Journal of Animal Science, 2020, Vol. 98, No. 10, 1–8

doi:10.1093/jas/skaa300
Advance Access publication September 16, 2020
Received: 25 June 2020 and **Accepted:** 4 September 2020
Non ruminant nutrition

NON RUMINANT NUTRITION

Effects of zinc oxide nanoparticles on regulatory appetite and heat stress protein genes in broiler chickens subjected to heat stress

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Abstract

This study was conducted to explore the effect of the zinc oxide nanoparticles (**ZnONPs**) supplement on the regulatory appetite and heat stress (**HS**) genes in broiler chickens raised under high or normal ambient temperatures. In this study, 240 one-day-old male broiler chicks (Cobb 500) were randomly assigned to 48 battery cages. From day 1, these 48 cages were randomly subjected to four different treatment strategies: Control (wherein, their basal diet included 60 mg/kg of ZnO), ZNONPs 40 (wherein basal diet included 40 mg/kg of ZnONPs), ZnONPs 60 (basal diet included 60 mg/kg of ZnONPs), and ZnONPs 100 (basal diet included 100 mg/kg of ZnONPs). Thereafter, from day 22 to 42, the chickens from each dietary treatment group were subjected to different temperature stresses either normal (23 ± 1 °C constant) or HS (34 ± 1 °C for 6 h/d), which divided them into eight different treatment groups. Our findings revealed that dietary ZnONPs altered the gene expression of cholecystokinin (ileum), heat stress proteins (**HSP**) 70 (jejunum and ileum), and HSP 90 (duodenum, jejunum, and ileum). The gene expression of ghrelin was affected by the interaction between the ZnONPs concentration and temperature in the duodenum and stomach. More studies are required to elucidate its complex physiological and biochemical functions of the regulation of gene expression within the intestine in heat-stressed broiler chickens.

Key words: broiler chickens, gene expression, heat challenge, small intestine, zinc oxide nanoparticles

Introduction

The impact of climate change has a devastating consequence on the poultry production to sustain its productivity. High ambient temperature is of great concern in all types of poultry production, which can lead to heat stress (**HS**), thus affecting the productivity of the chickens (Habibian et al., 2016). HS is a significant factor

Abbreviations

CCK	cholecystokinin
Ct	cycle threshold
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HS	heat stress
HSP	heat stress proteins
PCR	polymerase chain reaction

influencing domestic animal production especially in the tropical climate of Malaysia (Khajavi et al., 2003).

The performance of chickens affected by HS leads to significantly impaired growth rates and feed intake due to the intestinal inflammation and injury, pathophysiological alterations in digestion and loss of absorptive surface area, and impairment of intestinal barrier integrity causes increased susceptibility to infectious diseases (Ebrahimi et al., 2015). Severe effects of HS may lead to pathological damage via mucosal epithelial cell exfoliation and villi fracture that mainly involved the duodenum, jejunum, and ileum (Ning et al., 2003).

Most of the intestinal hormones in broiler chickens which include appetite inhibitors, such as ghrelin and cholecystokinin (**CCK**), are released from the intestine. These inhibitors are widely distributed in the gastrointestinal tract and are important in the regulation of feed intake in broilers and layers (Lei et al., 2013). Both of these genes transmit satiety signals to the brain after feed consumption, resulting in the attenuation of appetite (Honda et al., 2017). A study has shown that heat-induced stress increased the mRNA expression of ghrelin in the duodenum and downregulated mRNA CCK expression in the duodenum and ileum of broiler chickens (He et al., 2018). The expression of downregulation of CCK and upregulation of ghrelin observed in the intestine tissue suggested anorexigenic effects of acute high ambient temperature in chickens (Lei et al., 2013). The response of ghrelin and CCK expression under dietary manipulation on the appetite regulatory genes and HS genes under the HS environment in avian species have not been addressed.

Heat shock proteins or heat stress proteins (**HSP**) are a group of highly conserved proteins that are rapidly synthesized when living organisms are induced with HS (Al-Aqil and Zulkifli, 2009). Varasteh et al. (2015) reported that during HS the mRNA HSP70 and HSP 90 levels were upregulated in both the jejunum and ileum; however, no HS-related alteration of these HSPs was observed in the duodenum and colon in chickens. This indicated that HSPs expressions vary in the individual organs of the intestine (Varasteh et al., 2015). However, the information on the relationship between the mRNA expression of HSP70 and HSP 90 activity in the stressful condition is rather scarce.

Sahin et al. (2009) stated that Zinc (Zn) was a vital component that was used in many poultry diets for alleviating the HS-related effects. The usage of higher levels of Zn excreted from animals fed with zinc oxide has raised concerns about environmental pollution (Feng et al., 2009). Thus, this problem opens an alternative for better bioavailable Zn sources and, if possible, to reduce the dosage of Zn usage into animal feed. Scientific reports related to the role of ZnONPs in HS poultry performance are very scarce. In our previous study, increased retention of Zn in the liver of chicken had alleviated the negative effects of HS via maximizing the antioxidant defense system and minimizing lipid peroxidation (Ramiah et al., 2019). Because of these considerations, this study was designed to examine the effects of Zinc oxide nanoparticles (**ZnONPs**) on the regulation of appetite-associated (ghrelin and CCK) and HSP genes (70 and 90) in broiler chickens reared under normal and high ambient temperature conditions.

Materials and Methods

Ethical note

In this study, all experimental procedures were carried out as per the guidelines mentioned in the Animal Ethical Code No. UPM/AICUC/AUP-R040/2017. These procedures were also approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia. All animal models were cared for and their health and welfare were also regularly monitored by a qualified veterinarian, who was one of the researchers in this study.

