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Surface wetting strategy prevents acute heat exposure–induced alterations of hypothalamic stress– and metabolic-related genes in broiler chickens[1]

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ABSTRACT: Heat stress (HS) is devastating to poultry production worldwide, yet its biology and molecular responses are not well defined. Although advances in management strategy have partially alleviated the negative impact of HS, productivity still continues to decline when the ambient temperature rises. Therefore, identifying mechanism-based approaches to decrease HS susceptibility while improving production traits is critical. Recently, we made a breakthrough by applying a surface wetting strategy and showing that it improves growth performance compared with the current conventional cooling system. In the present study, we aimed to further define molecular mechanisms associated with surface wetting in ameliorating HS productivity loss in broilers. Five-week-old broiler chickens were exposed to acute HS (35°C for 2 h) alone or in combination with surface wetting. A control group was maintained at thermoneutral conditions (25°C). Core body temperature (BT) and feed intake were recorded. Blood was collected and hypothalamic tissues (main site involved in the regulation of energy homeostasis) were harvested to determine the expression profile of stress- and metabolic-related genes. Surface wetting prevents HS from increasing BT and plasma corticosterone levels ($P < 0.05$) and improves feeding and drinking behaviors. At molecular levels, surface wetting blocks the activation of hypothalamic heat shock protein and adenosine monophosphate-activated protein-induced by HS and significantly modulates the expression of feeding-related hypothalamic neuropeptides (agouti-related protein, proopiomelanocortin, orexin, orexin receptor, and leptin receptor). Taken together, our data represent the first evidence that surface wetting alleviates systemic and intracellular stress induced by HS and preserves the intracellular energy status, which, in turn, may result in improved broiler well-being and growth performance.

Key words: acute heat stress, adenosine monophosphate–activated protein kinase, broilers, hypothalamus, neuropeptides, surface wetting

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INTRODUCTION

Poultry production is facing substantial challenges from a projected steep increase in global demand for animal proteins and the need to adapt to high environmental temperatures due to climate change (Mora et al., 2013). Heat stress (**HS**) is devastating to poultry production worldwide because of its strong negative effects on feed efficiency (**FE**), meat yield, and

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mortality (Deeb and Cahaner, 2002; Niu et al., 2009). These effects resulted in significant economic losses to the poultry industry (St-Pierre et al., 2003) and such effects are likely to be aggravated as more intense and frequent heat waves continue to rise.

To alleviate HS effects, several managerial approaches have been applied but without knowledge of the fundamental mechanism changes that might be induced by these techniques. Most commercial broiler houses are equipped with a combination of tunnel ventilation and evaporative cooling systems as the status quo to overcome HS. Although these strategies keep the birds cool during hot seasons, they significantly increase water usage and relative humidity (**RH**). High temperature, especially when coupled with high RH, imposes severe stress and is counterproductive to the bird's own physiological ability to remove heat by evaporation of water from the moist lining of the respiratory tract (Fedde, 1998).

A surface wetting (**SW**) technology has recently been implemented and evaluated in 5 flocks in the summer months over 3 yr. Of particular interest, SW not only reduced water usage but also improved FE compared with the conventional system (Liang et al., 2014), although the underlying signaling pathways remain unknown. To further our understanding and because FE is controlled by the hypothalamus, we sought to determine, in the present study, the effect of HS and SW on the hypothalamic expression of heat shock proteins (**HSP**) and neuropeptides, key molecules involved in the regulation of energy homeostasis and stress response.

MATERIALS AND METHODS

Animals

All experimental procedures involving animals used in this study were conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health, and the protocol was reviewed and approved by the University of Arkansas Animal Care and Use Committee.

Thirty-five day-old male broiler chickens (Cobb 500; 2,921.5 g BW; $n = 36$) were housed in individual floor pens in 3 controlled environmental chambers (12 birds/chamber) under RH of approximately 20%. Birds had ad libitum access to fresh water and feed (3.9 Mcal ME/kg and 180 g CP/kg). The day before the experiment, the chickens were equipped with a Thermochron temperature logger (iButton, DS1922L, Maxim Integrated, San Jose, CA) for continuous monitoring of core body temperature (**BT**). The environmental temperature and RH were also continuously recorded in each chamber. Acute HS was induced by exposing the chickens to a high ambient temperature of 35°C in 2 chambers (HS), whereas the ambient temperature in other chamber was maintained at 25°C (thermoneutral [**TN**]). Half of the chickens in each HS chamber were exposed to sprinkler cooling (10 mL of water/bird at regular 30-min intervals; heat stress plus surface wetting [**HS+S**]) following our previous operating algorithm (Liang et al., 2014). Feed intake was measured and the data logger-equipped chickens (6 birds/group) were humanely killed by cervical dislocation at the end of the 2-h experiment. Blood samples were aseptically collected from wing veins using vacutainers with plasma separation tubes gel and lithium heparin (BD, NJ, Thermo Fisher Scientific Inc., Rockford, IL), and plasma was separated after centrifugation (1,500 × g for 10 min at 4°C) and stored at −20°C for later analyses of circulating hormones and metabolites. Hypothalamus samples were dissected as we previously described (Piekarski et al., 2016) and frozen in dry ice and kept at −80°C for subsequent gene and protein expression analyses. The temperature logger devices were also retrieved from their final location in the ventriculus, and core BT records were downloaded.

