GENETICS AND MOLECULAR BIOLOGY

Influence of incubation, diet, and sex on avian uncoupling protein expression and oxidative stress in market age broilers following exposure to acute heat stress

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ABSTRACT Genetic selection for rapid growth in broilers has inadvertently resulted in increased susceptibility to heat stress, particularly in male birds. Increased oxidative stress associated with hyperthermia may be reduced by avian uncoupling protein (**avUCP**), which has been proposed to modulate free radical production. However, the relationship between avUCP expression and current heat stress management strategies is unclear. Embryonic acclimation or thermal manipulation (**TM**) and dietary fat source are 2 heat stress interventions that may alter avUCP expression and oxidative stress, but the literature is inconclusive. The objective of this trial was to investigate the effect of TM and dietary fat source on avUCP gene expression and oxidative damage in the breast meat of market age broilers before and after acute heat challenge. The influence of bird sex was also evaluated as broilers exhibit a high degree of sexual dimorphism in growth and stress susceptibility. Concentration of thiobarbituric acid reactive substances (**TBARS**) was measured as a marker of oxidative damage. Embryonic TM occurred from incubation d 7 to 16 for 12 h daily at 39.5°C. Dietary treatments were applied during the finisher period using either poultry fat, soya oil, or olive oil supplemented at 4.5% in the diet. Acute heat stress (**AHS**) occurred on d 43 at 32°C for 4 h. Bird performance was decreased by TM, but no significant differences were noted between dietary fat source treatments. Neither avUCP nor TBARS concentrations were significantly influenced by TM or dietary fat source. Downregulation of avUCP was observed following AHS, concurrent with an increase in TBARS concentration. Male birds exhibited higher levels of both avUCP expression and TBARS compared to females and a significant interaction was noted for heat stress by sex, with avUCP expression being greatest in males prior to AHS. The increase in avUCP expression and TBARS concentrations in male birds may be associated with an increased susceptibility to stress arising from the increased growth rate noted for male broilers.

Key words: thermal manipulation, dietary fat source, acute heat stress, avian uncoupling protein, oxidative stress

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INTRODUCTION

Genetic selection for rapid growth in broilers has inadvertently resulted in increased susceptibility to heat stress, particularly in male birds (Cahaner and Deeb, 2000; Havenstein et al., 2003). Poor performance in broilers exposed to heat stress is commonly associated with reduced feed intake; however oxidative damage from reactive oxygen species (**ROS**) formation induced by heat stress can also decrease bird production (Altan et al., 2003; Lin et al., 2006). Excessive ROS are attenuated by proton leak into the mitochondrial matrix; this process is facilitated by mitochondrial uncoupling proteins (**UCP**) (Mailloux and Harper, 2011). Avian uncoupling protein (**avUCP**) is the only UCP identified in birds and is primarily expressed in skeletal muscle (Raimbault et al., 2001; Evock-Clover et al., 2002). Elevated ROS upregulates avUCP, suggesting a role for avUCP in oxidative stress management (Abe et al., 2006; Rey et al., 2010; Zhang et al., 2010). In broilers, heat stress is strongly associated with elevated ROS formation and oxidative damage (Mujahid et al., 2005; Azad et al., 2010);

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however, impact on avUCP expression is less clear, as reports indicate avUCP is downregulated (Mujahid et al., 2006; Kikusato and Toyomizu, 2013), unchanged (Al-Zghoul et al., 2015) or even upregulated (Dridi et al., 2008; Azad et al., 2010) in response to heat stress. If avUCP can be reliably upregulated during heat stress, oxidative damage may be attenuated, and bird performance sustained. Work concerning the relationship between avUCP expression and established heat stress interventions is limited, but the potential for a synergetic improvement in heat stressed broiler performance is worth further investigation.

One strategy for managing heat stress is embryonic acclimation to post-hatch temperature challenges through periods of controlled exposure to similar conditions, or thermal manipulation (**TM**) (Moraes et al., 2003). Midway during incubation, the hypothalamo-pituitary-thyroid and -adrenocortical axes (**HPT** and **HPA**, respectively) coalesce, and increased temperature exposure during this period may alter development of these thermoregulatory systems, improving heat stress tolerance post-hatch (Nichelmann and Tzschentke, 2002; Yahav et al., 2004; Piestun et al., 2008; Loyau et al., 2013). However, modification of the HPT and HPA axes may indirectly lower avUCP levels as triiodothyronine (Collin et al., 2003a) and the β-adrenergic system (Bugajski et al., 1991; Joubert et al., 2011) have both been proposed as regulators of avUCP mRNA expression. The current literature concerning the influence of TM on avUCP has been inconclusive (Loyau et al., 2014; Al-Zghoul et al., 2015, 2019), and further investigation is warranted to elucidate its possible role in TM adaptation.

