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Heat Stress Biomarker Amino Acids and Neuropeptide Afford Thermotolerance in Chicks

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With global warming, heat stress is becoming a pressing concern worldwide. In chickens, heat stress reduces food intake and growth, and increases body temperature and stress responses. Although it is believed that young chicks do not experience heat stress as they need a higher ambient temperature to survive, our series of studies in young chicks showed that they are sensitive to heat stress. This review summarizes current knowledge on amino acid metabolisms during heat stress, with special emphasis on the hypothermic functions of L-citrulline (L-Cit) and L-leucine (L-Leu), and the functions of neuropeptide Y (NPY) in terms of body temperature and heat stress regulation in chicks. Amino acid metabolism is severely affected by heat stress. For example, prolonged heat stress reduces plasma L-Cit in chicks and L-Leu in the brain and liver of embryos. L-Cit and L-Leu supplementation affords thermotolerance in young chicks. NPY expression is increased in the brains of heat-exposed chicks. NPY has a hypothermic action under control thermoneutral temperature and heat stress in chicks. The NPY-sub-receptor Y5 is a partial mediator of the hypothermic action of NPY. Further, NPY stimulates brain dopamine concentrations and acts as an anti-stress agent in heat-exposed fasted, but not fed chicks. In conclusion, young chicks can serve as a model animal for the study of heat stress in chickens. L-Cit, L-Leu, and NPY were identified as biomarkers of heat stress, with the potential to afford thermotolerance in chicks.

Key words: chicks, L-citrulline, L-leucine, neuropeptide Y, thermotolerance

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Introduction

The earth's surface temperature in 2017 ranked the second warmest since 1880 according to an analysis by the National Aeronautics and Space Administration, USA (NASA, 2017). The potential of such increasing ambient temperature to enhance heat stress issues is a serious concern globally. Heat stress threatens the commercial poultry sector in tropical and sub-tropical countries as well as in temperate countries exposed to summer heat waves. It can reduce food intake, live weight gain, and food efficiency in broilers (Howlider and Rose, 1987; Siegel, 1995; Niu *et al*., 2009; Azad *et al*., 2010), and can affect egg production in laying hens (Marsden *et al*., 1987; Peguri and Coon, 1991; Yahav *et al*., 2000; Sterling *et al*., 2003; Lin *et al*., 2004; Franco-Jimenez and Beck, 2007; Ajakaiye *et al*., 2010). In chickens, high ambient temperature (HT) can lead to an increase in deep body (rectal) temperature (Yahav and Hurwitz, 1996) and may cause heat stress (Bartlett and Smith, 2003; Soleimani *et al*., 2010). It is generally believed that young chicks are not prone to heat stress as they need HT for their survival. However, limited attention has been paid to young layer chicks with respect to heat stress issues, and several of our recent studies suggested that both layer and broiler chicks are sensitive to HT (Chowdhury *et al*., 2012a, b, 2014, 2017; Ito *et al*., 2014, 2015; Han *et al*., 2017, 2018, Eltahan *et al*., 2017). When young chicks were exposed to 35℃ for 3 h, their food intake decreased and their rectal temperature increased (Bahry *et al*., 2017). Further, 3- and 5-day-old chicks were less sensitive to heat stress (40℃ for 4 h) when the control thermoneutral temperature (CT) was 30℃, while chicks of 7 days or older were sensitive to heat stress (Chowdhury *et al*., 2012b).

Mammals are also very sensitive to heat stress. For example, like chickens, pigs do not have functional sweat glands (Ensminger *et al*., 1990). In pigs, thermoregulatory responses for avoiding heat stress are activated above 25℃ (Quiniou and Noblet, 1999). Heat stress increases rectal temperature in swine (Dou *et al*., 2017), cattle (Kamal *et al*., 2018), and chickens (Chowdhury *et al*., 2014). Further, a

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significant reduction in voluntary feed intake is generally observed in swine (Dou *et al*., 2017), cattle (Kamal *et al*., 2018), and chickens (Chowdhury *et al*., 2012) during heat stress. This response is considered the main adaptation mechanism for reducing metabolic heat production (Nyachoti *et al*., 2004), which has negative effects on growth performance in mammals (D'Allaire *et al*., 1996; Kamal *et al*., 2018) and chickens (Mashaly *et al*., 2004).