Feeding and Drinking Behaviors

Feeding and drinking behaviors were simultaneously recorded by 2 video cameras (Canon XA25, Canon, Tuscaloosa, AL) and Sony HD Handycam PJ304 (Sony Electronics Inc, Park Ridge, NJ) placed in front of feeders and drinkers in each TN, HS, and HS+S chamber. Video recordings were watched on a screen monitor at normal and slower speeds to distinguish 4 behavioral states: time spent eating (pecking in the feeders), time spent drinking (pecking in the drinker nipples), feeding frequency (number of visits to feeders), and drinking frequency (number of visits to drinkers). Two consecutive visits to feeders or drinkers with a <15 s break were considered 1 event

Plasma Metabolites and Hormone Measurements

Plasma glucose, triglyceride, cholesterol, lactate dehydrogenase, and uric acid levels were measured as previously described (Nguyen et al., 2015) using an automated spectrophotometer. Corticosterone concentration was measured by a commercially available ELISA kit (catalog number ADI-900-097; Enzo Life Sciences, Inc., Farmingdale, NY), according to manufacturer's instructions. Plasma levels of total 3,5,3'-triiodothyronine ($\mathbf{T_3}$) and thyroxine ($\mathbf{T_4}$) were determined using coated tube RIA kits (MP Biomedicals, Solon, OH), with some modifications. The sensitivity of both standard curves were extended to 0.03 ng/mL (T_3) and 1.5

ng/mL (T_4) by performing a series of 2-fold dilutions with steroid-free serum (MP Biomedicals), samples were diluted 1:5 (T_3 only) using steroid-free serum (MP Biomedicals), and tubes were incubated for 16 h at 4°C following addition of tracer. Radioactivity retained in each tube was counted for 1 min with a γ counter (Wallac 1470 Wizard Automatic Gamma Counter; PerkinElmer, Waltham, MA). Intra-assay CV values were 5.1 and 5.9% for T_3 and T_4, respectively.

Ribonucleic Acid Isolation, Reverse Transcription, and Real-Time Quantitative PCR

Total RNA was extracted from chicken hypothalamus samples by using Trizol reagent (Thermo Fisher Scientific Inc.) according to manufacturer's recommendations. Ribonucleic acid integrity and quality was assessed using 1% agarose gel electrophoresis, and RNA concentrations and purity were determined for each sample by a Take 3 Micro-Volume Plate using Synergy HT multimode micro plate reader (BioTek Instruments, Inc., Winooski, VT). The RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) and 1 μg RNA was reverse transcribed using a qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). The reverse transcription reaction was performed at 42°C for 30 min followed by an incubation at 85°C for 5 min. Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system; Thermo Fisher Scientific Inc.) was performed using 5 μL of 10x diluted cDNA, 0.5 μ*M* of each forward and reverse specific primer, and SYBR Green Master Mix (Thermo Fisher Scientific Inc.) in a 20-μL-total reaction. Oligonucleotide primers used for chicken hypothalamic neuropeptide Y (*NPY*), agouti-related peptide (*AgRP*), proopiomelanocortin (*POMC*), cocaine and amphetamine regulated transcript (*CART*), orexin (*ORX*), orexin receptor 1/2 (*ORXR1/2*), leptin receptor (*Ob-R*), *HSP60* and *HSP70*, heat shock factor (**HSF**) 1 through 4 (*HSF1–HSF4*), adenosine monophosphate-activated protein kinase (**AMPK**; *AMPKα1/2*, *AMPKβ1/2*, and *AMPKγ1/2/3*), mechanistic target of rapamycin (*mTOR*), p70 ribosomal protein S6 kinase 1 (*S6K1*), and ribosomal 18S as a housekeeping gene were previously published (Lassiter et al., 2015; Nguyen et al., 2015). Oligonucleotide primers used for chicken HSP90 are forward, 5′-TGACCTTGTCAACAATCTTGGTACTAT-3′, and reverse, 5′-CCTGCAGTGCTTCCATGAAA-3′, amplifying a fragment of 68 bp. The quantitative PCR cycling conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of a 2-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system (Thermo Fisher Scientific Inc.) to exclude contamination with unspecific PCR products. The PCR products were also confirmed by 2% agarose gel and showed only 1 specific band of the predicted size. For negative controls, no cDNA templates were used in the quantitative PCR and verified by the absence of gel-detected bands. Relative expressions of target genes were normalized to the expression of 18S rRNA and calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The TN group was used as a calibrator.

Western Blot Analysis

Hypothalamic tissues were homogenized in lysis buffer (10 m*M* Tris base, pH 7.4; 150 m*M* NaCl; 1 m*M* ethylenediaminetetraacetic acid; 0.1% Triton X-100; 0.5% NP-40; protease and phosphatase inhibitor cocktail). Total protein concentrations were determined using a Synergy HT multimode microplate reader (BioTek Instruments, Inc.) and a Bradford assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) with BSA as a standard. Proteins (100 μg) were run on 4 to 12% Novex Bis–Tris gels (Life Technologies, Grand Island, NY). The transferred membranes were blocked for 1 h at room temperature and incubated with primary antibodies (diluted 1:500–1:1,000) at 4°C overnight. The rabbit polyclonal anti-phospho AMP-activated protein kinase α (AMPKα1/2) ^{Thr172} (number 2531), anti-AMPKα1/2 (number 2795), anti-HSP90 (number PA5-17610), goat polyclonal anti-HSP60 (number sc-1052), and mouse monoclonal anti-HSP70 (number MAI-91159) were used. Protein loading was assessed by immunoblotting with the use of rabbit anti-β actin (number 4967). Prestained molecular weight marker (Precision Plus Protein Dual Color; Bio-Rad Laboratories, Inc.) was used as a standard. All primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA), except for the anti-HSP70 and anti-HSP90, which were purchased from Pierce Thermo Scientific (Rockford, IL), and anti-HSP60, which was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The secondary antibodies were used (1:5,000) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus; GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by a FluorChem M MultiFluor System (ProteinSimple, Santa Clara, CA). Image acquisition and analysis were performed using AlphaView software (version 3.4.0, 1993–2011; ProteinSimple).