Increased dietary fat is a customary intervention employed to support bird performance during heat stress as fat improves diet energy density at a lower heat increment compared to carbohydrates or proteins (Dale and Fuller, 1979; Tetter and Belay, 1996). Fat supplementation may also enhance avUCP expression (Collin et al., 2003b; Joubert et al., 2011), with diets rich in unsaturated fatty acids (**USFA**) increasing avUCP mRNA expression more than saturated fatty acids (**SFA**; Seifi et al., 2018). Supplementation with olive oil (containing USFA) during heat stress upregulates avUCP and reduces ROS production and oxidative damage (Mujahid et al., 2009). However, olive oil is not a cost-efficient ingredient for broiler diets, and heat stress interventions must be practical as well as effective to be commercially relevant. Soya oil may be a more conventional USFA fat source, but it requires further evaluation to ascertain if it can improve avUCP expression during heat stress.

Because genetic selection for rapid growth has amplified sexual dimorphism in modern broilers, studies must consider the influence of bird sex during hyperthermia (Zuidhof et al., 2014; Nogueira et al., 2019). The higher growth rate in male broilers may inadvertently increase ROS production, contributing to observations of heat stress susceptibility in males (Cahaner and Leenstra, 1992; Settar et al., 1999) Brothers et al. (2019). report that male broilers exhibit higher expression of genes involved in oxidative stress response under normal rearing conditions; however, the influence of bird sex on avUCP is not confirmed.

The aim of this study was to investigate the effects of heat stress interventions TM and dietary fat source on avUCP mRNA expression and oxidative damage in the breast tissue of market age broilers exposed to acute heat stress. Malondialdehyde (**MDA**) accumulation was used as an estimate of oxidative stress and the breast was chosen as the sample site due to the expression pattern of avUCP mRNA (Evock-Clover et al., 2002), as well as its importance as a premium portion. The influence of bird sex was also evaluated to determine if sexual dimorphism exists in either avUCP mRNA expression or oxidative damage.

MATERIALS AND METHODS

Incubation and rearing occurred at the North Carolina Department of Agriculture's Piedmont Research Station, in collaboration with North Carolina State University. All procedures used within this study were reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University as well as the Research Ethics committee of the Faculty of Natural and Agricultural Sciences, University of Pretoria (NAS200/2020).

Incubation and Rearing

Hatching eggs were obtained from a 43-wk-old Ross 708 broiler breeder flock (Aviagen, Inc., Huntsville, AL) with an average egg weight of 68.47 ± 1.04 g and stored at 18°C and 75% relative humidity (**RH**) for 2 to 5 d prior to incubation. Eggs were distributed between the 2 incubation treatment groups, with 1,740 eggs set into 12 replicate trays per treatment (3,480 eggs total). Pre-incubation occurred in a forced ventilation cabinet for 12 h at 26°C, after which all trays were placed into a single incubator (Natureform Inc., Jacksonville, FL) set at 37.5°C and 56% RH. Tray placement within the incubator was arranged in a generalized randomized complete block design, with 3 trays per treatment in each block (4 blocks in total) to reduce position effect within the machine. Turning occurred through 90° hourly.

At incubation day (**E**) 7, trays in the TM treatment were transferred to a second incubator set at 39.5°C and 65% RH. Relative humidity in the TM incubator was increased to prevent excessive moisture loss from the eggs during exposure to increased temperatures and limit evaporative cooling (Meijerhof and Van Beek, 1993). After 12 h of high temperature exposure, the TM trays were returned to the initial incubator for 12 h and this cycle was repeated at the same time daily from E7 to E16 (Piestun et al., 2008). Trays in the control group (**CN**) remained in the original setter at a constant temperature throughout incubation. The TM incubator was adjacent to the CN incubator to reduce

transport stress. A plastic tent was erected over both machines and heated to 37.5°C to minimize heat loss by the eggs during transfer between machines. Trays in the CN group were also handled twice daily during TM transfer so that any handling stress would be similar between groups. The TM exposure continued until E16 and at E17.5 eggs were transferred to hatching baskets and both groups was placed in a single hatcher until completion of incubation.

