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SHORT COMMUNICATION

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Gender and heat stress effects on hypothalamic gene expression and feed intake in broilers

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Abstract

Our study aims to evaluate gender and heat stress effects on animal performance and on the expression of five hypothalamic genes related to feed consumption: neuropeptide Y (*NPY*), ghrelin (*GHRL*), pro-opiomelanocortin (*POMC*), AMP-activated protein kinase (*AMPKα-1*), and liver kinase B1 (*LKB1*). To assay these effects, 42-day-old male and female broilers were maintained in thermal comfort or were subjected to heat stress (HS, 38°C for 24 hours). All animals were fed with diets formulated to meet their nutritional requirements. Broilers subjected to HS showed lower weight gain (p=0.0065) and tended to have lower feed intake (p=0.0687) than broilers kept in comfortable conditions. We observed gender and heat stress interaction effects on *NPY* (p=0.0225), *AMPKα-1* (p=0.0398), and *POMC* expression (p=0.0072). The highest *NPY* gene expression was observed in male broilers from the thermal comfort group. Male broilers exposed to HS showed the highest *AMPKα-1* gene expression levels. Comparing *POMC* expression between males and females at the comfortable temperature, we observed that females showed higher *POMC* expression levels than male broilers. A gender effect was also observed on *LKB1* and *AMPKα-1* gene expression (p=0.0256 and p=0.0001, respectively); increased expression was observed in male broilers. Our results indicate that the expression of some hypothalamic genes related to food consumption may contribute to the observed differences in voluntary feed intake between animals of different gender exposed to different environmental conditions.

Additional keywords: female broilers, *Gallus gallus*; orexigenic genes; anorexigenic genes; heat stress.

Abbreviations used: AMP (adenosine monophosphate); *AMPKα-1* (AMP-activated protein kinase); ARC (hypothalamic arcuate nucleus); ATP (adenosine triphosphate); CRH (corticotropin-releasing hormone); *GHRL* (ghrelin); HS (heat stress); *LKB1* (liver kinase B1); MSH (α-, β and γ melanocyte-stimulating hormone); mTOR (rapamycin target protein); *NPY* (neuropeptide Y); *POMC* (pro-opiomelanocortin); STK11 (serine/threoninekinase11).

Authors' contributions: Conceived and designed the experiments, and analyzed the data: EG, APDV and ASK. Wrote the paper: ASK, APDV, EG and MAMS. All authors performed experiments, read and approved the final manuscript.

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Introduction

The control of voluntary feed intake by animals involves a series of mechanisms that operate at the intersection of the central nervous system and the peripheral tissues of the body (Richards *et al.*, 2010). These mechanisms can be influenced by several factors, including gender and ambient temperature (Ferket & Gernat, 2006). Studies evaluating the expression of hypothalamic genes related to feed consumption, such as the neuropeptide Y (*NPY*), ghrelin (*GHRL*), and pro-opiomelanocortin (*POMC*), have shown that heat stress (HS) can influence the expression of these genes (Song *et al.*, 2012). Gender differences in food consumption may also be explained by different expression patterns of orexigenic/anorexigenic genes present in the hypothalamus of chickens of different genders (Merckaert & Vandesande, 1996). Thus, according to a previous study (Rondelli *et al.*, 2003), birds of different genders may also exhibit differences in feed intake and consequently, may differ in performance.

During pre-prandial and post-prandial periods, hormonal signals generated from the peripheral tissues of the body, and non-hormonal signals generated from nutrients are conducted to the hypothalamus, which, in turn, recognizes and interprets the signals generating adequate stimulatory or inhibitory consumption responses (Xue & Kahn, 2006). The hypothalamic arcuate nucleus (ARC) contains neuronal cells responsible for the synthesis and release of orexigenic neuropeptides, such as neuropeptide Y (NPY), and anorexigenic neuropeptides, such as pro-opiomelanocortin (POMC) (Minor *et al.*, 2009). Similar to the hypothalamic neuropeptides, AMP-activated protein kinase (AMPK) and the ghrelin hormone also have a close relationship with the feed intake control pathway (Xue & Kahn, 2006). AMPK is activated when physiological energy levels (ATP) are lower than normal, and there is an increase in the AMP: ATP ratio (Towler & Hardie, 2007). The phosphorylation and subsequent activation of AMPK may occur through several enzymes, including protein liver kinase B1 (LKB1), considered the primary AMPK activation protein (Towler & Hardie, 2007). Regarding ghrelin, studies have shown an inhibitory effect of ghrelin on feed intake in broilers. This inhibitory effect can be mediated by corticotropin-releasing hormone (CRH) (Saito *et al.*, 2005).