Statistical Analysis

Data were subjected to one-way ANOVA as a completely randomized design with individual bird as the experimental unit and experimental groups (TN, HS, and HS+S) as the fixed effects. If ANOVA revealed signif-

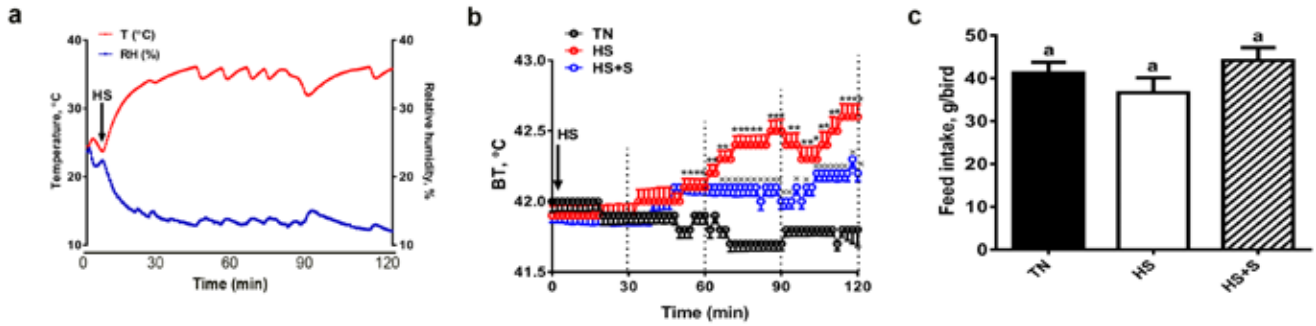

Figure 1. Surface wetting reverses heat stress (HS)–related effects on core body temperature (BT) and feed intake in broiler chickens. Surface wetting prevents HS (a)-increasing core body temperature (b) and decreasing feed intake (c) in broiler chickens. Data are presented as mean ± SEM (n = 12 for feed intake and n = 6 for core BT). *,[a]Different letters indicate significant difference at $P < 0.05$. HS+S = heat stress plus surface wetting; RH = relative humidity; T = air temperature; TN = thermoneutral. The arrow indicates the start of heat stress.

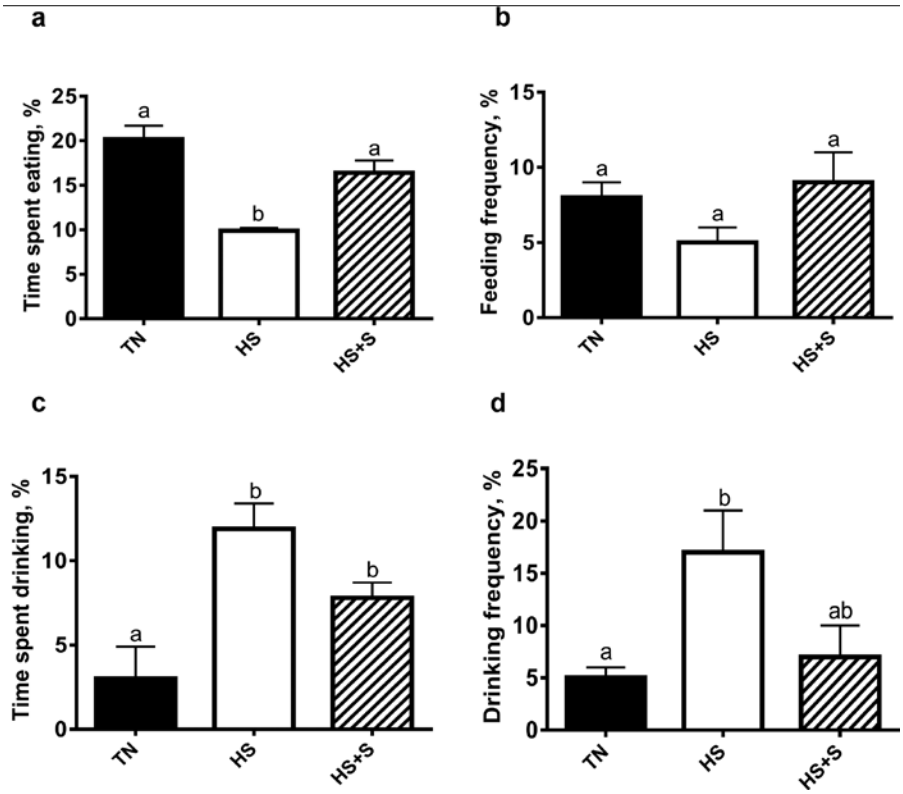

Figure 2. Surface wetting prevents feeding and drinking behavior alteration induced by acute heat stress (HS) in broiler chickens. Time spent eating (a), feeding frequency (b), time spent drinking (c), and drinking frequency (d) were recorded using video camera. Data are presented as mean ± SEM (n = 12). [a,b]Different letters indicate significant difference at $P < 0.05$. HS+S = heat stress plus surface wetting; TN = thermoneutral.

icant effects, the means were compared by Tukey multiple range test using GraphPad Prism version 6.00 for Windows (GraphPad Software, Inc., La Jolla, CA), and differences were considered significant at $P < 0.05$.

RESULTS

Surface Wetting Modulates Feeding/Drinking Behaviors and Reduces Core Body Temperature Increased by Acute Heat Stress in Broilers

The ambient temperature was increased within 10 min to reach 35°C in the chambers; however, the RH was gradually decreased to approximately 12% (Fig. 1a). As illustrated in Fig. 1b, after 45 min of heat exposure, the heat-stressed group exhibited higher core BT (0.8–1°C; $P < 0.05$) compared with their counterparts maintained at TN conditions. Surface wetting reduced core BT increased by HS by approximately 0.4 to 0.5°C (Fig. 1b). Neither acute heat exposure nor SW altered feed intake (Fig. 1c). To gain further insights into feeding and drinking behaviors, time spent eating/drinking and feeding/drinking frequencies were determined. As shown in Fig. 2a, acutely heat-stressed birds spent less time eating ($P < 0.05$) compared with the TN group. On the other hand, heat-stressed broilers spent more time drinking ($P = 0.002$) and drank more frequently ($P = 0.02$) compared with the TN group (Fig.