Chickens, housing, diets, and experimental design

The materials and methods used in this study have been previously outlined in the recently published parallel paper (Ramiah et al., 2019), including the composition of the basal diet during the starter (day 1 to 21) and finisher (day 22 to 42) periods (Table 1); 240 one-day-old male broiler chicks (Cobb 500) were randomly allotted to 48 battery cages, which were maintained in six similar environmentally controlled chambers. Feed and water were provided ad libitum. From day 1, the 48 cages were randomly subjected to four different treatments, that is, **Control** (the chicks were given a basal diet with 60 mg/kg ZnO), **ZnONPs 40** (wherein basal diet included 40 mg/kg ZnONPs), **ZnONPs 60** (basal diet of 60 mg/kg ZnONPs), and **ZnONPs 100** (basal diet included 100 mg/kg ZnONPs). The premix minerals used in this study were specially formulated by Peters Lab Sdn Bhd (Nilai, Negeri Sembilan, Malaysia) without inclusion of Zn in the treated diets. The nano zinc oxide (nano-ZnO) particles used in this study were acquired from the US Research Nanomaterials, Inc. (Houston, USA). The nano-ZnO has 99+% purity with nearly spherical particles of size ranged from 35 to 45 nm. On day 22, the chickens were subjected to different heat treatments, that is, normal ambient temperature or HS, which formed two groups. Thus, the chickens were divided into different groups with 4 diets × 2 temperatures. Each treatment was carried out on six replicates, wherein each subgroup consisted of five chickens.

Heat challenge

After 21 d of the adaptation period, the chickens were assigned at random to two groups. During the finisher period, chickens from each dietary treatment were assigned to normal ambient temperature (23 ± 1 °C throughout) or high ambient temperature (34 ± 1 °C for 6 h/d from 10:00 a.m. to 4:00 p.m.) resulting in eight treatment groups. The relative humidity ranged from 70% to 80%.

Samples collection for real-time polymerase chain reaction analysis

From each treatment group, 10 chickens were randomly selected. The chickens were slaughtered according to the halal slaughter method at the end of the experimental period (Farouk et al., 2014). The whole intestinal tract was removed from the broiler chickens. Using normal saline, the contents of the gut were flushed. Thereafter, 0.5-cm tissues were extracted from the center of the glandular stomach, duodenum (center of the duodenum), jejunum (between the entry of the bile duct and Meckel's diverticulum), and ileum (center between Meckel's

diverticulum and ileocecal junction) (Awad et al., 2008). After excision, all the tissue samples were frozen using liquid nitrogen and then stored at –80 °C. These samples were later used for RNA extraction.

RNA extraction

For extracting and purifying the total RNA from the tissue samples, the RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the kit instructions. The total RNA concentrations were quantified by determining the optical density. The results for the ratio of absorption at 260/280 nm for all samples ranged from 1.8 to 2.2.

Table 1. Composition of the diet during the starter (day 1 to 21) and finisher periods (day 22 to 42)

Item	Starter (day 1 to 21)	Finisher (day 22 to 42)
Ingredient composition, %		
Corn	54.05	58.21
Wheat bran	2.70	2.50
Soybean meal	32.9	30.4
Gluten meal	3.30	0.00
Palm oil	3.10	5.20
Dicalcium phosphate	1.81	1.47
Limestone	0.95	0.82
Salt (NaCl)	0.42	0.40
Vitamin premix[1]	0.07	0.05
Mineral premix[1]	0.10	0.10
DL-Methionine	0.25	0.21
L-Lysine HCl	0.24	0.05
L-Threorine	0.04	0.55
Choline CI, 70%	0.07	0.04
Nutrient composition (%DM, unless stated otherwise)[2]		
Crude protein	22.00	20.5
Metabolizable energy, kcal/kg	3,080	3,150
Crude fat	7.27	7.50
Phosphorus	0.45	0.42
Calcium	1.00	0.90
Methionine	0.55	0.50
Lysine	1.20	1.00
Na	0.20	0.15

[1]Premixes contributed the following nutrients per kilogram of complete feed: vitamin A, 2,300 IU; vitamin D_3, 400 IU; vitamin E, 1.8 mg; vitamin B_{12}, 3.5 mg; riboflavin, 1.4 mg; panthotenic acid, 2 mg; nicotinic acid, 7 mg; pyridoxine, 0.25 mg; folic Acid, 0.15 mg; menadione, 0.3 mg; thiamin, 0.15 mg; manganese oxide, 35 mg; ferrous sulfate, 35 mg; copper sulfate, 60 mg; cobalt carbonate, 5 mg; potassium iodine, 0.6 mg; selenium vanadate, 0.09 mg. Mineral premix was free of zinc.
[2]Calculated values.

Real-time polymerase chain reaction

QuantiTect Rev Transcription Kit (Qiagen, Hilden, Germany) was used for reverse transcription of mRNA; 5 µg of RNA from each sample was reverse transcribed according to the manufacturer's protocol. β-actin and glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) gene markers were used as reference genes for normalization. Real-time polymerase chain reaction (**PCR**) reaction was carried out using the Bio-Rad CFX96 Touch Real-time PCR System (Bio-Rad, USA), with the Quantitect primer assays for CCK. The remaining primers were purchased (i.e., HSP 70, HSP 90, and ghrelin) from 1st BASE Oligonucleotide Synthesis (1st Base, Singapore). The primers are listed in detail in Table 2.

The final concentration of the reaction was 1X QuantiFast SYBR green PCR kit (Qiagen, Hilden, Germany), 1 µg of cDNA, 400/500 nM of each forward and reverse primers, and 8.5 µL of RNase-free water. RT-quantitative PCR reaction conditions were set at initial activation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, followed by annealing at different temperatures according to targeted genes for 20 s and finally extension at 72 °C for 20 s. The amplification procedure was done in a 20-µL reaction volume. For developing the standard curve for each gene and checking the efficiency of primers, the respective cDNA templates were titrated using six serial dilutions in the PCR experiment. Each sample was run in triplicate, and the averaged triplicates were used to assign cycle threshold (**Ct**) values. In each run, template control was included. The transcription levels were calculated using the ΔΔCt method as described by Vandesompele et al. (2002). Thereafter, the real-time PCR data were normalized using a geometric average of the two reference genes (i.e., β-actin and GAPDH). The transcription levels of the HSP 70, HSP 90, ghrelin, and CCK genes in the treated and control groups were compared. Transcriptions levels in the control group were always expressed as 1-fold gene expression.