The present study was performed to test the hypothesis that factors such as gender and environmental conditions can influence animal performance through the expression of genes related to feed intake. The purpose of this study was to evaluate the effects of gender and heat stress on animal performance and on the expression of hypothalamic genes related to consumption: neuropeptide Y (*NPY*), ghrelin (*GHRL*), pro-opiomelanocortin (*POMC*), AMP-activated protein kinase (*AMPKα-1*), and liver kinase B1 (*LKB1*) in 42-day-old male and female broilers maintained in comfortable conditions or subjected to HS at 38°C for 24 hours.

Material and methods

The guidelines of the Committee on Animal Care at the Universidade Estadual de Maringá, Brazil, were followed while performing this experiment.

Experimental design and animals

A total of 60 male and 60 female broilers (Cobb 500) (*Gallus gallus*) at 22 days of age were used in the experiment. The experiment was a completely randomized factorial design with two thermal environments (thermal comfort at 19ºC or heat stress at 38°C for 24 h with a humidity of 60%) × two genders (male and female). The animals were separated by gender in collective cages (10 animals per cage), which served as the experimental units (n=6).

All of the animals were raised in two climate-controlled rooms in the thermal comfort zone (according to the Cobb guide) until 41 d of age. Then, 60 animals (30 of each gender) were acutely stressed with heat at 38°C for 24 h. After 24 h, the animals from both groups (thermal comfort and heat stress) were slaughtered by cervical dislocation at 42 d. During the experimental period, the animals had free access to water and feed. Their diet was balanced to meet their nutritional requirements (Rostagno *et al.*, 2011), and consisted of a feed based on soybean and corn with 19.70% crude protein and 3170 kcal/kg of metabolizable energy. The feed intake was calculated as the difference between the amount of feed offered at day 41 and the residues at the end of the experiment (day 42) for the birds of both genders in both environments. To calculate the weight gain of the broilers from the thermal comfort and HS groups, the animals (males and females) were weighed on days 41 and 42.

Gene expression

For the analysis of gene expression levels, hypothalamus samples were collected from six animals from the four treatments into liquid nitrogen, and stored in a -80ºC freezer until the total RNA was extracted. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (1 mL per 100 mg of tissue). All of the materials used had been previously treated with RNase inhibitor (RNase AWAY, Invitrogen, Carlsbad, CA, USA). The total RNA concentration was measured with a spectrophotometer at a wavelength of 260 nm. The RNA integrity was analyzed using a 1% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions to remove possible genomic DNA contamination. A SuperScript III First-Strand Synthesis Super Mix kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis, according to the manufacturer's instructions. The samples were stored at -20ºC until further use.

Real-time PCR reactions were performed using the fluorescent dye SYBR GREEN (PCR Master Mix, Applied Biosystems, Carlsbad, CA, USA). The primers used in the *NPY, AMPKα-1, POMC, LKB1* and *GHRL* amplification reactions were designed based on the gene sequences deposited at www.ncbi.nlm.nih.gov

(accessions: M87294, DQ302133, NM_001031098, NM_001045833, and AB075215, respectively; Table 1). Two endogenous controls, *β-actin* and *GAPDH*, were used, and *β-actin* (accession number L08165) was selected because the amplification of *β-actin* was more efficient. All of the analyses were performed in duplicate, each in a volume of 25 µL. The primers used in the gene expression study proved to be adequate for real-time PCR analysis. The amplification efficiency was similar for the genes of interest, at 90 to 110%. Analysis of the dissociation curves did not reveal the presence of unspecific products or the formation of primer dimers, demonstrating the reliability of the data for the estimation of the mRNA expression of the evaluated genes. The *β-actin* gene used as an endogenous control did not show any statistically significant differences across treatments, demonstrating the validity of its use as the endogenous control.

Statistical analysis

The $2^{-\Delta CT}$ method was used for relative quantification analysis, and the data were expressed in arbitrary units (AU). The results are expressed as averages and standard deviations (SD). The UNIVARIATE procedure was applied to evaluate the normality of the data. The experiment was conducted using a completely randomized factorial design, with two environments (thermal comfort and heat stress) and two genders (male and female). Data were analyzed by two-way ANOVA, with all effects considered as fixed, and the averages were compared using the Tukey test ($p<0.05$) (SAS, 2002, vers 9.00).

