as the formation of primer dimers, which demonstrated the reliability of the data in the estimated mRNA expressions of the evaluated genes. The *β-actin* used as the endogenous

Table 1. Primer sequences used for quantitative real-time polymerase chain reactions

Gene	Amplicon (pb)	Annealing temperature (°C)	Primer sequence (5'–3')	Reference
FAS	107	60	CTATCGACACAGCCTGCTCCT CAGAATGTTGACCCCTCCTACC	Lei and Lixian (2012)
LKB1	158	60	TGAGAGGGATGCTTGAATACGA ACTTGTCCTTTGTTTCTGGGC	Lei and Lixian (2012)
AMPKα-1	266	60	CGGAGATAAACAGAAGCACGAG CGATTCAGGATCTTCACTGCAAC	Lei and Lixian (2012)
ADIPOQ	86	60	GCCAGGTCTACAAGGTGTCA CCATGTGTCCTGGAAATCCT	Jiang et al. (2014)
APOA-I	217	60	GTGACCCTCGCTGTGCTCTT CACTCAGCGTGTCCAGGTTGT	Jiang et al. (2014)
APOB	196	60	GACTTGGTTACACGCCTCA TAACTTGCCTGTTATGCTC	Jiang et al. (2014)
β-actin	136	60	ACCCCAAAGCCAACAGA CCAGAGTCCATCACAATACC	

pb = base pairs, *FAS* = fatty acid synthase, *LKB1* = liver kinase B1, *AMPKα-1* = AMP-activated protein kinase α-1, *ADIPOQ* = adiponectin, *APOA-I* = apolipoprotein A-I, *APOB* = apolipoprotein B

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Table 2. Feed intake and weight gain of male and female broilers (in kg)

	Gender		*P*-value	n^1
	male	female		
Feed intake	$2.54^a \pm 0.24$	$1.71^b \pm 0.15$	0.0421	6
Weight gain	1.72 ± 0.16	1.25 ± 0.17	0.1178	6

[a,b]means within a row with different superscripts significantly differ by Tukey's test ($P < 0.05$); results are means ± SE

[1]collective cages (10 birds/cage) were considered as experimental units

Table 4. Feed intake and weight gain of broilers at 21 and 42 days of age (in kg)

	Age		*P*-value	n^1
	21 days	42 days		
Feed intake	$0.76^b \pm 0.01$	$1.84^a \pm 0.09$	0.0003	10
Weight gain	$0.64^b \pm 0.01$	$1.42^a \pm 0.01$	< 0.0001	10

[a,b]means within a row with different superscripts significantly differ by Tukey's test ($P < 0.05$); results are means ± SE

[1]collective cages (10 birds/cage) were considered as experimental units

control did not show any statistically significant differences among the treatments, which verified the efficiency of *β-actin* as an endogenous control.

Statistical analysis. The $2^{-\Delta Ct}$ method (Livak and Schmittgent 2001) was used to analyze relative expression. The results are expressed as the means and standard deviations. The Shapiro–Wilk test was applied to evaluate the normality of the data. The experiments were conducted in a completely randomized design: Experiment 1 evaluated the gender effect (male and female), and Experiment 2 evaluated the effect of two ages (21 and 42 days of age). The results were submitted to ANOVA, and when the effect was significant, the averages were compared using Tukey's test ($P < 0.05$) by the SAS software (Statistical Analysis System, Version 9.0, 2002).

RESULTS

Experiment 1. Gender effects on performance and gene expression are presented in Table 2 and Table 3, respectively. A significant gender effect on feed intake was found ($P = 0.0421$), as male broilers had greater feed intake than females. There was no difference between weight gain of male and female broilers.

Regarding gene expression, females presented greater expression of *FAS* than males (3.504 vs 1.442 arbitrary units (AU)). Females also had lower expression of *ADIPOQ* (0.038 vs 0.098 AU) and *AMPKα-1* (0.377 vs 0.914 AU) than male broilers. There was no significant gender effect on *APOA-I*, *APOB* or *LKB1* gene expression (Table 3).

Experiment 2. Age had a significant effect on feed intake ($P = 0.0003$) and weight gain ($P < 0.0001$) (Table 4). At 42 days of age broilers had greater feed intake and weight gain than 21 day-old birds.