Statistical analysis

The general linear model of the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA) software (SAS, 2005) was used to analyze the gene expression data by selecting the heat, diet treatments, and their interaction as the main effects. When these main effects showed a significant interaction, they were

Table 2. Primer sequences used for real-time PCR analysis

Gene	Primers set	Size	Primer Melting Temperature	References
HSP 70	F: 5′-CGTCAGTGCTGTGGACAAGAGTA-3′ R: 5′-CCTATCTCTGTTGGCTTCATCCT-3′	145	60	Uerlings et al. (2018)
HSP 90	F: 5′-GAGTTTGACTGACCCGAGCA-3′ R: 5′-TCCCTATGCCGGTATCCACA-3′	107	60	Uerlings et al. (2018)
Ghrelin	F: 5′-CCTTGGGACAGAAACTGCTC-3′ R:5′-CACCAATTTCAAAAGGAACG-3′	203	60	Lei et al. (2013)
GAPDH	F:TGAAAGTCGGAGTCAACGGATT-3' R:CCACTTGGACTTTGCCAGAGA-3′	81	60	Ojano-Dirain et al. (2007)
β-Actin	F: 5′-ATGAAGCCCAGAGCAAAAGA-3′ R-:5′-GGGGTGTTGAAGGTCTCAAA-3′	223	62	König et al. (2008)

compared with each experimental variable. The mean values were compared using the Tukey's test, and the values were seen to be statistically significant at $P \leq 0.05$.

Results

Table 3 shows the effects of dietary ZnONPs supplementation and temperature on the fold changes in the expression of genes in the duodenum of broiler chickens at 42 d of age. There were significant interactions between diet and temperature for ghrelin ($P = 0.0048$) and HSP 70 ($P = 0.0330$) genes. Chickens fed with ZnONPs 60 diet had higher ghrelin fold changes under normal temperature compared with HS conditions. However, there were no differences in ghrelin fold changes between temperatures in chickens fed with Control, ZnONPs 40, or ZnONPs 100. Also, chickens fed with ZnONPs 100 diet had higher fold changes of the ghrelin gene under HS conditions compared with other diets. Under normal temperature, however, fold changes of the ghrelin gene were higher in chickens fed with ZnONPs 100 and ZnONPs 60 diets compared with those fed with Control and ZnONPs 40 diets. Unlike chickens fed with Control and ZnONPs 40 diets, fold changes of HSP 70 gene highly increased when heat-stressed broilers fed with ZnONPs 100 and ZnONPs 60 diets compared with their counterparts fed the same diets under normal temperature. The temperature had no effect on the fold changes of HSP 90 and CCK genes. However, chickens fed with 100 mg/kg of ZnONPs have the highest fold changes of HSP 90 at 4.55 ($P < 0.05$) compared with control groups. Diet had no effect on the fold changes of the CCK gene.

The effects of dietary ZnONPs supplementation and temperature on the fold changes in the expression of genes in the jejunum of broiler chickens at 42 d of age are presented in Table 4. No significant interactions ($P > 0.05$) were observed between diet and temperature for fold changes of ghrelin, CCK, HSP 70, or HSP 90 in the jejunum. The gene expression of HSP 70 was found to be higher ($P < 0.05$) under normal temperature compared with HS conditions. The fold changes of HSP 70 were significantly ($P < 0.001$) higher in chickens fed with ZNONPs 60 and 100 compared with other treatment diets. In this study, the gene expression of HSP 90 was not affected by temperature conditions. The chickens fed with ZNONPs 40 had greater fold changes of HSP90 gene compared with chickens fed with the control diet.

Table 5 presented the effects of the dietary ZnONPs supplementation and temperature stress on the fold changes in the ileum gene expression in the broiler chickens. Significant interactions were noted between the diet and temperature stresses for the mRNA expressions of the ghrelin ($P = 0.0018$) and CCK ($P = 0.0047$) genes. According to the results, the treated chickens reared under the HS conditions showed no effect on the fold changes of the ghrelin gene. At normal temperature, when the chickens were fed the ZnONPs 100 diet, a significant increase was seen in the fold changes of the ghrelin gene in comparison to the other treatment diets. In the mRNA expression of the CCK gene, the temperature had no effect on the fold changes of CCK gene in chickens fed with the Control, ZnONPs 60, or ZnONPs 100 diets. However, feeding chickens with ZnONPs 40 significantly increased the fold changes of the CCK gene under HS conditions compared with normal temperature. The gene expression of HSP 70 and HSP 90 was not affected by temperature. Regardless of temperature, fold changes of HSP 70 gene were significantly ($P < 0.001$) higher in chickens fed with ZnONPs 60 diet compared with other diets. Chickens fed with the Control diet had fewer fold changes of HSP 70 compared with their counterparts fed with ZnONPs 40 and ZnONPs 100 diets. The expression of HSP 90 gene was significantly higher in chickens fed with ZnONPs 40 diet compared with their counterparts fed with the Control diet.

Table 6 presents the data regarding the effects of the dietary ZnONPs supplementation and temperature stress on the fold changes in the expression of the glandular stomach genes,

Table 3. Effects of dietary ZnONPs supplementation and temperature on the fold changes in the expression of genes in the duodenum of broiler chickens at 42 d of age