Hatchability was decreased by approximately 12% in the TM treatment as compared to the CN group (Brannan et al., 2021). Chicks were assessed by trained technicians and first grade chicks were characterized by having a healed navel, sound legs, clean beak, and eyes, as well as being overall active and alert. First grade chicks were feather sexed and placed in a 60 pen environmentally controlled broiler house. Treatment pens were assigned in a randomized complete block design with 6 treatments (2 incubation profiles × 3 finisher diets) in 10 blocks. Each block contained 6 pens, representing each treatment combination for a total of 10 replicates per treatment. An equal number of chicks for both sexes were placed in each pen for a total of 18 chicks per pen and body weights per pen were taken prior to placement. All pens (1.2 × 1.5 m) used wood shavings for bedding and contained one tube feeder as well as 5 drinking nipples. A supplemental feed pan and chick water font was included in each pen for the first week of rearing and removed at 7 d. Standard commercial lighting and temperature profiles (Aviagen, 2018) were implemented until the acute heat challenge at 43 d, when temperatures within the house were gradually increased from 21°C to 32°C for 4 h. Following heat stress, standard temperature profiles were implemented until the end of the study at 49 d.

Birds were fed a standard commercial starter and grower diet until 14 d and 28 d, respectively, after which dietary treatments were applied during the finisher period from 28 to 49 d (Table 1). Treatment diets differed in fat source, which consisted of a monounsaturated fatty acid source (olive oil, **OO**), a polyunsaturated fatty acid source (soya oil, **SO**), and a saturated fatty acid source (poultry fat, **PF**). Fat sources were analyzed for free fatty acids (method Ca 5a-40, AOCS, 2017) as well as SFA and USFA concentrations (method 996.06, AOAC, 2012) and energy values were determined for each using the Wiseman equation for birds older than 21 d (Wiseman and Salvador, 1991; Wiseman et al., 1998). Diets were formulated based on the energy contribution of each fat source at an inclusion level of 4.5%, with final dietary ME being 3,180 Kcal for the PF diet, 3,203 Kcal for the SO diet, and 3,208 Kcal for the OO diet (Table 1). All diets were formulated to meet or exceed NRC (1994) requirements. The fatty acid profiles and nutritional parameters of each fat source are shown in Table 2. All diets were manufactured at the North Carolina State University Feed Mill.

Body weight (**BW**) and feed intake (**FI**) were recorded by pen on a weekly basis. Feed conversion ratio (**FCR**) was calculated as was production efficiency factor (**PEF**) using the following formulae.

Table 1. Composition of the starter, grower, and base finisher diet.

Ingredients (%)	Starter	Grower	Finisher
Corn	49.93	58.89	62.65
Soybean meal, 48%	34.65	26.50	21.57
Distillers dried grains with solubles	7.50	8.00	8.00
Fat[1]	3.50	3.43	4.50
Salt	0.29	0.26	0.24
Limestone	1.14	1.20	1.17
Dicalcium phosphate, 18.5%	1.54	0.85	0.46
DL-methionine, 99%	0.32	0.23	0.25
L-lysine-HCl, 78.8%	0.27	0.21	0.29
Choline chloride, 60%	0.18	0.18	0.18
Sodium bicarbonate	0.27	0.18	0.21
L-threonine, 98%	0.05	0.02	0.07
Coccidiostat[2]	0.05	0.05	0.05
Mineral premix[3]	0.23	0.20	0.20
Vitamin premix[4]	0.10	0.10	0.10
Phytase[5]	0.01	0.01	0.01
Calculated nutrient content			
Metabolizable energy, kcal/kg	2,950	3,050	*
Crude protein, %	22.00	18.95	17.11
Calcium, %	1.05	0.85	0.76
Available phosphorus, %	0.45	0.40	0.37
Digestible lysine, %	1.22	1.05	0.93
Sodium,%	0.24	0.23	0.19
Chloride, %	0.29	0.31	0.26

[1]Starter and grower diets included only poultry fat. Finisher diets included either poultry fat (PF), soybean oil (SO), or olive oil (OO) at 4.5 %.

[2]Coban 90 (Monesin) (Elanco Animal Health, Greenfield, IN) at 90 g / ton of feed.

[3]Trace minerals provided per kg of premix: manganese (MnO_2), 220 g; zinc (ZnO and $ZnSO_4$), 250 g; iron ($FeCO_3$), 75 g; copper (CuSO4 and $CuCl_2$), 10 g; iodine ($Ca(IO_3)_2$), 5 g; selenium (Na_2SeO_3), 1 g.