Amino acids play important roles in growth (Li and Wu, 2018) and might be critical in controlling food intake (Tran *et al*., 2015, 2016) and behavior (Kabuki *et al*., 2011; Ikeda *et al*., 2014; Tran *et al*., 2015). Several free amino acids were found to be significantly increased in the blood, brain, and skeletal muscle of chicks within 15 or 30 min of exposure to HT (35℃; Ito *et al*., 2014); however, most of these amino acids in the brain and plasma declined when chicks were exposed to prolonged HT (35℃ for 48 h; Chowdhury *et al*., 2014). Based on these findings, we subsequently found that L-citrulline (L-Cit), which was increased in the plasma of chicks following short-term heat exposure and decreased after long-term heat exposure, has a hypothermic function when administered orally in chicks (Chowdhury *et al*., 2015, 2017). In addition, thermal manipulation (TM) during embryogenesis resulted in a reduction in brain and liver concentrations of L-leucine (L-Leu). Interestingly, *in ovo* feeding of L-Leu affords thermotolerance in broiler chicks (Han *et al*., 2017, 2018). Thus, certain amino acids that decrease or increase in chicks depending on the length of exposure to HT and its magnitude may serve as biomarkers of heat stress.

The central nervous system is thought to be a critical center of behavioral motivation. Therefore, brain function has been meticulously studied. Hypothalamic neuropeptides are important in the regulation of food intake (Furuse, 2007; Cline and Furuse, 2012) and stress (Carrasco and Van de Kar, 2003; Madaan and Wilson, 2009; Alldredge, 2010; Schank *et al*., 2012; Lin, 2012; Catena-Dell'Osso *et al*., 2013). Hypothalamic neuropeptides are also important in the regulation of body temperature. The thermoregulatory center is widely reported to be located in the hypothalamus. In particular, the hypothalamic preoptic area (POA) contains neurons that are sensitive to warmth and trigger hypothermia (Hammel, 1968). Tan *et al*. (2016) reported that brain-derived neurotrophic factor and pituitary adenylate cyclase-activating polypeptide have important roles in body temperature regulation in the POA. Recent studies have focused on hypothalamic neuropeptide Y (NPY) in an attempt to unravel its role in the regulation of body temperature and heat stress in chicks (Bahry *et al*., 2017; Eltahan *et al*., 2017).

In this review, I will summarize the effects of heat stress on amino acid metabolism, with special emphasis on the hypothermic functions of L-Cit and L-Leu. Moreover, the functions of NPY in terms of body temperature and heat stress regulation in chicks will be described.

Amino Acid Metabolism in Embryos and Chicks under Heat Stress

Twenty amino acids are commonly found in animal proteins. In growing chicks, 11 of these (arginine (Arg), histidine, isoleucine, leucine (Leu), lysine, methionine, phenylalanine, threonine, tryptophan, valine, and glycine) are essential amino acids, while the others are nonessential amino acids (Banerjee, 1998). These 20 amino acids which have codon, cannot be stored as free molecules and must follow anabolic routes to peptides, proteins, hormones, and other bioactive molecules or catabolic pathways to glucose, ketone bodies, or uric acids in birds. Heat stress causes catabolic activity to increase in organisms (Maeda *et al*., 2017) to provide energy to counter the heat stress. Protein turnover has a high energy cost: it requires 4.5−7 mol of ATP per mole of peptide bond formed and 1−2 mol of ATP per mole of peptide bond breakage (Bequette, 2003). Therefore, the increase or decrease in free amino acids that occurs under heat stress (Ito *et al*., 2014, 2015; Chowdhury et al., 2014; Eltahan *et al*., 2017) can be assumed to be the result of catabolic processes. Inter-organ amino acid flux is important for determining the sites where amino acids are ultimately used (Seal and Parker, 2000). All tissues contain enzymes for amino acid catabolism and synthesis, but their expression and activity levels vary depending on the metabolic needs or functions of the tissue. Catabolism involves deamination/deamidation reactions followed by either reamination of the resulting carbon skeleton to form non-essential amino acids, or channeling of the carbon skeleton into the tricarboxylic acid cycle, where it is oxidized, channeled towards gluconeogenesis via pyruvate carboxylase, or converted from pyruvate into acetate for fatty acid synthesis. Amino acids act not only as constituents of proteins but also as regulators of various physiological and/or pharmacological functions. Substantial attention has been paid to the regulation of physiology and behavior, including stress responses (Asechi *et al*., 2006; Hamasu *et al*., 2009a, b; Kurauchi *et al*., 2010; Kurata *et al*., 2011; Erwan *et al*., 2014). Bird diets have been supplemented with amino acids, especially essential amino acids, to overcome the problems caused by heat stress (Mendes *et al*., 1997; Rose and Uddin, 1997; Brake *et al*., 1998; Daghir *et al*., 2003; Willemsen *et al*., 2011; Dai *et al*., 2012). Although there have been fewer investigations into the changes in amino acid concentrations in chickens under heat stress, Ito *et al*. (2014) reported that while several free essential as well as nonessential amino acids increased in the blood, brain, and skeletal muscle following short-term heat stress (less than 30 min), the levels of some other amino acids were significantly reduced.