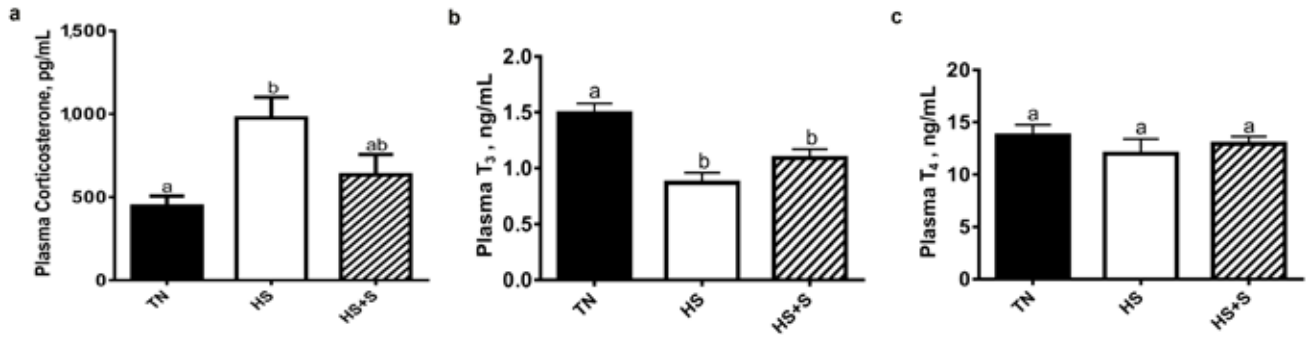

Figure 3. Effects of acute heat stress (HS) and surface wetting on plasma hormone levels in broiler chickens. Plasma corticosterone levels were measured using ELISA (a), and circulating 3,5,3'-triiodothyronine (T_3; b) and thyroxine (T_4; c) concentrations were measured using RIA as described in Materials and Methods. Data are presented as mean ± SEM (n = 12). [a,b]Different letters indicate significant difference at $P < 0.05$. HS+S = heat stress plus surface wetting; TN = thermoneutral.

Table 1. Effects of acute heat stress (HS) and surface wetting on circulating metabolite levels in broiler chickens

	Experimental groups[2]				
Metabolites[1]	TN	HS	HS+S	F (DFn, DFd)[3]	Value
Glucose, mg/dL	275 ± 5.8	259.1 ± 21	275.6 ± 5.7	0.4	0.6
Cholesterol, mg/dL	159.6 ± 8.4	133.5 ± 9.9	152.3 ± 6.8	2.5	0.1
Triglycerides, mg/dL	21.6 ± 1.8	26 ± 5.4	33.1 ± 1.7	2.8	0.08
LDH, units/L	150 ± 32	133.5 ± 17	149.1 ± 11	0.1	0.8
UA, mg/dL	4.1 ± 0.3	2.9 ± 0.4	3.8 ± 0.3	3.5	0.05

[1]LDH = lactate dehydrogenase; UA = uric acid.

[2]TN = thermoneutral; HS+S = heat stress plus surface wetting.

[3]F = F distribution; DFn = degree of freedom numerator; DFd = degree of freedom denominator.

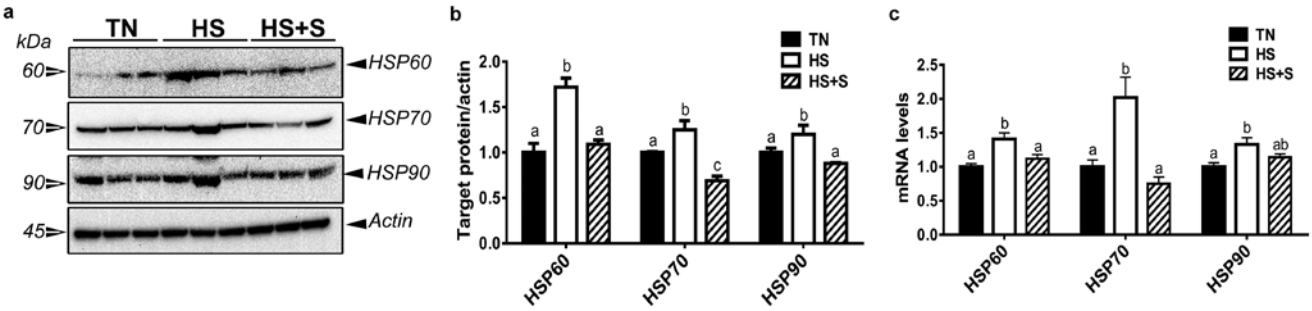

Figure 4. Surface wetting prevents heat stress (HS) upregulating heat shock protein (HSP) 60/HSP70/HSP90 expression in broiler hypothalamus. Protein levels of HSP60, HSP70, and HSP90 were assessed by Western blot (a) and presented as normalized ratio to the housekeeping protein β-actin (b). Relative mRNA abundance of HSP60, HSP70, and HSP90 (c) was determined by quantitative PCR as described in Materials and Methods. Data are presented as mean ± SEM (n = 6). [a,b]Different letters indicate significant difference at $P < 0.05$. HS+S = heat stress plus surface wetting; TN = thermoneutral.

2c and 2d). Surface wetting reversed these effects, that is, increased feeding- and decreased drinking-related behaviors (Fig. 2a, 2c, and 2d).

Surface Wetting and Heat Stress Modulate Circulating Hormone and Blood Parameter Levels

As shown in Fig. 3a, plasma corticosterone levels increased by 2-fold ($P < 0.05$) during HS, and this effect was prevented by the SW strategy (Fig. 3a). Plasma T_3 concentrations were significantly reduced in the HS group ($P < 0.05$) as well as in the HS+S group ($P < 0.05$) compared with the TN group (Fig. 3b). No significant difference ($P > 0.05$) was observed between all groups for plasma T_4 and blood parameter (glucose, cholesterol, lactate dehydrogenase, triglyceride, and uric acid) levels (Table 1).