[4]Vitamins provided per kg of premix: vitamin A, 18,739,292 IU; vitamin D3, 6,613,868 IU; vitamin E, 66,139 IU; vitamin B12, 33 mg; riboflavin, 22,046 mg; niacin, 88,185 mg; d-pantothenic acid, 30,865 mg; menadione, 3,968 mg; folic acid, 2,646 mg; vitamin B6, 7,716 mg; thiamine, 5,512 mg; biotin, 176 mg.

[5]Quantum Blue 5G 5 at 0.20 lbs/ton (100 g / ton) to provide 500 FYT (AB Vista, Marlborough, UK) delivering 0.13% of available P, 0.06% of calcium and 0.03% of sodium.

[*]The Wiseman equation (Wiseman and Salvador, 1991; Wiseman et al., 1998) was used to calculate the energy value for each fat and final dietary ME was 3,180 kcal/kg (PF), 3,203 kcal/kg (SO), and 3,208 kcal/kg (OO).

$$\text{FCR} = \frac{\textit{Feed intake}}{\textit{Body weight gain}}$$

$$\text{PEF} = \frac{\textit{Live weight (kg) x Livability (\%)}}{\textit{Age (d) x FCR}} \textit{ x } 100$$

Table 2. Fatty acid profile and nutritional parameters of the dietary fat sources[1].

Fatty acid (g/100 g)	Poultry fat	Soya oil	Olive oil
C16:0	23.35	11.58	10.90
C16:1	6.17	0.18	0.17
C18:0	7.76	5.85	4.24
C18:1	40.80	22.78	29.07
C18:2	19.61	51.34	47.82
C18:3	0.81	7.11	7.03
Omega fatty acids (g/100 g)			
Omega-3	1.16	7.11	7.03
Omega-6	19.61	51.34	47.82
Omega-9	41.08	23.00	29.22
Nutritional parameters (%)			
Total saturated fatty acids	31.86	18.37	15.67
Total unsaturated fatty acids	68.14	81.62	84.24
Unsaturated/saturated ratio	2.14	4.44	5.38
Total monounsaturated fatty acids	47.37	23.17	29.39
Total polyunsaturated fatty acids	20.77	58.45	54.85
Free fatty acids	5.46	0.55	0.77

[1]Each fat source included at 4.5% to the base finisher diet.

Breast muscle tissue samples were taken at 42 d (pre-AHS) and 43 d (post-AHS) to evaluate lipid peroxidation through MDA levels and avUCP mRNA expression. Tissue samples were taken from one male and one female bird selected from each pen by BW to be close to the average BW of the treatment pen for each sex. Sample birds were euthanized via cervical dislocation by trained technicians and tissue was collected from the center of the left breast muscle (*Pectoralis* major), approximately 2.5 cm from the keel bone. Tissue samples were flash frozen in liquid nitrogen immediately upon collection and stored at −80°C until analysis.

Thiobarbituric Acid Reactive Substance Assay

Lipid peroxidation, as determined through measuring the amount of MDA that reacts with 2-thiobarbituric acid, was used to estimate oxidative stress (Armstrong and Browne, 1994; Yagi, 1998). A thiobarbituric acid reactivate substance (**TBARS**) assay kit was used to evaluate MDA levels in the breast tissue samples taken pre- and post-heat stress (42 and 43 d, respectively). Samples were analyzed within 3 wk of collection to prevent any changes in MDA levels due to long-term storage. Approximately 50 mg (±5 mg) of tissue from each sample was mixed with 500 μL of homogenization buffer, which contained 50 mM TrisHCl pH 8, 150 mM NaCl, 1% NP-40, 1 mM Na3VO4, 0.1% SDS, 2 uM leupeptin, 2 uM pepstatin, 10 KIU/mL aprotinin, and 400 uM PMSF, and then homogenized with a Tissue Tearor (Model 985370, Biospec Products Inc., Bartlesville, OK) before being centrifuged at 1,600 × *g* for 10 min at 4°C. The resultant supernatant was subjected to bicinchoninic acid assay to determine protein concentration, and samples were normalized to the lowest concentration, with each sample having a final total volume of 100 μL. The colorimetric version of the TBARS assay was performed per kit instructions (item no. 700870, Cayman Chemical, Ann Arbor, MI) and absorbance was read at 532 nm (Bio-Rad xMark, Bio-Rad Laboratories Inc., Hercules, CA). Results are presented here as MDA relative to the amount of sample protein (nmol/mg).