The reason for the increases in various amino acids is still unknown, but it is possible that the increased plasma amino acids may have been derived from proteins from the liver or some other soft tissues that are more labile than myofibrillar proteins in skeletal muscle. Interestingly, the levels of free amino acids induced during short-term heat stress (35℃, 15 or 30 min) were nearly inversely correlated with those induced by long-term heat stress (35℃, 24 or 48 h), upon which most of the free amino acids were reduced. For instance, tryptophan, Cit, and Orn were reduced in the plasma of chicks exposed to long-term heat stress (Chowdhury *et al*.,

2014), whereas they increased during short-term heat stress (Ito *et al*., 2014).

Various free amino acids in the chick brain and breast muscle are also altered upon heat exposure. Interestingly, the free amino acids in the brain and skeletal muscle were mostly different from those found in the plasma. All the altered free amino acids in the various parts of the brain, except for proline and cystathionine, were different. These findings indicate that alterations in free amino acid contents may be tissue-specific, which is in accordance with the fact that enzymatic activity related to amino acid metabolism and protein synthesis is tissue-specific. In chick breast muscle, the concentration of 3-methyl histidine, a marker of proteolysis (Young and Munro, 1978; Nishizawa, 1983), was significantly ($P<0.05$) declined upon short-term heat stress (Ito *et al*., 2014), suggesting that protein degradation was reduced in this condition. As protein synthesis and degradation are balanced in the body, it can be predicted that protein synthesis would decrease and the pool of free amino acids in the tissue would increase during heat stress.

TM is applied during embryogenesis to increase the incubation temperature, resulting in the acquisition of thermotolerance by neonatal chicks (Moraes *et al*., 2003) and chickens (Loyau *et al*., 2014) under HT. Recently, we found that several amino acids, including Leu, lysine, and phenylalanine, were significantly reduced in the brain and liver in embryos that were exposed to TM (Han *et al*., 2017). In summary, amino acid metabolic activity can be affected by heat stress in embryos and chicks.

Hypothermic Amino Acids in Heat-Exposed Chicks

Intracerebroventricular (i.c.v.) administration of L-Cit, L-Orn, and L-Arg did not cause a reduction in rectal temperature (Chowdhury *et al*., 2015). However, orally administered L-Cit, but not L-Arg or L-Orn, did reduce rectal temperature (Chowdhury *et al*., 2015). Further, it caused a significant ($P<0.0001$) reduction in rectal temperature in heat-exposed chicks, comparable to that observed in non-heat-exposed control chicks (Chowdhury *et al*., 2017). These findings suggested L-Cit has a hypothermic function.

In mammals, nearly all L-Arg supplied via the food is withdrawn from the portal blood by the liver for conversion to urea (Curis *et al*., 2005). However, L-Cit can bypass the liver, as the liver is unable to uptake L-Cit from the portal circulation (Windmueller and Spaeth, 1981). This bypassed L-Cit is converted to L-Arg in the kidneys and is released into the blood to make it available for the whole body. Birds lack carbamyl phosphate synthetase, one of the enzymes of the urea cycle necessary for the synthesis of L-Cit from L-Orn (Tamir and Ratner, 1963). Hence, they cannot synthesize L-Cit or L-Arg from L-Orn, although they can synthesize L-Orn from L-Arg (Suenaga *et al*., 2008). Fig. 1 shows the L-Cit metabolic pathways in chickens.