Diet[1]	Temperature[2]	*n*	Ghrelin	CCK	HSP 70	HSP 90
Interaction effects						
Control	HS	6	1.00^{b} ± 0.00	1.00 ± 0.00	1.00^{b} ± 0.00	1.00 ± 0.00
Control	Normal	6	1.00^{b} ± 0.00	1.00 ± 0.00	1.00^{b} ± 0.00	1.00 ± 0.00
ZnONPs 40	Heat stress	6	1.64^{b} ± 0.38	13.0 ± 8.29	1.75^{b} ± 0.21	2.42 ± 0.73
ZnONPs 40	Normal	6	1.92^{b} ± 0.39	5.64 ± 0.59	1.62^{b} ± 0.31	2.33 ± 0.54
ZnONPs 60	Heat stress	6	2.23^{b} ± 0.33	5.74 ± 1.95	25.59^{a} ± 8.35	2.72 ± 0.53
ZnONPs 60	Normal	6	5.43^{a} ± 0.48	4.04 ± 0.45	2.64^{b} ± 0.34	1.38 ± 0.12
ZnONPs 100	Heat stress	6	4.78^{a} ± 0.88	2.01 ± 0.49	25.6^{a} ± 11.7	3.97 ± 1.53
ZnONPs 100	Normal	6	4.29^{a} ± 0.89	4.96 ± 0.69	3.76^{b} ± 0.79	5.13 ± 2.43
Main effects						
Control		12	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00^{b} ± 0.00
ZnONPs 40		12	1.78 ± 0.26	9.34 ± 4.12	1.69 ± 0.18	2.38^{ab} ± 0.43
ZnONPs 60		12	3.83 ± 0.56	4.89 ± 0.99	14.1 ± 5.28	2.05^{b} ± 0.33
ZnONPs 100		12	4.54 ± 0.60	3.48 ± 0.60	14.7 ± 6.49	4.55^{a} ± 1.38
	HS	24	2.41 ± 0.38	5.45 ± 2.22	13.49 ± 4.19	2.53 ± 0.47
	Normal	24	3.16 ± 0.45	3.91 ± 0.44	2.26 ± 0.31	2.46 ± 0.67
ANOVA (*P*-value)						
Diet			<0.0001	0.0616	0.0092	0.0178
Temp			0.0516	0.4783	0.0034	0.9332
Diet × Temp			0.0048	0.3908	0.0330	0.7256

[1]Control, basal diet containing 60 mg/kg zinc oxide; ZnONPs 40, basal diet containing 40 mg/kg zinc oxide nanoparticles; ZnONPs 60 basal diet containing 60 mg/kg zinc oxide nanoparticles; ZnONPs 100 basal diet containing 100 mg/kg zinc oxide nanoparticles.
[2]Equal numbers of chickens from each diet were subjected to either 23 ± 1°C throughout (normal temperature) or 34 ± 1 °C for 6 h per day (HS) from 22 to 42 d of age.
[a,b]Means within a column-subgroup with no common superscripts are significantly different at $P < 0.05$.

Table 4. Effects of dietary ZnONPs supplementation and temperature on the fold changes in the expression of genes in the jejunum of broiler chickens at 42 d of age

Diet[1]	Temperature[2]	*n*	Ghrelin	CCK	HSP 70	HSP 90
Interaction effects						
Control	HS	6	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Control	Normal	6	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
ZnONPs 40	HS	6	2.51 ± 0.73	4.83 ± 1.29	1.34 ± 0.23	5.54 ± 2.33
ZnONPs 40	Normal	6	14.3 ± 8.89	3.41 ± 0.49	2.23 ± 0.27	1.72 ± 0.31
ZnONPs 60	HS	6	1.70 ± 0.44	1.64 ± 3.43	2.12 ± 0.84	3.08 ± 0.57
ZnONPs 60	Normal	6	4.53 ± 1.11	4.20 ± 0.64	4.18 ± 1.04	2.54 ± 0.37
ZnONPs 100	HS	6	2.05 ± 0.59	1.92 ± 0.39	3.29 ± 1.15	2.10 ± 0.31
ZnONPs 100	Normal	6	1.89 ± 0.49	4.19 ± 0.63	5.23 ± 0.41	2.89 ± 0.48
Main effects						
Control		12	1.00 ± 0.00	1.00 ± 0.00	1.00^{b} ± 0.00	1.00^{b} ± 0.00
ZnONPs 40		12	8.40 ± 4.61	3.05 ± 0.67	1.78^{b} ± 0.22	3.63^{a} ± 1.26
ZnONPs 60		12	3.12 ± 0.71	4.49 ± 1.67	3.15^{a} ± 0.71	2.81^{ab} ± 0.33
ZnONPs 100		12	1.97 ± 0.37	3.23 ± 0.46	4.26^{a} ± 0.65	2.49^{ab} ± 0.29
	HS	24	1.82 ± 0.27	2.69 ± 0.91	1.94^{b} ± 0.38	2.93 ± 0.66
	Normal	24	5.43 ± 2.36	3.20 ± 0.36	3.16^{a} ± 0.44	2.04 ± 0.22
ANOVA (*P*-value)						
Diet			0.1107	0.0939	<0.0001	0.0387
Temp			0.1177	0.5935	0.0115	0.1627
Diet × Temp			0.2173	0.8132	0.3572	0.0643

[1]Control, basal diet containing 60 mg/kg zinc oxide; ZnONPs 40, basal diet containing 40 mg/kg zinc oxide nanoparticles; ZnONPs 60 basal diet containing 60 mg/kg zinc oxide nanoparticles; ZnONPs 100 basal diet containing 100 mg/kg zinc oxide nanoparticles.
[2]Equal numbers of chickens from each diet were subjected to either 23 ± 1 °C throughout (normal temperature) or 34 ± 1 °C for 6 h per day HS from 22 to 42 d of age.
[a,b]Means within a column-subgroup with no common superscripts are significantly different at $P < 0.05$.