Surface Wetting Prevents Heat Stress–Inducing Hypothalamic Expression of Heat Shock Proteins and Heat Shock Factors

Acute HS significantly upregulated the hypothalamic expression of *HSP60* and *HSP70* mRNA (Fig. 4c) and HSP60, HSP70, and HSP90 protein levels compared with TN condition (Fig. 4a and 4b), and these effects were prevented by SW application (Fig. 4a–4c). Surface wetting also inhibited the increased

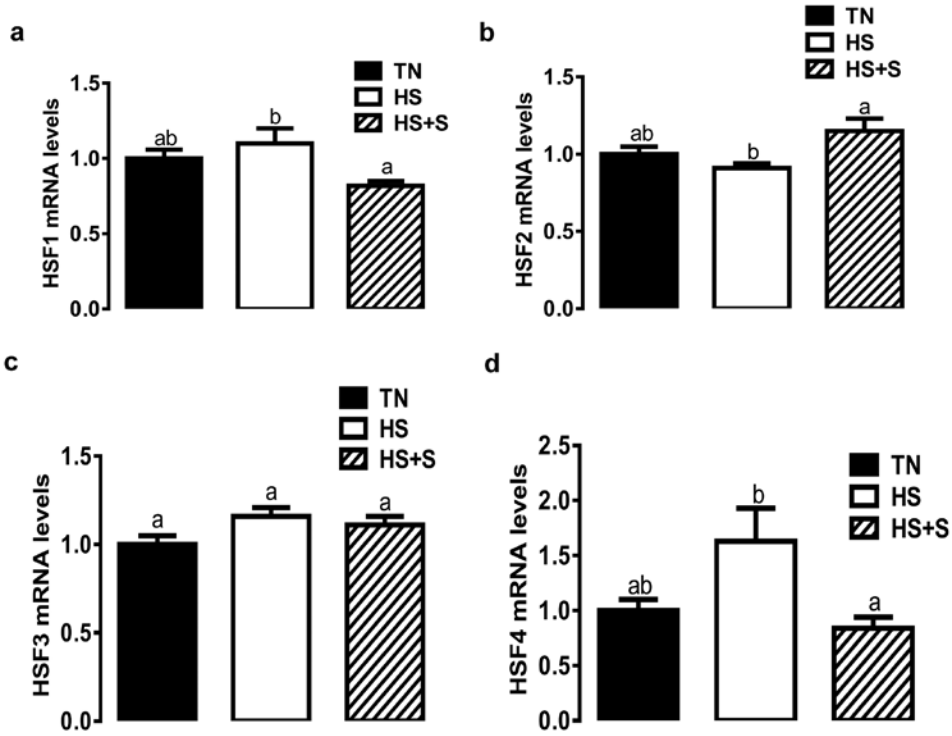

Figure 5. Effects of acute heat stress (HS) and surface wetting on the hypothalamic expression of heat shock factors (HSF) 1 through 4 in broiler chickens. Relative expression of HSF1 (a), HSF2 (b), HSF3 (c), and HSF4 (d) was determined by quantitative PCR. Data are presented as mean ± SEM (n = 6). [a,b]Different letters indicate significant difference at $P < 0.05$. HS+S = heat stress plus surface wetting; TN = thermoneutral.

expression of hypothalamic *HSF4* induced by HS (Fig. 5d). Heat stress did not elicit any change in the mRNA abundance of *HSF1*, *HSF2*, and *HSF3* compared with TN conditions (Fig. 5a–5c).

Surface Wetting Modulates the Expression of Feeding-Related Hypothalamic Neuropeptides Altered by Heat Stress

Acute HS up regulated the hypothalamic expression of *AgRP*, *POMC*, *CART*, *ORX*, *ORXR1/2*, and *Ob-R* compared with the TN condition ($P < 0.05$; Fig. 6b–6h). Surface wetting inhibited these effects and specifically maintained the mRNA levels of hypothalamic *AgRP*, *POMC*, *ORX*, *ORXR2*, and *Ob-R* similar to that of the TN group (Fig. 6b, 6c, 6e, 6g, and 6h); however, it did not affect *CART* and *ORXR1* expression (Fig. 6d and 6f). Neither HS nor SW (HS + S) affected the hypothalamic expression of *NPY* compared with the TN group (Fig. 6a).

Surface Wetting Prevents Adenosine Monophosphate–Activated Protein Kinase Activation Induced by Heat Stress

As shown in Fig. 7a and 7b, HS significantly increased the levels of AMPKα1/2 phosphorylated at the Thr172 site, and SW inhibited this effect. At mRNA levels, SW significantly downregulated the hypothalamic expression of *AMPKγ1* compared with the TN group. Neither HS nor SW affected the hypothalamic expression of *AMPKα1/2*, *AMPKβ1/β2*, and *AMPKγ2/3* (Fig. 7c–7i).

DISCUSSION

As the average global temperature increases, heat waves will become more frequent, more intense, and longer lasting (Alley et al., 2005; Mora et al., 2013). Although several managerial strategies exist, environmental HS still impacts every aspect of animal lives and their very existence (Chen et al., 2011). It can result in heat-related discomfort (stress), illness, and multiple organ damage and, under extreme conditions, can cause spiraling hyperthermia leading to death. In broiler chickens, HS has deleterious effects mainly through reducing feed intake, growth, FE, and meat yield and increasing mortality rate, which, in turn, leads to a significant financial burden (Cahaner and Leenstra, 1992; Leenstra and Cahaner, 1992; St-Pierre et al., 2003). There is, therefore, an urgent need to define the biology of how HS imperils poultry productivity and to develop mechanism-based approaches to prevent its adverse effects.