Nitric oxide (NO), produced during the conversion of L-

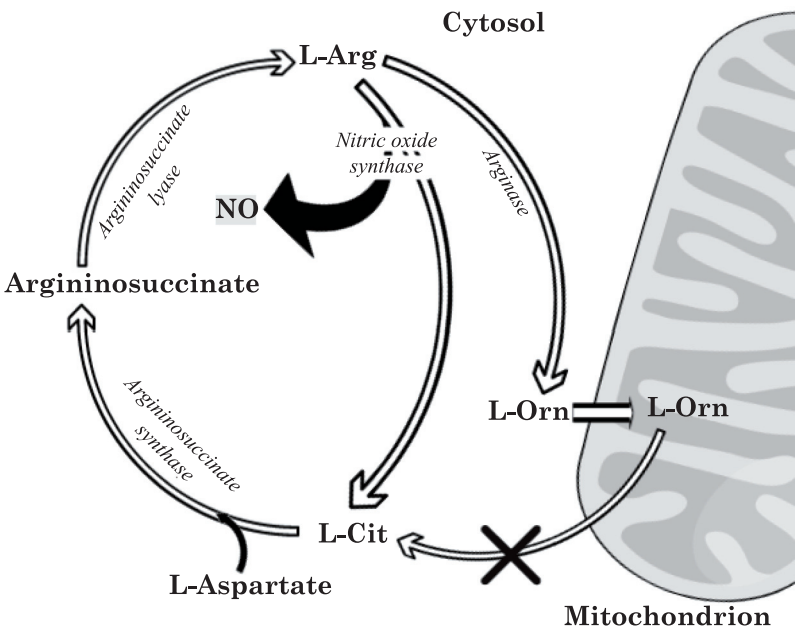

Fig. 1. **Diagram showing the L-Cit metabolic pathway.** Enzymes for all steps of the biochemical pathway are shown. Notably, chickens lack carbamoyl phosphate synthetase, which is necessary for the synthesis of L-Cit from L-Orn (Tamir and Ratner, 1963). Therefore, chickens cannot synthesize L-Cit from L-Orn (indicated with a cross). L-Arg, L-arginine; L-Cit, L-citrulline; NO, nitric oxide. This image was reprinted from Journal of Thermal Biology, 69: 163−170 with permission from Elsevier as the authors' right.

Arg to L-Cit by the enzyme NO synthase (Palmer *et al*., 1987), may act as a hypothermic agent in chicks, and thermoregulation has been proposed as one of the main physiological functions of NO (Szabo, 1996). However, we recently found that NO may not be the main factor in L-Cit-dependent hypothermia and thermotolerance (Chowdhury *et al*., 2017). Plasma glucose has been found to be lower in L-Cit-treated chicks at both 30 and 90 min. Somehow, L-Cit causes an abrupt reduction in plasma glucose, and this may be connected in some way with hypothermia (Chowdhury *et al*., 2017). Hypothermia reportedly is related to hypoglycemia in mammals and amphibians. In mammals, an acute reduction in circulating glucose normally causes the body temperature to decrease (Mayer-Gross and Berliner, 1942; Freinkel *et al*., 1972; Buchanan *et al*., 1991). Similarly, hypoglycemia causes hypothermia and is considered to be an adaptive response in rats (Buchanan *et al*., 1991). Doerfler *et al*. (1998) reported that hypoglycemia occurred in turkeys when hypothermia was detected. It is worth mentioning here that the blood glucose level may be connected in some way with the high body temperature in birds. For example, the blood glucose level in domestic canary (*Serinus canaria domestica*), whose body temperature is 42℃, is ~236 mg/100 m*l*. The common swift (*Apus apus*), a medium-sized bird, has a blood glucose level of ~305 mg/100 m*l* and a body temperature of 44℃. The house sparrow (*Passer domesticus*) has 288 mg/100 m*l* blood glucose and a body temperature of 41.4℃, which is close to that of chickens, whose body temperature is 41.5℃ and blood glucose ~260 mg/100 m*l* (Flindt, 2002). In comparison, the blood glucose level in humans is ~100 mg/100 m*l* and body temperature 37℃. Further experiments are needed to clarify this potential link. We can conclude that NO production may not contribute significantly to L-Cit-dependent hypothermia; instead, hypoglycemia appears to be one of the factors that play a role in this process. Because oral administration of L-Cit affords thermotolerance in chicks, this amino acid might be a novel nutritional candidate for enabling poultry to cope with heat stress.