Table 5. Effects of dietary ZnONPs supplementation and temperature on the fold changes in the expression of genes in the ileum of broiler chickens at 42 d of age

Diet[1]	Temperature[2]	*n*	Ghrelin	CCK	HSP 70	HSP 90
Interaction effects						
Control	HS	6	1.00^{b} ± 0.00	1.00^{c} ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Control	Normal	6	1.00^{b} ± 0.00	1.00^{c} ± 0.00	1.00 ± 0.00	1.00 ± 0.00
ZnONPs 40	HS	6	1.33^{b} ± 0.15	4.93^{a} ± 1.19	2.45 ± 0.76	2.91 ± 0.66
ZnONPs 40	Normal	6	3.41^{b} ± 0.19	2.63^{b} ± 0.37	2.46 ± 0.52	2.69 ± 0.43
ZnONPs 60	HS	6	2.11^{b} ± 0.09	1.50^{bc} ± 0.18	6.21 ± 1.18	2.23 ± 0.68
ZnONPs 60	Normal	6	1.22^{b} ± 0.14	2.79^{b} ± 0.07	4.11 ± 0.55	1.63 ± 0.21
ZnONPs 100	HS	6	3.03^{b} ± 0.76	1.65^{bc} ± 0.33	1.85 ± 0.35	3.36 ± 1.71
ZnONPs 100	Normal	6	12.3^{a} ± 3.48	1.74^{bc} ± 0.27	2.86 ± 0.36	1.24 ± 0.09
Main effects						
Control		12	1.00 ± 0.00	1.00 ± 0.00	1.00^{c} ± 0.00	1.00^{b} ± 0.00
ZnONPs 40		12	2.37 ± 0.65	3.78 ± 0.69	2.46^{b} ±0.44	2.79^{a} ± 0.38
ZnONPs 60		12	1.66 ± 0.16	2.14 ± 0.22	5.16^{a} ±0.69	1.93^{ab} ± 0.35
ZnONPs 100		12	7.65 ± 2.19	1.69 ± 0.20	2.36^{b} ±0.28	2.20^{ab} ± 0.80
	HS	24	1.87 ± 0.24	2.27 ± 0.44	2.88 ± 0.54	2.33 ± 0.45
	Normal	24	4.47 ± 1.29	2.04 ± 0.18	2.61 ± 0.30	1.64 ± 0.18
ANOVA (*P*-value)						
Diet			<0.0001	<0.0001	<0.0001	0.0468
Temp			0.0084	0.4956	0.5247	0.1114
Diet × Temp			0.0018	0.0047	0.0760	0.3677

[1]Control, basal diet containing 60 mg/kg zinc oxide; ZnONPs 40, basal diet containing 40 mg/kg zinc oxide nanoparticles; ZnONPs 60 basal diet containing 60 mg/kg zinc oxide nanoparticles; ZnONPs 100 basal diet containing 100 mg/kg zinc oxide nanoparticles.
[2]Equal numbers of chickens from each diet were subjected to either 23 ± 1 °C throughout (normal temperature) or 34 ± 1 °C for 6 h per day HS from 22 to 42 d of age.
[a,b,c]Means within a column-subgroup with no common superscripts are significantly different at $P < 0.05$.

which were extracted from the 42-d-old broiler chickens. A significant diet × temperature interaction was noted for the CCK mRNA ($P = 0.0319$) and HSP 90 mRNA ($P = 0.0014$) genes. The chickens, which were fed the ZnONPs 40 diet, showed a higher fold change in the CCK gene expression when they were reared in normal temperatures in comparison to those reared under an HS condition. However, the temperature had no effect on the expression of CCK when chickens were fed with Control, ZnONPs 60, or ZnONPs 100 diets. The gene expression of HSP 90 was significantly upregulated under heat conditions in chickens fed with ZnONPs 60 diet. However, the temperature had no effect on the expression of HSP 90 and

Table 6. Effects of dietary ZnONPs supplementation and temperature on the fold changes in the expression of genes in the glandular stomach of broiler chickens at 42 d of age

Diet[1]	Temperature[2]	*n*	Ghrelin	CCK	HSP 70	HSP 90
Interaction effects						
Control	HS	6	1.00 ± 0.00	1.00^{c} ± 0.00	1.00 ± 0.00	1.00^{b} ± 0.00
Control	Normal	6	1.00 ± 0.00	1.00^{c} ± 0.00	1.00 ± 0.00	1.00^{b} ± 0.00
ZnONPs 40	HS	6	2.43 ± 0.31	1.58^{c} ± 0.23	2.90 ± 0.71	2.34^{b} ± 0.62
ZnONPs 40	Normal	6	7.78 ± 3.26	7.11^{a} ± 2.47	5.48 ± 0.53	3.02^{b} ± 0.28
ZnONPs 60	HS	6	1.99 ± 0.63	5.60^{ab} ± 0.84	2.16 ± 0.92	7.51^{a} ± 1.28
ZnONPs 60	Normal	6	2.36 ± 1.18	5.90^{ab} ± 1.31	4.96 ± 0.55	1.85^{b} ± 0.34
ZnONPs 100	HS	6	6.12 ± 1.37	2.88^{bc} ± 0.98	6.19 ± 4.31	3.45^{b} ± 1.22
ZnONPs 100	Normal	6	4.58 ± 0.87	4.70^{ab} ± 1.22	3.86 ± 0.44	3.43^{b} ± 1.39
Main effects						
Control		12	1.00^{b} ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
ZnONPs 40		12	5.11^{a} ± 1.76	3.43 ± 1.17	4.19 ± 0.57	2.68 ± 0.34
ZnONPs 60		12	2.17 ^{b} ± 0.64	5.68 ± 0.67	3.56 ± 0.66	4.68 ± 1.06
ZnONPs 100		12	5.35^{a} ± 0.81	3.79 ± 0.79	5.03 ± 2.09	4.44 ± 0.88
	HS	24	2.89 ± 0.54	2.77 ± 0.48	3.07 ± 1.12	3.58 ± 0.68
	Normal	24	3.93 ± 0.99	3.96 ± 0.81	3.82 ± 0.42	2.33 ± 0.39
ANOVA (*P*-value)						
Diet			0.0053	0.0002	0.0889	0.0009
Temp			0.2890	0.0088	0.5098	0.0412
Diet × Temp			0.0864	0.0319	0.3476	0.0014

[1]Control, basal diet containing 60 mg/kg zinc oxide; ZnONPs 40, basal diet containing 40 mg/kg zinc oxide nanoparticles; ZnONPs 60 basal diet containing 60 mg/kg zinc oxide nanoparticles; ZnONPs 100 basal diet containing 100 mg/kg zinc oxide nanoparticles.
[2]Equal numbers of chickens from each diet were subjected to either 23 ± 1 °C throughout (normal temperature) or 34 ± 1 °C for 6 h per day HS from 22 to 42 d of age.
[a,b,c]Means within a column-subgroup with no common superscripts are significantly different at $P < 0.05$.

ghrelin in chickens fed with the ZnONPs 40 or ZnONPs 100 diets. The gene expression of ghrelin in the glandular stomach was upregulated in chickens fed with ZnONPs 40 or ZnONPs 100 diets compared with Control and ZnONPs 60.