In addition and in line with our previous work (Liang et al., 2014), SW reduced core BT increased by acute HS, which might explain its beneficial effects on growth performances in broilers (Liang et al., 2014). When the environmental temperature is within the TN zone (13–24°C),

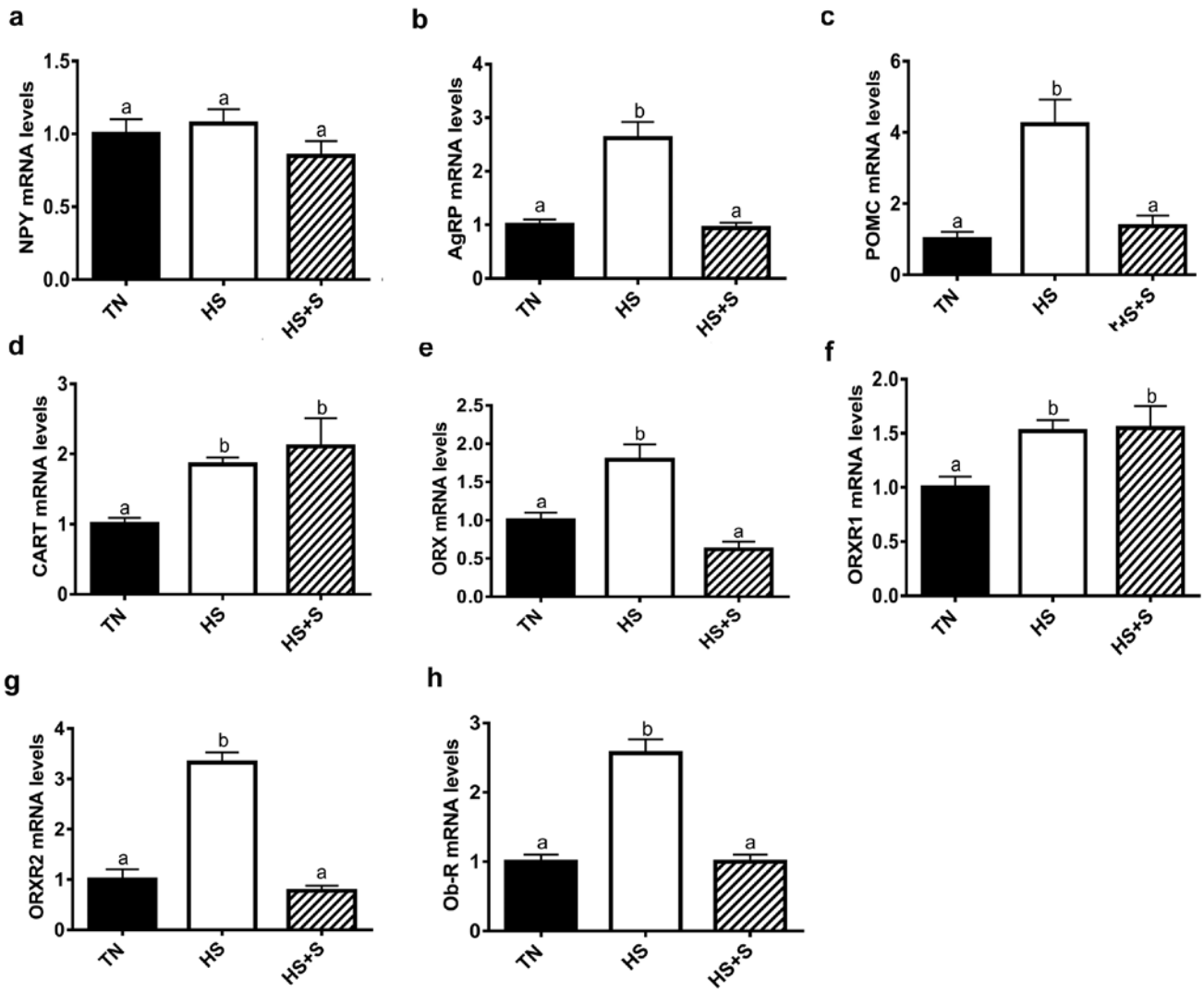

Figure 6. Effects of acute HS and surface wetting on the hypothalamic expression of anorexigenic and orexigenic neuropeptides in broiler chickens. Relative expression of NPY (a), AgRP (b), POMC (c), CART (d), ORX (e), ORXR1 (f), ORXR2 (g), and OB-R mRNA (h) was determined by quantitative PCR. Data are presented as mean ± SEM ($n = 6$). [a,b]Different letters indicate significant difference at $P < 0.05$. HS+S = heat stress plus surface wetting; TN = thermoneutral.

birds dissipate body heat to the surrounding environment by radiation, conduction, and convection, which together refer to sensible heat loss (Genc and Portier, 2005). Using these avenues for thermoregulation, birds do not need to alter their behavioral, physiological, and immune patterns or metabolism (Mitchell, 1985). However, when the ambient temperature rises above their comfort zone (>26.5°C), birds rely on hyperventilation (panting) to lose heat by evaporation of water from the moist lining of the respiratory tract (Fedde, 1998). Panting itself is energy demanding and generates body heat, and when it is coupled with high metabolic rate and lack of sweat glands, it results in increased BT (Cooper and Washburn, 1998). It is likely that SW acts as artificial sweat for the bird and, when it evaporates, helps remove body heat (Flamenbaum et al., 1986; Mutaf et al., 2008).

Although the underlying molecular mechanisms are not well defined, at least in avian species, reduced feed intake during HS is a highly conserved response among species and presumably represents an attempt to decrease metabolic heat production and minimize heat increment associated with feed (Baumgard and Rhoads, 2012; Syafwan et al., 2012). In this study, SW prevented HS from reducing the time spent eating. As expected and to compensate water loss induced by hyperventilation, acutely heat-stressed birds showed higher drinking frequency and spent more time drinking, which resulted in greater water intake (Lott, 1991; Belay et al., 1993). Surface wetting reversed these effects, indicating that this strategy may alleviate the adverse effects of HS.