We observed an age effect on *APOA-I* ($P = 0.0010$), *APOB* ($P = 0.0060$), *ADIPOQ* ($P = 0.0120$), *LKB1* ($P = 0.0030$), and *AMPKα-1* ($P = 0.0012$) gene expression (Table 5). Broilers at day 21 presented greater expression of *APOA-I* (3729.709 AU), *ADIPOQ* (0.106 AU), *LKB1* (1.873 AU), and *AMPKα-1* (0.963AU), and lower *APOB* gene expression, than broilers at 42 days of age. There was no age effect on *FAS* gene expression.

Table 3. *FAS, APOA-I, APOB, ADIPOQ, LKB1,* and *AMPKα-1* gene expression in the liver of male and female broilers

	Gender		*P*-value	n^1
	male	female		
FAS	$1.442^b \pm 0.379$	$3.504^a \pm 0.752$	0.0343	6
APOA-I	2335.226 ± 185.107	1983.827 ± 94.725	0.1219	6
APOB	433.514 ± 18.406	529.171 ± 72.932	0.2323	6
ADIPOQ	$0.098^a \pm 0.008$	$0.038^b \pm 0.003$	< 0.0001	6
LKB1	1.115 ± 0.188	1.147 ± 0.058	0.8751	6
AMPKα-1	$0.914^a \pm 0.138$	$0.377^b \pm 0.096$	0.0095	6

FAS = fatty acid synthase, *APOA-I* = apolipoprotein A-I, *APOB* = apolipoprotein B, *ADIPOQ* = adiponectin, *LKB1* = liver kinase B1, *AMPKα-1* = AMP-activated protein kinase α-1 (expressed as arbitrary units (AU))

[1]birds were considered as experimental units

[a,b]means within a row with different superscripts significantly differ by Tukey's test ($P < 0.05$); results are means ± SE

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Table 5. Expression of *FAS*, *APOA-I*, *APOB*, *ADIPOQ*, *LKB1* and *AMPKα-1* genes in the liver of 21- and 42-day-old broilers

	Age		*P*-value	n^1
	21 days	42 days		
FAS	4.635 ± 1.694	4.504 ± 1.253	0.9510	6
APOA-I	3729.709^a ± 122.913	2298.827^b ± 212.322	0.0010	6
APOB	304.343^b ± 20.143	564.671^a ± 54.740	0.0060	6
ADIPOQ	0.106^a ± 0.026	0.039^b ± 0.003	0.0120	6
LKB1	1.873^a ± 0.157	1.234^b ± 0.070	0.0030	6
AMPKα-1	0.963^a ± 0.076	0.424^b ± 0.074	0.0012	6

FAS = fatty acid synthase, *APOA-I* = apolipoprotein A-I, *APOB* = apolipoprotein B, *ADIPOQ* = adiponectin, *LKB1* = liver kinase B1, *AMPKα-1* = AMP-activated protein kinase α-1 (expressed as arbitrary units (AU))

^{a,b}means within a row with different superscripts significantly differ by Tukey's test ($P < 0.05$); results are means ± SE

[1]birds were considered as experimental units

DISCUSSION

Feed intake and weight gain are determined by genetic potential, environment, nutrition, and other factors such as gender and age. In line with the results from literature, in our study males presented greater feed intake than females, and older broilers had greater feed intake and weight gain than younger broilers (Musundire et al. 2017).

Nowadays, besides the best performance provided by the animal breeding, broilers also have increased body fat deposition (Tumova and Teimouri 2010). Fat is one of the main problems that occurs in the poultry industry and may represent a significant source of loss in carcass yield. Increased fat content can also reduce acceptance by consumers (Gaya et al. 2006). Body fat deposition is the result of absorption, synthesis, and oxidation of lipids (Smink et al. 2010) which are determined by the balance between lipogenesis and lipolysis (β-oxidation) (Nelson and Cox 2011). These reactions are dependent on the transport of lipids in the plasma.