Real-Time Quantitative PCR

The expression of avUCP mRNA in the breast tissue was determined using RT-qPCR. Methodology was performed as described by Saini et al. (2019) and followed the Minimum Information for Publication of Quantitative Real-Time Experiments (**MIQE**) guidelines (Bustin et al., 2009). Trizol reagent (item no. 15596026, Invitrogen, Carlsbad, CA) was used for total RNA isolation according to the manufacturer's instructions. Sample RNA purity was confirmed by spectrophotometry (NanoDrop 200, Thermo Scientific, Waltham, MA) and RNA integrity was evaluated by gel electrophoresis. Isolated RNA was stored at −80°C. Synthesis of cDNA was performed using 1 μg of RNA with random hexamer primers and ImProm-II Reverse Transcriptase (item no. A3803, Promega, Madison, WI) diluted to a concentration of 1 μg/μL, prepared and synthesized as per manufacturer's instructions. Following synthesis, cDNA was stored at −20°C.

In preparation for RT-qPCR analysis, cDNA was added to a PCR reaction mix that included 2 μM of both forward and reverse primers (IDT, Coralville, IA), as well as 2× SYBR Green Supermix (item no. 1725271, Bio-Rad, Madison, WI). The forward and reverse primers used for avUCP (GenBank accession no. AF433170.2) were GTCACTTCGTGGCTGCCTTC and CTCGGCACATTGCGGTACTG, respectively. Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**, GenBank accession no. NM_204305.1) served as the reference gene and the GAPDH primer sequences were forward ATGACCACTGTCCATGCCATCA and reverse AGGGATGACTTTCCCTACAGCCTT. Dilution of cDNA occurred at a 1:10 concentration for avUCP and 1:1,000 for GAPDH so that Ct values between the target and reference genes would be no more than 5 Ct values apart. Amplification efficiency for all primers was between 90 and 110%. RT-qPCR was conducted using a PCR Thermal Cycler (Bio-Rad CFX384 Real-Time System, Bio-Rad Laboratories Inc.) and cycles consisted of 95°C for 2 min followed by 45 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Incorporation of the fluorescent dye was measured at the end of each amplification cycle and an analysis of the melting curve occurred upon the completion of the final cycle to confirm amplification specificity. Samples were run in triplicate and target gene expression was normalized to GAPDH. Mean relative expression change was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) for statistical analysis. Abundance of GAPDH was not affected by bird sex, heat exposure, incubation, or dietary treatment.

Statistical Analysis

All data were subjected to the Shapiro-Wilk test to assess normality and Levene's test was used to verify the equality of variances. Performance data (BWG, FI, FCR, and PEF) were analyzed as a two-way ANOVA (incubation × diet) using the fit-model platform of JMP Pro 13 (SAS Institute, Cary, NC) with rearing pen as the experimental unit. A forward selection stepwise regression with Bayesian information criterion (**BIC**) was conducted to determine the optimal model for evaluating the effect of incubation, diet, sex, and heat stress on avUCP gene expression and TBARS levels. Heat stress and sex were selected to be significant variables, while incubation and dietary treatments were removed from the model. Subsequently, gene expression and TBARS data were analyzed as a two-way ANOVA with sex and heat stress as the main effects. Concentrations of TBARS were not normally distributed and were, therefore, log-transformed for analysis while the untransformed values are presented in the results

Table 3. Weekly average body weight gain (BWG), feed conversion ratio (FCR), and feed intake (FI) as influenced by incubation treatment and dietary fat source during the finisher period, as well as their interactions pre- and post-heat challenge[1].