At molecular levels, a series of highly integrated regulatory mechanisms, not yet completely defined in avian species, exists for the control of feed intake at both peripheral and central sites. Within the central nervous system, the hypothalamic satiety and hunger centers play key roles in the regulation of these processes (Morgane, 1961; Elmquist et al., 1999). Two separate populations of neuronal cell types are located in the

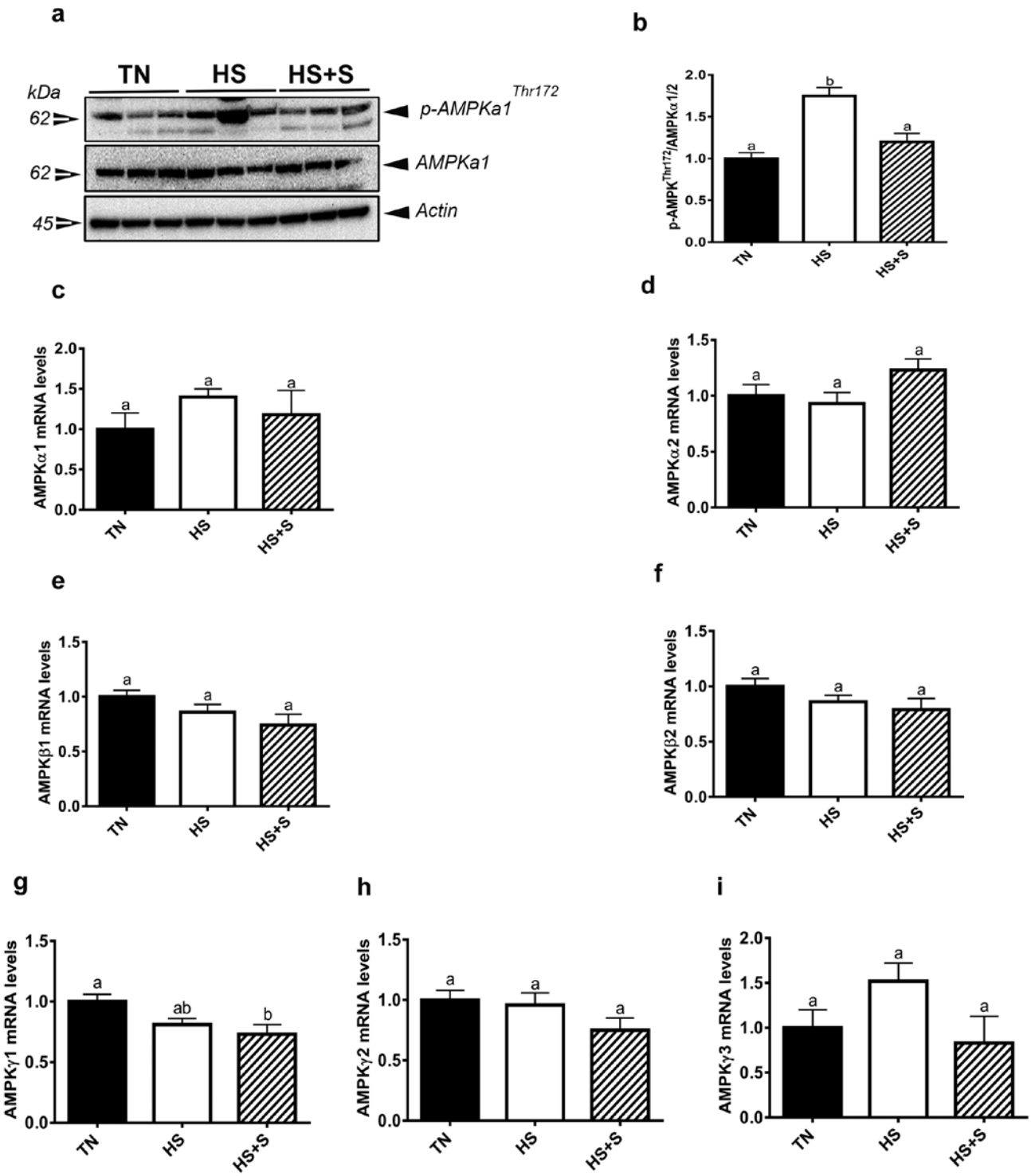

Figure 7. Surface wetting prevents heat stress (HS) from inducing hypothalamic Adenosine monophosphate-activated protein kinase (AMPK) activation in broiler chickens. Phosphorylated and pan levels of AMPKα1/2 were determined using Western blot (a) and their relative expression was presented as normalized ratio of phosphorylated:pan AMPKα1/2 protein (b). Protein loading was assessed by immunoblotting with the use of the housekeeping β-actin. Relative abundance of AMPKα1 (c), AMPKα2 (d), AMPKβ1 (e), AMPKβ2 (f), AMPKγ1 (g), AMPKγ2 (h), and AMPKγ3 (i) mRNA was measured by quantitative PCR. Data are presented as mean ± SEM ($n = 6$). [a,b]Different letters indicate significant difference at $P < 0.05$. HS+S = heat stress plus surface wetting; TN = thermoneutral.

mammalian arcuate nucleus (the equivalent of infundibular nucleus in chickens); one synthesizes the powerfully orexigenic peptides (NPY and AgRP) whereas the other produces the anorexigenic proopiomelanocortin and cocaine and amphetamine regulated transcript (Friedman and Halaas, 1998). Both neurons project to multiple hypothalamic and extrahypothalamic sites where they communicate with second-order neurons involved in the regulation of energy balance (Balthasar et al., 2005). In attempt to further our understanding of the mechanisms by which acute HS alters feeding behavior, the expression profile of feeding-related hypotha-

lamic neuropeptides were determined. Specifically, the mRNA abundances of *AgRP*, *POMC*, *CART*, *Ob-R*, and *ORX* and its related receptors *ORXR1/2* were higher in heat-stressed birds compared with their TN counterparts. However, *NPY*, the potent stimulator of appetite and the most studied in chickens (Kuenzel et al., 1987; Blomqvist et al., 1992), remained unchanged among all groups in our experimental conditions. Interestingly, SW was able to block the HS-related effects only for *AgRP*, *POMC*, *ORX*, *ORXR2*, and *Ob-R*. Previous studies showed that heat exposure for 7 d upregulated the hypothalamic expression of *CART* but not *NPY*, *POMC*, and *AgRP* in 24-wk-old laying hens (Song et al., 2012). In broilers, Tu et al. (2016) reported that acute HS for 2 h increased the hypothalamic expression of *NPY*; however, Lei et al. (2013) showed that 6 h of heat exposure did not elicit any changes in the expression of *NPY*, *AgRP*, *POMC*, and *CART*. The discrepancies between these findings might be related to numerous factors including chicken strains (layer vs. broilers), age, duration and severity of HS, and/or experimental conditions (diet composition, stress, density, and feeding system). Electrophysiological data suggested that AgRP neurons send direct GABAergic projections to POMC neurons and speculated that POMC neurons are one of the key downstream mediators of AgRP (Cowley et al., 2001; Cone, 2005). However, recently, the Sternson laboratory elegantly showed that POMC neurons play a minimal role in mediating AgRP action on acute feeding, although they receive indeed direct GABAergic projections from *AgRP* neurons (Atasoy et al., 2012). Whether POMC or AgRP is predominantly involved in mediating the effect of acute HS on feeding behavior is not known at this time and warrants further investigation.