Discussion

In the present study, results showed elevated levels of mRNA ghrelin in the duodenum and stomach together with the increased level of mRNA CCK in the ileum after heat exposure in broiler chickens. Studies have shown that intracerebroventricular (i.c.v.) and peripheral injection of ghrelin inhibits feed intake in chicks (Saito et al., 2005; Chen et al., 2018). In chickens, the stomach is the major source of the endogenous ghrelin (Richards et al., 2006), and CCK transcripts are dispersed throughout the small intestine, particularly around the proximal ileum (Reid and Dunn, 2018). Our study is consistent with an earlier study, which indicated that the ghrelin mRNA levels significantly increased in the glandular stomach and duodenum tissues of broiler chickens that were exposed to an acute HS condition (Lei et al., 2013). In addition, He et al. (2018) observed that high ambient temperature could significantly upregulate the ghrelin and CCK mRNA expression in the duodenum of broilers that reduced the feed intake. Our present results coincided with the previous study by Ramiah et al. (2019) with a general trend observed in heat-stressed broilers of decreased feed intake. A study by Richards et al. (2006) indicated that the intestinal hormones such as CCK and ghrelin mediated signals in the gut and transmitted these to the hypothalamus that caused the reduction in feed intake in poultry. Hence, in this study, the elevated expression of ghrelin and CCK seem to be a result of decreased feed consumption in broiler chickens. The proteins encoded by ghrelin and CCK probably inhibit the appetite via a decrease in intestinal motility, which exerts the comparable effects on feed intake in broiler chickens (Song et al., 2012).

It was observed that ZnONP supplementation had some amplified effects on these elevating levels of mRNA ghrelin and CCK in the intestine. In our previous study (Ramiah et al., 2019), the feed intake was substantially reduced with the decrease of ZnONP concentration during the finisher and overall periods. We found that ZnONPs may not have eliminative the effects on the appetite regulatory genes under HS. The reduced feed consumption in ZnONP diets may be due to increased synthesis of intestinal metallothionein in which is associated with reduced zinc absorption (Jahanian et al., 2008). The role of metallothionein in the regulation of absorption and excretion of Zn in the gut by limitation of Zn absorption at high Zn concentration and enhances Zn absorption at Zn discrepancy (Tran et al., 2011).

A recent study found that dietary nanosized ZnO significantly increased the Zn content in the eggshells, Zn concentration serum, ghrelin and the IgG levels in aged layers compared with dietary ZnO (Mao and Lien, 2017). On the other hand, Hu et al. (2016) stated that decreased ghrelin mRNA expression of the hypothalamus tissue in Zn-supplemented broiler chickens. Yin et al. (2009) noted that when the gastric mucosal piglet cells were provided with a varying ZnO supplementation, ranging between 0 and 0.5 mM, ZnO-supplemented cells showed a higher ghrelin concentration. Although zinc supplementation has positive impacts on animal performances, however, the molecular mechanism describing the Zn effect on the appetite regulation genes in poultry remains unclear. The contradictory results could arise because of the various levels and forms of Zn supplementation were utilized.

In heat environments, compared with HSP 90, the HSP 70 gene expression plays a greater role in cellular functions (Cedraz et al., 2017). In the present results, HSP 70 expression was elevated in an HS environment compared with normal

temperature in the duodenum of broiler chickens fed higher concentrations of ZnONPs (60 and 100 mg/kg ZnONPs). One school of thought suggested that high HSP expression is associated with HS tolerance (Wang and Edens, 1993). On the other hand, the downregulation expression of HSP genes could be due to the adaptation of chickens to environmental temperature stress (Rajkumar et al., 2017). In contrast to our results, the chickens exposed to HS showed a significant upregulation of HSP 70 in mRNA levels in both jejunum and ileum (Varasteh et al., 2015). The discrepancies of the results might be partly due to the stability of tissues to high ambient temperature to protect the body tissues from being exposed to stress (Rajkumar et al., 2017). The mRNA expression of HSP 70 varied between the heart, liver, and stomach upon HS in pigs, indicating tissue specificity (Zhang et al., 2012). In this study, the disparity of HSP 70 mRNA expression varied in the different parts of the chickens' gut because severe HS causes pathological damage to the duodenum, jejunum, and ileum, which mainly involved mucosal epithelial cell exfoliation and villi fracture as reported by (Ning et al., 2003).

HSPs play a vital role when the animals are exposed to oxidative stress (Zhao et al., 2014). Zn supplementation could help in suppressing free radicals, thereby decreasing their production due to HS (Prasad and Kucuk, 2002). Results showed that the HSP 90 mRNA expression was higher in the duodenum and glandular stomach tissue samples in the broiler chickens that were fed the highest ZnONP levels. Similar results were reported by Ramiah et al. (2019), who showed that a diet containing 100 mg/kg ZnONPs increased the serum corticosterone levels. Hence, a low Zn level was absorbed because of lower feed intake. The results showed that the intestinal stress induced a higher HSP 90 expression, which could protect the intestinal mucosa against some severe tissue injuries. No HS-related alterations were observed in the level of expression of HSP 90 in the jejunum or ileum. This suggested the differences in the susceptibility of the individual parts of the intestines exposed to HS (Varasteh et al., 2015).