Leu is significantly reduced in the embryonic brain and liver as a result of TM. Supplementation of L-Leu through *in ovo* feeding led to hypothermia in both male and female chicks at hatching; however, females could better reduce their body temperature at hatching than males (Han *et al*., 2017). Metabolic activity was significantly ($P<0.05$) increased following *in ovo* administration of L-Leu during embryogenesis. Lipid metabolism in embryos and in male but not female chicks significantly ($P<0.05$) increased as a result of *in ovo* feeding of L-Leu. The enhanced lipid metabolic rate might have been the result of increased mitochondrial activity, as Liang *et al*. (2014) reported that L-Leu and its metabolites [α-ketoisocaproate and β-hydroxy-β-methylbutyrate] are able to stimulate mitochondrial biogenesis and oxidative activities. Levels of plasma tryacylglycerol (TG), non-esterified fatty acids (NEFA), and ketone bodies were higher in L-Leu-treated male chicks under heat stress than in heat-exposed control chicks. Broiler chicks would benefit from L-Leu *in ovo* feeding because fat produces less heat, and the beneficial effects of fats in hot-weather feeding programs are well documented (Daghir, 2008). Han *et al*. (2018) have suggested that L-Leu-dependent prenatal imprinted lipid metabolic memory might have a gender-specific metabolic response to active lipid metabolic functions. Methylation or acetylation on the 'Z' chromosome as a result of *in ovo* feeding of L-Leu might have a stronger influence in males (ZZ) than in females (ZW), explaining the differences in lipid metabolism that afford gender-specific thermotolerance. Further research is needed to clarify this matter.

The high level of plasma ketone bodies found in chicks fed L-Leu *in ovo* indicates that the liver generates and releases more ketone bodies into the bloodstream (Han *et al*., 2018). Consequently, there might be increased demand for acetyl-CoA in the liver, which might in turn stimulate the β-oxidation of fatty acids to produce more acetyl-CoA (Fig. 2). Unlike fatty acids, ketone bodies can cross the blood-brain barrier to provide energy to the brain when glucose is limited (Botham and Mayes, 2015). Fatty acid oxidation is highly exergonic in comparison with glucose oxidation, yielding numerous ATPs (Voet and Voet, 1995). The high levels of plasma NEFA and TG might support the lipid metabolism and ketogenesis that take place in the liver, similar to the findings during embryogenesis. Yahav (2015) suggested that reduced energy investment in high meat-producing broilers could be the reason for hyperthermia under heat stress; thus, the greater availability of energy produced by lipid metabolism in male chicks injected with L-Leu might be beneficial by affording thermotolerance under heat stress. In summary, *in ovo* feeding of L-Leu stimulates O_2 consumption, HP, and lipid metabolism during embryogenesis, possibly causing a prenatal sex-specific metabolic imprinting to activate lipid metabolism in male, but not female broiler chicks under heat stress. Future study is needed to reveal the molecular mechanisms by which sex-dependent L-Leu activity confers thermotolerance.

Neuropeptide Expression in Heat-Exposed Chicks

Heat stress causes profound alterations at the cellular level (Morera *et al*., 2012). These alterations include changes in gene expression and biochemical adaptation responses, and are characterized by metabolic reprogramming of cells (Lindquist, 1986). Brobeck (1960) reported that hypothalamic neurons are able to perceive body temperature increases and as a result, influence the cells that are responsible for controlling food intake. Neuropeptides are important in the regulation of food intake in chicks (Cline and Furuse, 2012). For example, central injection of ghrelin has been found to inhibit food intake in birds (Furuse *et al*., 2001; Saito *et al*., 2002). A significant increase in brain ghrelin mRNA expression has been observed in layer chickens chronically exposed to heat (31±1.5℃, 7 days) (Song *et al*., 2012). Thus, heat stress might upregulate anorexigenic peptide expression, which in turn suppresses food intake. In an attempt to verify this, first we examined the mRNA expres-