Lipid molecules are transported by lipoproteins, which contain apolipoproteins such as apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB). Apolipoprotein A-I is the major constituent of the protein fraction of high density lipoprotein (HDL), and thus, apoA-I acts in the reverse transport process of cholesterol, from extrahepatic peripheral cells to the liver where it is metabolized (Spady 1999). According to Zhuo et al. (2015), a decreased *APOA-I* gene expression may affect the formation of HDL, thereby impairing the process of cholesterol reverse transport. This suggests that decreased *APOA-I* expression could result in increased accumulation of body fat.

Apolipoprotein B (apoB) is required for the synthesis and secretion of chylomicrons and very low density lipoproteins (VLDL) (Ginsberg 2002). Amongst other functions, it has a role in the absorption and secretion of lipids (Cruz et al. 2015). Moreover, apoB is the major protein component of low density lipoprotein (LDL), which transports lipids from the liver to the body's cells (Novak and Bydlowski 1996). Although the detailed mechanism governing the deposition of fat in the carcass is not fully understood, studies show that abdominal fat accumulation has been associated with a higher content of lipoprotein particles containing apoB-48 and apoB-100, as well as increased *APOB* gene expression (Zhang et al. 2006). We observed lower *APOA-I* and greater *APOB* gene expression in broilers of 42 days of age, which may help explain, at the level of transcription, the greater body fat observed in older birds (Zerehdaran et al. 2005).

Regarding the differences between male and female broilers, we observed that females had higher *FAS* gene expression than males. *FAS* is a gene related to lipids synthesis. The synthesis of lipids starts by the action of the multifunctional enzyme complex ACC. After ACC action, lipids synthesis occurs by a sequence of repetitive reactions mediated by the action of multienzyme system FAS (Nelson and Cox 2011). Studies have shown that *FAS* gene expression is positively correlated

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to the body fat content in animals (Mildner and Clarke 1991). Also, according to Nogalska and Swierczynski (2001), the maximum lipid synthesis capability in body tissues is determined by the level of *FAS* gene synthesis.

We also evaluated the expression of genes related to lipid oxidation, including the adiponectin gene (*ADIPOQ*). After ligation of adiponectin to its receptor, the phosphorylation of AMPK occurs (Yamauchi et al. 2003), which in turn phosphorylates and inactivates the ACC enzyme. This mechanism stimulates lipid oxidation, with a consequent reduction in lipid synthesis, since it regulates the expression of genes involved in lipogenesis such as *ACC* and *FAS* (Wang et al. 2009). The reduced *ADIPOQ* expression observed in birds at 42 days of age and in female broilers may indicate that the higher body fat content usually observed in females (Silva 2012) and older birds (Zerehdaran et al. 2005) may be related to the lower *ADIPOQ* gene expression observed in these animals, since adiponectin is inversely related to fat in the carcass (Whitehead et al. 2005).

Another gene related to oxidation of lipids evaluated in this study is AMP-activated protein kinase α-1 (*AMPKα-1*), which has been recognized as an important protein able to regulate lipid metabolism due to its action on lipogenic enzymes, such as ACC and FAS (Zhou et al. 2001). The action of AMPK reduces the lipogenesis process and increases lipid oxidation (Winder and Hardie 1999). Activation of AMPK occurs when the level of organic energy (ATP) is reduced, and its activation may occur due to the phosphorylation of the 172 threonine residue present in the alpha catalytic subunit of AMPK (Hawley et al. 2003). Liver kinase B1 (LKB1) is considered the major route of AMPK activation, since an LKB1 deficiency results in an almost complete loss of AMPK activity (Shaw et al. 2005). Our results show that male broilers had higher *AMPKα-1* gene expression than females, and 21-day-old broilers had greater *LKB1* and *AMPKα-1* gene expression than older broilers. The increased expression of these genes may be related to the increased *ADIPOQ* expression also observed in those birds, since adiponectin activates AMPKα by the LKB1 pathway, thereby promoting an increase in lipid oxidation rate and reduction in lipid synthesis. These results may contribute to the lower fat deposition in the carcass of males and of 21-day-old broilers.

CONCLUSION

The results obtained in this study provide another possible explanation at the molecular level for the differences usually observed in body fat content of broilers of different genders and ages; they may occur due to an increased expression of genes related to lipid deposition, such as *FAS* and *APOB*, to a lower *APOA-I* gene expression and to a lower expression of genes related to lipid oxidation such as *ADIPOQ*, *LKB1*, and *AMPKα-1*.

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