	29 to 42 d, pre-heat stress				43 to 49 d, post-heat stress			
Treatment	BWG,	FI,	FCR,	PEF	BWG,	FI,	FCR,	PEF
	g/bird	g/bird	g:g		g/bird	g/bird	g:g	
Incubation[2]								
CN	1,213.9[A]	2,058.5[A]	1.79	456.5	669.9	1,405.5[A]	2.09	189.3
TM	1,154.5[B]	1,974.6[B]	1.71	439.8	652.0	1,329.9[B]	2.04	201.4
SEM[3]	12.2	18.0	0.01	6.5	11.0	16.0	0.03	5.5
P-value	0.001	0.002	0.292	0.072	0.256	0.001	0.227	0.124
Diet[4]								
Olive	1,186.4	2,028.0	1.71	456.8	637.1	1,357.9	2.11	187.4
Poultry	1175.7	2016.3	1.71	445.4	680.2	1,381.6	2.04	200.4
Soya	1,190.4	2,005.3	1.68	442.3	665.5	1,363.6	2.06	198.1
SEM	15.0	22.0	0.01	7.9	13.4	19.3	0.03	6.7
P-value	0.774	0.765	0.189	0.403	0.081	0.670	0.196	0.352
Incubation × diet								
CN-Olive	1,213.7	2,057.0	1.70	466.5	627.6	1,396.8	2.18[A]	174.2[b]
CN-Poultry	1,204.6	2,045.1	1.70	456.7	716.0	1,411.1	1.97[B]	208.3[a]
CN-Soya	1,223.4	2,073.5	1.69	446.4	665.9	1,408.5	2.12[AB]	185.5[ab]
TM-Olive	1,159.1	1,999.1	1.72	447.1	646.6	1,319.0	2.05[AB]	200.7[ab]
TM-Poultry	1,146.8	1,987.4	1.73	434.1	644.4	1,352.2	2.10[AB]	192.6[ab]
TM-Soya	1,157.5	1,937.2	1.68	438.2	665.1	1,318.6	1.99[B]	210.7[a]
SEM	21.2	31.1	0.02	11.2	18.8	27.3	0.04	9.7
P-value	0.962	0.353	0.287	0.795	0.054	0.850	0.005	0.049

[1]Acute heat stress occurred at 32°C for 4 h at 43 d.
[2]TM, thermal manipulation at 39.5°C and 65% RH for 12 h from E7 to E16; CN = control remained at 37.5°C and 56% RH.
[3]SEM, Standard error of mean.
[4]Poultry = poultry fat; Soya = soya oil; Olive = olive oil added at 4.5% to finisher diet.
[A,B]Means in a column that possess different superscripts differ significantly ($P < 0.01$).
[a,b]Means in a column that possess different superscripts differ significantly ($P < 0.05$).

section. Differences between means were separated using Tukey's HSD test (Tukey, 1949) and significance was determined at $P < 0.05$.

RESULTS

Rearing Performance

The effect of incubation and dietary treatments, as well as their interactions, on broiler performance during the finisher phase is shown in Table 3. Decreased BWG and FI were noted for the TM birds prior to the heat challenge ($P < 0.001$ and $P < 0.002$, respectively). Although BWG was shown to be similar following AHS, FI remained lower for the TM group ($P < 0.001$). Differences in FCR and PEF scores between the incubation treatments were not observed, regardless of heat challenge. Dietary fat source was not shown to influence broiler performance.

A significant interaction was observed for FCR and PEF during the post-heat stress period. Birds in the CN-PF and TM-SO exhibited improved feed efficiency while the CN-OO group demonstrated the poorest ($P = 0.005$). Likewise, CN-PF and TM-SO resulted in the highest PEF scores while CN-OO had the lowest ($P = 0.049$). A similar trend ($P = 0.054$) was noted for BWG, with CN-PF exhibiting the highest BWG and CN-OO the lowest.

TBARS

Stepwise regression analysis revealed no influence of dietary or incubation treatment on TBARS levels (Table S1). Concentration of TBARS as influenced by bird sex and acute heat stress are shown in Figure 1. Male broilers exhibited higher levels of TBARS in comparison to females ($P = 0.0001$) and heat stress was shown to increase TBARS for all birds ($P < 0.0001$). Significant differences were not apparent for interactions between bird sex and heat challenge ($P = 0.3540$).

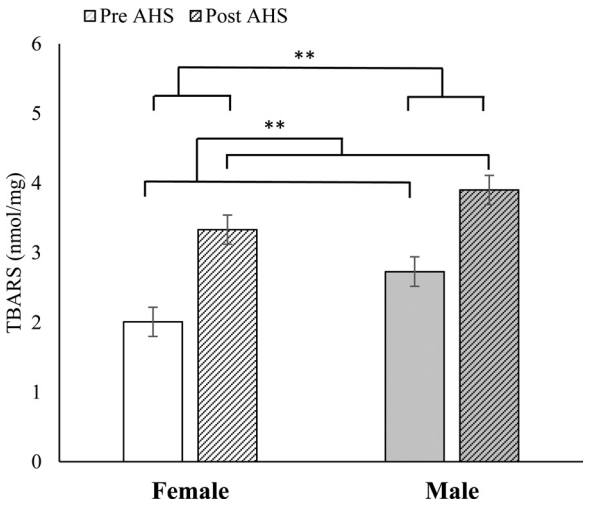

Figure 1. Concentration of thiobarbituric acid reactive substances (TBARS) in pectoralis major of broiler chickens as influenced by broiler sex ($P = 0.0001$) and exposure to acute heat stress (AHS) ($P < 0.0001$). Heat challenge occurred at 43 d for 4 h at 32°C. Data were subjected to log transformation for statistical analysis. Values are presented as untransformed means and standard error of means. $N = 60$ individuals per treatment interaction. ** Means differ significantly ($P \leq 0.0001$).