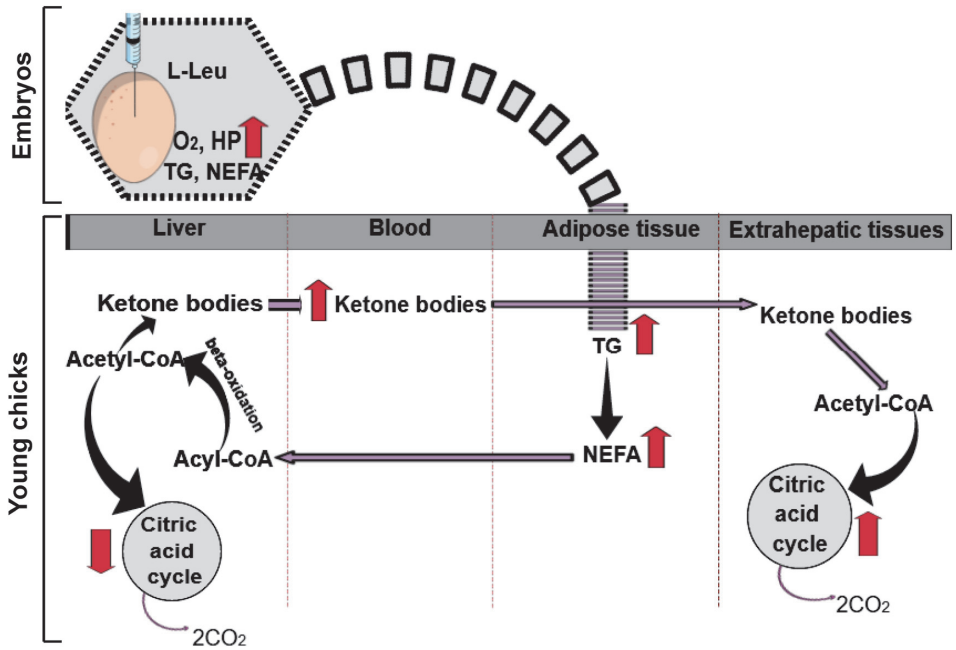

Fig. 2. **Schematic overview of a possible imprinting in the lipid metabolism as a result of *in ovo* feeding of L-Leu between the embryonic and young stages in broiler chicks.** The broken curved line shows a possible imprinting of lipid metabolic memory from the embryo to the young chick. Arrows indicate the progression (→), increase (↑), or decline (↓) in metabolites and metabolic processes. L-Leu, L-leucine; TG, tryacylglycerol; NEFA, non-esterified fatty acid; HP, heat production; O_2, oxygen; CO_2, carbon dioxide.

sion of a number of anorexigenic neuropeptide genes, including pro-opiomelanocortin (POMC)-derived melanocortin peptides (Kawakami *et al*., 2000; Honda *et al*., 2012), cholecystokinin (CCK; Furuse *et al*., 2000), ghrelin (Furuse *et al*., 2001), and corticotropin-releasing hormone (CRH; Furuse *et al*., 1997b), in the acute heat-exposed chick brain. Second, we examined the orexigenic neuropeptides NPY (Kuenzel *et al*., 1987; Kuenzel and McMurtry, 1988; Furuse *et al*., 1997a) and gonadotropin-inhibitory hormone (GnIH, Tachibana *et al*., 2005; McConn *et al*., 2014, 2016). The abundant amounts of POMC, CCK, ghrelin, and CRH precursor mRNAs were not affected by heat stress (40℃, 5 h). However, diencephalic NPY and GnIH mRNA expression was increased by heat stress in chicks (Chowdhury *et al*., 2012a; Ito *et al*., 2015). Surprisingly, when food intake was also suppressed, there was no significant alteration in the plentiful supply of anorexigenic neuropeptides in the acute heat-exposed chick brain (Ito *et al*., 2015). Interestingly, however, hypothalamic NPY precursor mRNA expression was upregulated when food intake was suppressed under heat stress. Similarly, we observed elevated expression of hypothalamic GnIH precursor mRNA in heat-exposed (35±1℃, 24 or 48 h) chicks (Chowdhury *et al*., 2012a). We theorized that the increased GnIH expression could be a consequence of food-intake suppression during heat stress (Chowdhury *et al*., 2012a), because Boswell *et al*. (1999) demonstrated that food restriction in growing broilers was associated with an increase in the steady-state abundance of NPY mRNA in the hypothalamus. However, alternative functions of NPY and GnIH were considered, as reduced food intake is a physiological adaptive mechanism of chicks experiencing heat stress. Hence, it was subsequently found that in addition to their role in food-intake regulation, NPY and GnIH play major roles in stress regulation.