Although the physiological functions of the newly identified chicken leptin is still unknown (Seroussi et al., 2016), chicken leptin receptor has been reported to be associated with growth and FE in broiler chickens (El Moujahid et al., 2014). In addition, immunization against chicken leptin receptor has been reported to stimulate feed intake and alter the expression of feeding-related hypothalamic neuropeptides in growing chickens (Lei et al., 2015). Interestingly, HS has been shown to increase leptin secretion in mice (Morera et al., 2012) and alter the expression of leptin receptor in bovine peripheral blood mononuclear cells (Lacetera et al., 2009). Together, these data in combination with our present results suggest that leptin receptor might be involved in HS responses in chickens and that SW might prevent HS-induced neuroendocrine changes by downregulation of the hypothalamic leptin receptor expression.

Although some avian neuropeptides have effects similar to that described in mammals, several other peptides have the opposite or no effects. For instance, orexins/hypocretins are potent orexigenic agents in mammals but are without any apparent effects on feed intake in chicks (Sakurai et al., 1998; Furuse et al., 1999). The emerging evidence from mammalian studies identifying orexin as a stress modulator via its interaction with the hypothalamic–pituitary–adrenal axis and corticosterone release (Bonnavion et al., 2015) prompted us to determine the expression of physiological hallmarks of the stress response. First, we measured, at the systemic level, the plasma corticosterone concentration (Quinteiro-Filho et al., 2012; Furukawa et al., 2016) and found that SW repressed HS-increasing circulating corticosterone levels. Second, the hypothalamic expression of HSP and their related transcription factors were determined at intracellular levels.

Heat shock proteins were first described as heat shock–inducible proteins and were originally thought to be a generalized cellular defense response through chaperoning, protein folding, degradation targeting, sequestration, and scaffolding (Gething and Sambrook, 1992; Hartl et al., 1992; Becker and Craig, 1994; Hartl, 1996). However, investigations into their molecular mechanisms are still yielding new surprises, and HSP are now understood to be involved in a variety of cellular processes from transcriptional control to inflammation and cell death signaling (Rutherford and Zuker, 1994; Moseley, 1998; Samali and Orrenius, 1998). Although HSP have multiple levels of regulation, their stress-induced transcription lies primarily under the control of HSF, transcription factors that, when bound to heat shock response elements in the upstream promoter regions, are activated and upregulated target HSP (Shuey and Parker, 1986). At central levels and in agreement with our results, HSP and HSF have been shown to be induced by heat shock (Nowak et al., 1990; Higashi et al., 1995; Manzerra and Brown, 1996; Sun et al., 2015) and are well poised to transmit and coordinate stress signals following cerebral ischemia and to function in neuroprotective capacities (Matsumori et al., 2005, 2006). Together, HS increases circulating corticosterone levels and induces HSP activation and surface wetting prevents this.

As HS has been reported to alter the cellular energy status in mammals (i.e., a decrease in the concentration of ATP and phosphocreatine and an increase in the concentration of adenosine monophosphate; Ghussen and Isselhard, 1984), we sought to determine in this study the expression profile of hypothalamic AMPK, the master energy sensor (Hardie, 2007). Adenosine monophosphate-activated protein kinase is an evolutionary conserved serine/threonine kinase with a catalytic α-subunit and regulatory β- and γ-subunits, forming a heterotrimeric complex that not only senses cellular energy levels but also controls and responds to altered energy status in most eukaryotic cells (Carling et al., 2012). When energy

status is compromised, AMPK is activated through phosphorylation at the threonine 172 residue of the α-kinase subunit (Stein et al., 2000) and activates catabolic pathways and switches off anabolic pathways. It stands at the crossroads of various cellular processes and can be a guardian or a killer depending on the physiological status of the organism, the severity of the stressor, and the cell type (Xu and Si, 2010; Ju et al., 2011). Although the upstream signaling cascades modulated by acute HS to activate AMPK are not known at this time, its inactivation by SW indicates that this strategy may preserve the cellular ATP stores and utilization. It is possible that the activation of hypothalamic AMPK is due to reduction of feed intake induced by HS (Minokoshi et al., 2004). As HS induces redistribution of blood flow, which is being diverted from certain internal organs including the brain to dilated blood vessels of the peripheral tissues (skin) to facilitate heat loss (Mutaf et al., 2009), the activation of hypothalamic AMPK may due to inadequate and insufficient flow of nutrients associated with reduction of blood supply. In addition, because AMPK has been shown to orchestrate the metabolic effects of several hormones, it is reasonable to hypothesize that ORX (Wu et al., 2013), corticosterone (Zhao et al., 2008), and/or Ob-R (Minokoshi et al., 2004; Kwon et al., 2016) may mediate the effect of HS/SW on hypothalamic AMPK expression. However, further experimental evidence for this hypothesis and mechanistic studies are needed.

In conclusion, the present study is the first to report molecular evidence of the beneficial effect of a SW strategy in preserving the central cellular energy status and in mitigating systemic and intracellular stress induced by acute HS, which thereby may result in improvement of poultry well-being and productivity. Further broad studies including additional metabolically important peripheral tissues and longer HS exposure are warranted to further our understanding of HS/SW responses.

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