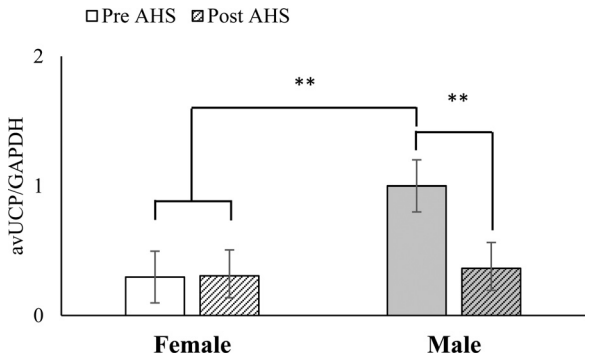

Figure 2. avUCP mRNA expression in pectoralis major of broiler chickens as influenced by the interaction between broiler sex and exposure to acute heat stress (AHS) ($P = 0.0001$). Pre-AHS samples were taken on 42 d and post AHS samples were taken immediately following a heat challenge at 32°C for 4 h on 43 d. Values are presented as means and standard error of means. $N = 60$ birds per treatment interaction group. ** Means differ significantly ($P < 0.0001$).

Avian UCP mRNA Expression

Gene expression of avUCP was not shown to be significantly affected by dietary or incubation treatments (Table S1), as determined by stepwise regression analysis. The effect of bird sex, heat challenge, and their interaction on avUCP expression is displayed in Figure 2. Increased avUCP expression was noted for males ($P < 0.0001$) while acute heat stress resulted in a significant down regulation of avUCP ($P = 0.0003$). A significant interaction was noted for sex and AHS, with males prior to heat exposure exhibiting the greatest abundance of avUCP mRNA ($P = 0.0001$).

DISCUSSION

Gene expression of avUCP and concentration of TBARS in broiler breast muscle were not significantly altered by TM exposure. Previous studies have reported similar findings for the influence of TM on avUCP mRNA expression in broiler skeletal muscle (Loyau et al., 2014; Al-Zghoul et al., 2015), although upregulation of avUCP has been observed in the liver of TM birds (Al-Zghoul et al., 2019). This disparity likely represents differences in tissue regulation as other work has demonstrated variations in avUCP expression between skeletal muscle and liver tissue (Vianna et al., 2001; Del Vesco et al., 2013). The breast portion was chosen for sampling in the current trial as skeletal muscle has been shown to exhibit the greatest abundance of avUCP mRNA in broilers, while liver and adipose tissues displayed the lowest (Evock-Clover et al., 2002; Dridi et al., 2004). Additionally, breast meat is considered a premium portion and ROS accumulation resulting from high ambient temperatures has been linked to undesirable meat characteristics that can result in economic loss at processing (Sandercock et al., 2001; Wang et al., 2009). A reduction in breast muscle oxidative damage, through either TM adaptation or avUCP expression, would presumably result in a corresponding reduction in losses associated with poor meat quality in heat stressed broilers. However, similar to avUCP expression, significant differences in TBARS concentrations in breast meat were not apparent between the incubation treatments. The decrease in BWG and FI for TM birds in the current results rather suggests that TM may have been detrimental to performance, although other authors have reported reduced mortality during heat stress as benefit of TM exposure (Piestun et al., 2008; Zaboli et al., 2017; Brannan et al., 2021). While the balance between bird performance and heat stress livability associated with TM remains to be elucidated, the present results do not support a role for avUCP gene expression or reduced oxidative damage in TM adaptation.