Anti-Stress and Hypothermic Functions of NPY in Heat-Exposed Chicks

It has been suggested that in mammals, NPY is an anti-stress agent (Heilig, 2004; Kormos and Gaszner, 2013; Reichmann and Holzer, 2016; Sabban *et al*., 2016) with a neuroprotective function (Malva *et al*., 2012), and that NPY can affect the concentration of monoamines in the brain. For example, central administration of NPY increased extracellular dopamine (DA) and its metabolites, norepinephrine (NE), 3, 4-dihydrooxyphenylacetic acid, and homovanillic acid (HVA), but did not affect serotonin (5-HT) or 5-hydroxyindoleacetic acid concentrations in rats (Matos *et al*., 1996). However, few studies have evaluated monoamine changes, or the regulatory influence of NPY on these changes, during heat stress. Recently, we reported that NPY does not in-

fluence rectal temperature in fed chicks. However, rectal temperature did change after i.c.v. injection of NPY in fasted chicks (Bahry *et al*., 2017). NPY caused a significant ($P<$ 0.001) increase in food intake under both CT and heat stress. Central NPY resulted in a significant decline in 5-HT ($P<$ 0.05) concentrations; however, 3-methoxy-4-hydroxyphenylglycol and HVA were significantly ($P<0.05$) increased in fed chicks. Interestingly, a high dose of NPY significantly ($P<0.05$) increased diencephalic DA concentrations in fasted chicks. Plasma NE and epinephrine (E) increased in chicks treated with NPY only under heat stress. NPY has been shown to reduce body temperature in neonatal chicks (Tachibana *et al*., 2006) and mammals (Szekely *et al*., 2004). NPY does not reduce rectal temperature in fed chicks but it does significantly reduce rectal temperature in fasted chicks under CT, but not under heat stress. However, food intake may have caused increased metabolic heat production, and this in turn might have masked an NPY-dependent reduction in body temperature because the metabolic rate affects body temperature (Webb, 1997). Furthermore, the hypothermic function of NPY under heat stress might be masked by norepinephrinergic activity because the sympathetic nervous system is stimulated under stress (Cockrem, 2007; Wang *et al*., 2013), and this could lead to an increase in body temperature (Szekely *et al*., 2004). Bahry *et al*. (2017) showed that NE and E concentrations were increased, while corticosterone levels were decreased in the plasma of fasted chicks under heat stress. Zhang *et al*. (2003) reported that central administration of NE led to a reduction in plasma corticosterone. Thus, it is possible that NPY and NPY-dependent increases in NE reduced the plasma corticosterone levels under heat stress (Bahry *et al*., 2017). This would be consistent with an anti-stress effect of NPY in chicks.

Eltahan *et al*. (2017) reported that NPY causes hypothermia under CT as well as under heat stress in fasted chicks (Fig. 3A). I.c.v. injection of NPY significantly ($P<0.05$) decreased the rectal temperature in fasted chicks under both CT and HT. The mRNA expression of NPY-sub receptors (NPYSRs)-Y5, -Y6, and -Y7 significantly ($P<0.05$) increased in the brain following NPY injection under both CT and HT. The NPY-induced decline in rectal temperature was significantly ($P<0.05$) suppressed by coinjection of CGP 71683 (Fig. 3B), an NPYSR-Y5 antagonist. Plasma glucose was significantly ($P<0.05$) reduced by NPY i.c.v. injection. Central NPY also reduced plasma corticosterone levels under heat stress.

NPYSRs carry out specific functions related to the stress response. For example, NPYSR-Y1 brings about an anxiolytic effect, whereas NPYSR-Y2 mediates anxiogenic functions (Reichmann and Holzer, 2016). The expression of