In summary, this study has compiled the current and past data regarding the role played by the ZnONPs in the health of the poultry exposed to HS. Heat exposure impairs the appetite, which may be correlated with the increased gene expression of appetite-related genes (CCK and ghrelin). In the present study, we found that supplementation of ZnONPs at 100 mg/kg-fed chickens upregulated HSP 70 (jejunum) and HSP 90 (duodenum and ileum). In our previous data, we found that supplementation of ZnONPs at 100 mg/kg reduced feed intake in broiler chickens due to increased synthesis of intestinal metallothionein, which is associated with reduced zinc absorption. Since the Zn absorption was low, it may not exert a positive influence on the broilers subjected to HS. Therefore, the upregulation of HSP genes as shown in this study may be one of the mechanisms to combat HS by protecting the tissues from being exposed to stress. The influence of ZnONPs on appetite regulatory and HSP genes in broilers under HS condition still not completely understood. Hence, future study is needed to understand the dynamics of this mineral on the overall productivity and health of broiler chickens.

Acknowledgments

We would like to express our gratitude to all staff from the Laboratory of Animal Production and Sustainable Biodiversity, Institute of Tropical Agriculture and Food Security (ITAFoS), for their support. This research is supported by the Ministry of Higher Education Malaysia through Higher Institutions Centres of Excellence (HiCoE) grant and Universiti Putra Malaysia.

Authors' Contributions

Conceptualization: S.K.R.; methodology: S.K.R., E.A.A., and M.E.; validation: S.K.R., E.A.A., and M.S.; formal analysis: S.K.R. and E.A.A.; investigation: S.K.R., O.J., M.J., and A.F.S.; data curation: S.K.R. and E.A.A.; writing-review and editing: S.K.R., E.A.A., M.S., and Z.I.; All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors declare that they have no conflict of interests.

Literature Cited

Al-Aqil, A., and I. Zulkifli. 2009. Changes in heat shock protein 70 expression and blood characteristics in transported broiler chickens as affected by housing and early age feed restriction. *Poult. Sci.* **88**:1358–1364. doi:10.3382/ps.2008-00554

Awad, W., K. Ghareeb, and J. Böhm. 2008. Intestinal structure and function of broiler chickens on diets supplemented with a synbiotic containing *Enterococcus faecium* and oligosaccharides. *Int. J. Mol. Sci.* **9**:2205–2216. doi:10.3390/ijms9112205

Cedraz, H., J. G. G. Gromboni, A. A. P. Garcia Jr, R. V. Farias Filho, T. M. Souza, E. R. Oliveira, E. B. Oliveira, C. S. D. Nascimento, C. Meneghetti, and A. A. Wenceslau. 2017. Heat stress induces expression of HSP genes in genetically divergent chickens. *PLoS One*. **12**:e0186083. doi:10.1371/journal.pone.0186083

Chen, C., H. Wang, H. Jiao, X. Wang, J. Zhao, and H. Lin. 2018. Feed habituation alleviates decreased feed intake after feed replacement in broilers. *Poult. Sci.* **97**:733–742. doi:10.3382/ps/pex358

Ebrahimi, R., M. Faseleh Jahromi, J. B. Liang, A. Soleimani Farjam, P. Shokryazdan, and Z. Idrus. 2015. Effect of dietary lead on intestinal nutrient transporters mRNA expression in broiler chickens. *Biomed Res. Int.* **2015**:149745. doi:10.1155/2015/149745

Farouk, M. M., H. M. Al-Mazeedi, A. B. Sabow, A. E. Bekhit, K. D. Adeyemi, A. Q. Sazili, and A. Ghani. 2014. Halal and Kosher slaughter methods and meat quality: a review. *Meat Sci.* **98**:505–519. doi:10.1016/j.meatsci.2014.05.021

Feng, M., Z. S. Wang, A. G. Zhou, and D. W. Ai. 2009. The effects of different sizes of nanometer zinc oxide on the proliferation and cell integrity of mice duodenum-epithelial cells in primary culture. *Pak. J. Nutr.* **8**:1164–1166. doi:10.3923/pjn.2009.1164.1166

Habibian, M., S. Ghazi, and M. M. Moeini. 2016. Effects of dietary selenium and vitamin E on growth performance, meat yield, and selenium content and lipid oxidation of breast meat of broilers reared under heat stress. *Biol. Trace Elem. Res.* **169**:142–152. doi:10.1007/s12011-015-0404-6

He, X., Z. Lu, B. Ma, L. Zhang, J. Li, Y. Jiang, G. Zhou, and F. Gao. 2018. Effects of chronic heat exposure on growth performance, intestinal epithelial histology, appetite-related hormones and genes expression in broilers. *J. Sci. Food. Agri.* **98**:4471–4478. doi:10.1002/jsfa.8971.

Honda, K., T. Saneyasu, and H. Kamisoyama. 2017. Gut hormones and regulation of food intake in birds. *J. Poult. Sci.* **54**:103–110. doi:10.2141/jpsa.0160100

Hu, X., A. Sheikhahmadi, X. Li, Y. Wang, H. Jiao, H. Lin, B. Zhang, and Z. Song. 2016. Effect of zinc on appetite regulatory peptides in the hypothalamus of *Salmonella*-challenged

broiler chickens. *Biol. Trace Elem. Res*. **172**:228–233. doi:10.1007/s12011-015-0582-2

Jahanian, R., H. N. Moghaddam, A. Rezaei, and A. Haghparast. 2008. The influence of dietary zinc-methionine substitution for zinc sulfate on broiler chick performance. *J. Biol. Sci*. **8**:321–327. doi:10.3923/jbs.2008.321.327

Khajavi, M., S. Rahimi, Z. M. Hassan, M. A. Kamali, and T. Mousavi. 2003. Effect of feed restriction early in life on humoral and cellular immunity of two commercial broiler strains under heat stress conditions. *Br. Poult. Sci*. **44**:490–497. doi:10.1080/00007166031000159832 8

König, B., J. Spielmann, K. Haase, C. Brandsch, H. Kluge, G. I. Stangl, and K. Eder. 2008. Effects of fish oil and conjugated linoleic acids on expression of target genes of PPARα and sterol regulatory element-binding proteins in the liver of laying hens. *Br. J. Nutr*. **100**:355–363. doi:10.1017/S0007114507883024