Dietary fat source was not shown to significantly alter avUCP gene expression or TBARS concentration in the present trial. The difference between the current results and previous work demonstrating significant avUCP upregulation associated with USFA (Mujahid et al., 2009; Seifi et al., 2018) are likely due to differences in trial design. The heat challenge implemented by Seifi et al. (2018) occurred for a period of 10 d and chronic heat stress has been reported by other authors to upregulate avUCP, independent of diet (Dridi et al., 2008; Azad et al., 2010). Similarly, the method of olive oil supplementation utilized by Mujahid et al. (2009) may have resulted in a greater difference in metabolizable energy between treatment diets when compared to the present trial diets. Higher levels of dietary fat have been shown to upregulate avUCP expression (Collin et al., 2003b), while the current trial rations were determined to be similar in energy content and representative of a commercially relevant diet. Levels of TBARS were also unaltered by dietary fat source which may be related to the similarities between diets in avUCP expression, based on the proposed role of avUCP as a modulator of oxidative stress. Overall, the current results do not support a role for dietary fat source in upregulating avUCP expression or reducing oxidative damage in broilers exposed to AHS.

Similarly, no advantage was noted in terms of bird performance for dietary fat source, although a significant interaction was noted to occur between the heat-stress interventions following AHS for FCR and PEF, concurrent with a similar trend in BWG. These differences were likely linked to the adverse effects of hyperthermia on broiler performance (Teeter and Belay, 1996) and may reflect increased variation in BWG, FI, and livability which fluctuated widely within the interactions following the heat challenge. Previous work in our group noted a numerically larger mortality rate for the CN-OO birds (Brannan et al., 2021), which likely contributed to the poorer PEF in the present results. However, the similarities between the interaction groups as well as the main effect treatments for avUCP mRNA expression and TBARS levels do not indicate a strong role for these factors in the heat stress interventions evaluated.

Heat stress and sex were shown to significantly influence avUCP mRNA expression and TBARS levels, both individually and through interaction effects. A significant increase in TBARS concentration was observed

concurrent with the downregulation of avUCP expression following AHS exposure, in contrast to pre-AHS values. As heat stress is well-established to induce free radical production, lipid peroxidation, and oxidative damage in broilers (Altan et al., 2003; Mujahid et al., 2005; Lin et al., 2006; Azad et al., 2010), the rise in TBARS post-AHS was to be expected. The increase in oxidative damage following heat stress may be linked to the downregulation of avUCP, as reported here as well as by other authors (Mujahid et al., 2006; Kikusato and Toyomizu, 2013). While these results reinforce the suggested function of avUCP as a modulator of oxidative stress, future work quantifying avUCP protein levels under similar conditions would contribute substantially to the understanding of this protein.

Independent of AHS, males exhibited a higher expression of avUCP, which may have been driven by the increased TBARS concentrations also noted in males as compared to female birds. Males have been shown to exhibit a greater susceptibility to oxidative stress, which has been proposed to arise from their rapid growth rate and heavier BW when compared to female broilers (Brothers et al., 2019; Nogueira et al., 2019). As free radical production is a normal product of increased mitochondrial activity (Hensley et al., 2000), male broilers may potentially generate higher levels of ROS than their female counterparts under standard conditions due to their increased metabolic rate (Meltzer, 1983). The increase in avUCP expression may be in response to the increased free radical generation but may not be sufficient to curb oxidative damage. Exposure to additional stress, such as AHS, may further exacerbate oxidative stress as avUCP is downregulated and result in decreased heat tolerance in male birds (Cahaner and Leenstra, 1992; Brothers et al., 2019).

To the authors' knowledge, this is the first report of differences in broilers between the sexes in avUCP gene expression and may explain the disparities of heat stress on avUCP expression in the literature. Previous work demonstrating avUCP downregulation in broilers exposed to heat stress exclusively sampled male birds (Mujahid et al., 2006, 2009; Kikusato and Toyomizu, 2013) while more ambiguous results occurred in mixed sex trials (Al-Zghoul et al., 2015). It may be speculated that in mixed sex studies the ratio of male to female birds sampled can have a greater effect on avUCP mRNA expression and should be considered as an influencing factor in future trials.

The current results indicate that avUCP mRNA expression and TBARS concentration in breast meat from market age broilers are influenced by heat stress and bird sex rather than the heat stress interventions TM and dietary fat source. The finding of sexual dimorphism in avUCP gene expression in broilers contributes to existing evidence that males may be more susceptible to oxidative stress than females (Brothers et al., 2019) and necessitates the inclusion of sex as a variable in future stress trials. The higher growth rate and heavier BW exhibited by male broilers is likely to continue to diverge from females as genetic selection continues to pursue maximal performance and may inadvertently further exacerbate oxidative stress differences between the sexes. Future research is needed to develop heat stress interventions that can address performance issues as well as oxidative damage equally for both sexes.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.101748.

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