Results and discussion

We observed that males tended to consume a larger amount of feed than females, although the differences were not statistically significant ($p=0.0668$). This observed gender difference in feed intake can be partially explained by the higher growth rate observed in males. Higher growth rates are related to greater feed intake capacity, which, in turn, is closely related to gender differences in nutritional requirements (Bertechini, 2012). Furthermore, although the differences were not statistically significant ($p=0.0687$), we also observed that animals subjected to HS tended to have a lower feed intake. Regarding weight gain, birds subjected to HS presented a lower weight gain than broilers at the comfortable temperature ($p=0.0065$; Table 2). Genetically selected broilers show better performance, but are also more sensitive to the effects of the environmental temperature. Studies have shown that broilers subjected to HS conditions may exhibit lower feed intake with a consequent reduction in weight gain (Mujahid *et al.*, 2007). Another possible explanation for the reduction in weight gain observed in birds experiencing heat stress would be an increase in plasma corticosterone levels, because this hormone has been associated with a higher degree of body protein breakdown (Yunianto *et al.*, 1997).

The results of gene expression analyses for *NPY, AMPKα-1, POMC, GHRL* and *LKB1* are presented in Table 3. *NPY* ($p=0.0225$), *AMPKα-1* ($p=0.0398$), and *POMC* gene expression ($p=0.0072$) was influenced by the interaction between gender and HS. The highest expression

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

Gene [1]	Amplicon (bp) [2]	Primer sequence (5'-3') [3]
NPY	101	F: GAGGCACTACATCAACCTCATCA R: CTGTTTTCTGTGCTTTCCCTCAA
AMPKα-1	266	F: CGGAGATAAACAGAAGCACGAG R: CGATTCAGGATCTTCACTGCAAC
POMC	88	F: CGCTACGGCGGCTTCA R: TCTTGTAGGCGCTTTTGACGAT
LKB1	158	F: TGAGAGGGATGCTTGAATACGA R: ACTTGTCCTTTGTTTCTGGGC
GHRL	203	F: CCTTGGGACAGAAACTGCTC R: CACCAATTTCAAAAGGAACG
β-actin	136	F: ACCCCAAAGCCAACAGA R: CCAGAGTCCATCACAATACC

[1]*NPY*: neuropeptide Y; *AMPKα-1*: AMP-activated protein kinase; *POMC*: pro-opiomelanocortin; *LKB1*: liver kinase B1; *GHRL*: ghrelin. [2]bp: base pairs. [3]F: forward; R: reverse. Annealing temperature for all primers was 60°C.

Table 2. Feed intake (FI) and weight gain (WG) of male and female broilers. Results shown are means ± SD.

		FI (kg)	WG (kg)
Male	Comfort	0.12±0.02	0.08±0.01
	Heat Stress	0.08±0.03	-0.04±0.03
Female	Comfort	0.08±0.01	0.06±0.01
	Heat stress	0.07±0.03	-0.01±0.08
Main effects			
Gender	Male	0.10±0.03	0.02±0.07
	Female	0.07±0.02	0.03±0.06
Environment	Comfort	0.10±0.03	0.07^{a}±0.02
	Heat stress	0.07±0.03	-0.02^{b}±0.06
***p*-value**			
Gender		0.0668	0.9316
Environment		0.0687	0.0065
Interaction		0.3685	0.3626

a,b Mean values within a column with different superscript letters are significantly different (p<0.05).

level of the *NPY* gene was observed in males from the comfortable temperature treatment. The ARC contains neuronal cells responsible for the synthesis and release of orexigenic neuropeptides that stimulate consumption, such as NPY, and anorexigenic neuropeptides that have an inhibitory effect on consumption, such as on POMC (Minor *et al.*, 2009). Thus, the balance between the actions of orexigenic and anorexigenic neuropeptides determines the organic energy status that modulates feed intake and body weight.

NPY is considered the most potent orexigenic peptide and it is widely expressed in the central nervous system (Eva *et al.*, 2006). Our results confirm the stimulatory effect of NPY on feed intake because the highest levels of expression were observed in animals that tended to have increased consumption. Regarding *AMPKα-1* expression, male broilers from the HS group presented the highest *AMPKα-1* expression levels. We could also observe a gender effect on *LKB1* and *AMPKα-1* expression (p=0.0256 and p=0.0001, respectively), with higher expression levels observed in male broilers. AMPK also plays a role in processes related to feed intake (Xue & Kahn, 2006). AMPK activation may occur due to environmental and metabolic stress, which can inhibit ATP synthesis or accelerate the use of ATP, causing an increase in the ratio of intracellular AMP: ATP (Richards *et al.*, 2010). LKB1, also known as serine/threonine kinase 11 (STK11), is a heterotrimeric complex with two accessory proteins (Boudeau *et al.*, 2003). These three units together form a biological unit that phosphorylates and activates AMPK (Towler & Hardie, 2007). According to Richards *et al.* (2010), AMPK, a conserved energy sensor, when activated in the hypothalamus by metabolic and environmental stresses that deplete cells of energy, can stimulate catabolic pathways in an attempt to restore the amount of energy available to the animal organism. Our results also confirm the functional relationship between LKB1 and AMPK in birds (Proszkowiec-Weglarz *et al.*, 2006) and suggest that males have a more efficient physiological mechanism of protection against ATP depletion by LKB1 and AMPK action than females.