Lei, L., L. Hepeng, L. Xianlei, J. Hongchao, L. Hai, A. Sheikhahmadi, W. Yufeng, and S. Zhigang. 2013. Effects of acute heat stress on gene expression of brain-gut neuropeptides in broiler chickens. *J. Anim. Sci*. **91**:5194–5201. doi:10.2527/jas.2013-6538

Mao, S. Y., and T. F. Lien. 2017. Effects of nanosized zinc oxide and γ-polyglutamic acid on eggshell quality and serum parameters of aged laying hens. *Arch. Anim. Nutr*. **71**:373–383. doi:10.1080/1745039X.2017.1355600

Ning, Z., S. Liu, D. Zhao, X. Tan, and S. Yu. 2003. The influence of heat stress on morphological and ultrastructure change of respiratory, digestive and endocrine tissues in broilers. *Acta. Veterinaria. et. Zootechnica. Sinica*. **34**:558–561.

Ojano-Dirain, C., M. Toyomizu, T. Wing, M. Cooper, and W. G. Bottje. 2007. Gene expression in breast muscle and duodenum from low and high feed efficient broilers. *Poult. Sci*. **86**:372–381. doi:10.1093/ps/86.2.372

Prasad, A. S., and O. Kucuk. 2002. Zinc in cancer prevention. *Cancer Metastasis Rev*. **21**:291–295. doi:10.1023/a:1021215111729

Rajkumar, U., A. Vinoth, M. Shanmugam, K. S. Rajaravindra, and S. V. Rama Rao. 2017. Effect of increased incubation temperature on Hsp 90 and 60 gene expressions in coloured broiler chickens. *J. Appl. Anim. Res*. **45**:298–303. doi:10.1080/09712119.2016.1174128

Ramiah, S. K., E. A. Awad, S. Mookiah, and Z. Idrus. 2019. Effects of zinc oxide nanoparticles on growth performance and concentrations of malondialdehyde, zinc in tissues, and corticosterone in broiler chickens under heat stress conditions. *Poult. Sci*. **98**:3828–3838. doi:10.3382/ps/pez093

Reid, A. M. A., and I. C. Dunn. 2018. Gastrointestinal distribution of chicken gastrin-cholecystokinin family transcript expression and response to short-term nutritive state. *Gen. Comp. Endocrinol*. **255**:64–70. doi:10.1016/j.ygcen.2017.10.009

Richards, M. P., S. M. Poch, and J. P. McMurtry. 2006. Characterization of turkey and chicken ghrelin genes, and regulation of ghrelin and ghrelin receptor mRNA levels in broiler chickens. *Gen. Comp. Endocrinol*. **145**:298–310. doi:10.1016/j.ygcen.2005.09.013

Sahin, K., N. Sahin, O. Kucuk, A. Hayirli, and A. S. Prasad. 2009. Role of dietary zinc in heat-stressed poultry: a review. *Poult. Sci*. **88**:2176–2183. doi:10.3382/ps.2008-00560

Saito, E. S., H. Kaiya, T. Tachibana, S. Tomonaga, D. M. Denbow, M. Kangawa, and M. Furuse. 2005. Inhibitory effect of ghrelin on food intake is mediated by the corticotrophin-releasing factor system in neonatal chicks. *Regul. Pept*. **125**:201–208. doi:10.1016/j.regpep.2004.09.003

SAS. 2005. *SAS/STAT software, version 9.4*. Cary (NC): SAS Inst. Inc.

Song, Z., L. Liu, A. Sheikhahmadi, H. Jiao, and H. Lin. 2012. Effect of heat exposure on gene expression of feed intake regulatory peptides in laying hens. *J. Biomed. Biotechnol*. **2012**. doi:10.1155/2012/484869

Tran, C. D., J. Cool, and C. J. Xian. 2011. Dietary zinc and metallothionein on small intestinal disaccharidases activity in mice. *World J. Gastroenterol*. **17**:354–360. doi:10.3748/wjg.v17.i3.354

Uerlings, J., Z. G. Song, X. Y. Hu, S. K. Wang, H. Lin, J. Buyse, and N. Everaert. 2018. Heat exposure affects jejunal tight junction remodeling independently of adenosine monophosphate-activated protein kinase in 9-day-old broiler chicks. *Poult. Sci*. **97**:3681–3690. doi:10.3382/ps/pey229

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. **3**:RESEARCH0034. doi:10.1186/gb-2002-3-7-research0034

Varasteh, S., S. Braber, P. Akbari, J. Garssen, and J. Fink-Gremmels. 2015. Differences in susceptibility to heat stress along the chicken intestine and the protective effects of galacto-oligosaccharides. *PLoS One*. **10**:e0138975. doi:10.1371/journal.pone.0138975

Wang, S., and F. W. Edens. 1993. Stress-induced heat-shock protein synthesis in peripheral leukocytes of turkeys, *Meleagris gallopavo*. *Comp. Biochem. Physiol. B*. **106**:621–628. doi:10.1016/0305-0491(93)90139-V

Yin, J., X. Li, D. Li, T. Yue, Q. Fang, J. Ni, X. Zhou, and G. Wu. 2009. Dietary supplementation with zinc oxide stimulates ghrelin secretion from the stomach of young pigs. *J. Nutr. Biochem*. **20**:783–790. doi:10.1016/j.jnutbio.2008.07.007

Zhang, M., Z. Yue, Z. Liu, A. Islam, B. Rehana, S. Tang, E. Bao, and J. Hartung. 2012. Hsp70 and HSF-1 expression is altered in the tissues of pigs transported for various periods of times. *J. Vet. Sci*. **13**:253–259. doi:10.4142/jvs.2012.13.3.253

Zhao, F. Q., Z. W. Zhang, J. P. Qu, H. D. Yao, M. Li, S. Li, and S. W. Xu. 2014. Cold stress induces antioxidants and Hsps in chicken immune organs. *Cell Stress Chaperones* **19**:635–648. doi:10.1007/s12192-013-0489-9