Unlike *NPY* and *AMPK*, the *POMC* gene encodes a substance considered to be anorexigenic, which after processing gives rise to bioactive peptide hormones such as melanocyte-stimulating hormone (MSH α, β and γ)

Table 3. *NPY* (neuropeptide Y), *AMPKα-1* (AMP-activated protein kinase), *POMC* (pro-opiomelanocortin), *GHRL* (ghrelin) and *LKB1* (liver kinase B1) genes expression in the hypothalamus of male and female broilers. Results shown are means ± SD.

		NPY	***AMPKα-1***	***POMC***	***GHRL***	***LKB1***[1]
Male	Comfort	11.57^{a} ± 2.05	0.45^{b} ± 0.04	0.04^{b} ± 0.01	0.03 ± 0.01	1.90 ± 0.65
	Heat stress	7.09^{b} ± 1.87	0.60^{a} ± 0.15	0.05^{ab} ± 0.01	0.02 ± 0.02	2.08 ± 0.56
Female	Comfort	9.19^{ab} ± 1.53	0.34^{bc} ± 0.05	0.05^{a} ± 0.01	0.03 ± 0.01	1.23 ± 0.44
	Heat stress	9.19^{ab} ± 3.10	0.31^{c} ± 0.13	0.04^{b} ± 0.01	0.02 ± 0.01	1.63 ± 0.58
Main effects						
Gender	Male	9.33 ± 3.00	0.53 ± 0.13	0.04 ± 0.01	0.03 ± 0.01	1.99^{a} ± 0.59
	Female	9.19 ± 2.33	0.32 ± 0.09	0.04 ± 0.01	0.03 ± 0.01	1.43^{b} ± 0.54
Environment	Comfort	10.38 ± 2.13	0.40 ± 0.07	0.04 ± 0.01	0.03 ± 0.01	1.56 ± 0.64
	Heat stress	8.14 ± 2.67	0.45 ± 0.20	0.04 ± 0.01	0.02 ± 0.01	1.85 ± 0.59
***p*-value**						
Gender		0.8803	0.0001	0.7128	0.8682	0.0256
Environment		0.0223	0.1967	0.4522	0.1322	0.2200
Interaction		0.0225	0.0398	0.0072	0.4736	0.6378

[1] Expressed in arbitrary units (AU). [a,b] Mean values within a column with different superscript letters are significantly different (p<0.05).

(Bicknell, 2008). The α-melanocyte-stimulating hormone (α-MSH), by binding to the melanocortin receptor 3 and/or 4 located in the central nervous system, promotes an inhibition of food consumption and an increase in body energy expenditure (Bicknell, 2008). In this study, we found that females at comfortable temperature had higher *POMC* gene expression levels. The increased expression of *POMC* may be related to the lower *AMPKα-1* expression, also observed in those birds. Lower AMPK activity has been linked to the activation of mTOR (rapamycin target protein), which in turn has been associated with *POMC* activation (Richards *et al.*, 2010). This cascade of events causes a reduction in consumption and an increase in the use of organic energy for maintenance, growth and reproduction (Richards *et al.*, 2010). The opposite actions of these two routes (AMPK and mTOR), which cause changes in food intake while maintaining the energy balance, may be key to achieving metabolic balance and promoting animal development. In addition to *POMC*, other substances such as ghrelin may also have an anorexigenic role in birds (Saito *et al.*, 2005). Although we did not observe any effect of heat stress treatment on *GHRL* expression, we believe that more studies should be carried out to better understand the role of this hormone in mediating the differences in feed intake between birds of different genders exposed to different temperature conditions. Our results indicate that feed intake is a function of many factors still unknown, and suggest that the hypothalamic genes evaluated in this study may be involved in the observed differences in voluntary feed intake between animals of different genders exposed to heat stress.

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