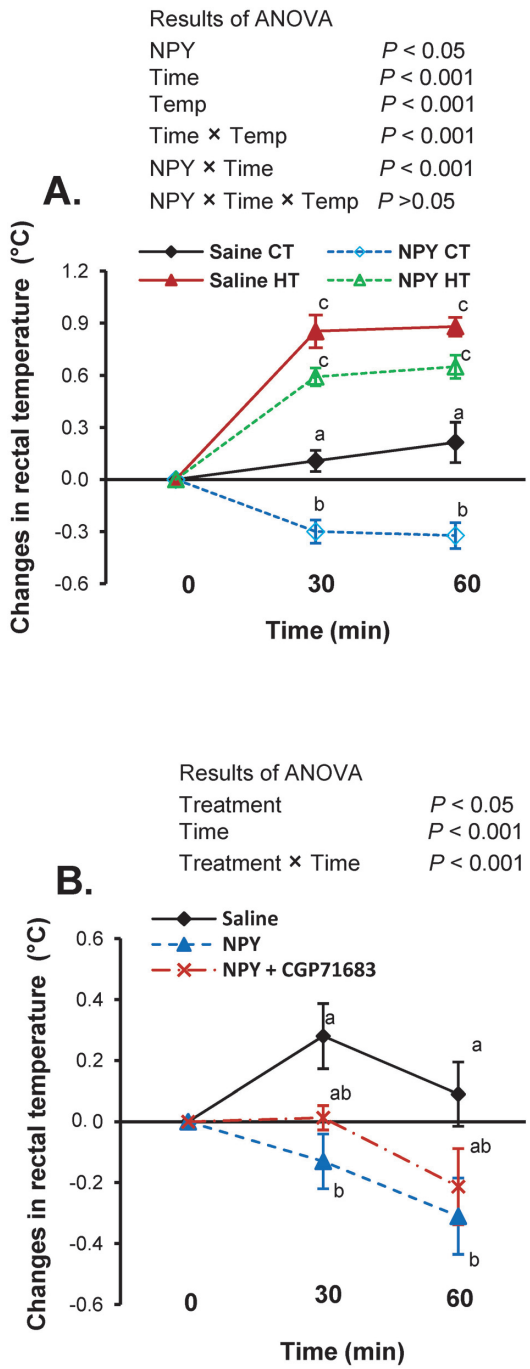

Fig. 3. **Effects of NPY and CGP71683 on rectal temperature in chicks.** (A) Rectal temperature in fasted chicks following central injection of NPY (375 pmol) or saline under control thermoneutral temperature (CT: 30±1℃) or high ambient temperature (HT: 35±1℃) for 1 h. (B) Rectal temperature in chicks following central injection of NPY (375 pmol), saline or NPY (375 pmol) plus CGP 71683 (3750 pmol) under CT for 1 h. Different letters indicate significant differences at $P<0.05$ between groups. Values are the mean±SEM of 12−15 chicks in A and 8−10 chicks in B. This image was reprinted from Physiological Reports, 5 (23): e13511. 69 with permission from Wiley Periodicals, Inc. on behalf of the Physiological Society and the American Physiological Society Wiley as the authors' right.

NPYSRs-Y5, -Y6, and -Y7, but not -Y1, -Y2, and -Y4, is stimulated by NPY, which indicates that the hypothermic functions of NPY are mediated by all or any of these receptors. The finding by Eltahan *et al*. (2017) that coinjection of CGP71683 with NPY slightly lessens NPY-induced hypothermia suggests that NPYSR-Y5 is partially, but not entirely, involved in hypothermia. The functions of NPYSRs-Y6 and -Y7 in chickens remain unknown. There are currently no antagonists are available for NPYSRs-Y6 and -Y7. Previous reports (Boswell *et al*., 1998; He *et al*., 2016; Gao *et al*., 2017) have indicated that the mRNA expression patterns of NPY and its receptors closely resemble those of protein expression; however, further analysis of such correlation will be needed in future.

Plasma glucose is lower in NPY-treated chicks, and this might be a result of the anabolic function of NPY. Hypoglycemia is a well-known phenomenon that occurs concomitantly with hypothermia in mammals (Buchanan *et al*., 1991) and amphibians (Branco, 1997; Rocha and Branco, 1998), as discussed above. Thaxton *et al*. (1974) reported that oral administration of glucose increased the body temperature of chicks that were exposed to a cold environment and the authors proposed the involvement of carbohydrate metabolism in the physiological regulation of body temperature. Kuenzel and McMurtry (1988) reported that central injection of NPY increased plasma insulin. Therefore, it is possible that central NPY injection causes an increase in peripheral insulin and reductions in blood glucose and body heat.

In summary, central injection of NPY has an anti-stress effect and causes hypothermia in fasted chicks. I.c.v. injection of NPY affords thermotolerance, along with increased mRNA expression of HSP-70 and -90 and of NPYSRs (-Y5, -Y6, and -Y7), in heat-exposed chicks. Results obtained with the NPYSR-Y5 antagonist CGP71683 suggest that NPYSR-Y5 may partially mediate NPY-induced hypothermia. Decreased levels of plasma glucose, corticosterone, and E further suggest that central NPY may control thermal stress and body temperature to afford protective thermotolerance.

Conclusions and Future Prospects

Heat stress affects amino acid metabolic activity and neuropeptide expression in chicks. Certain amino acids–namely, L-Cit and L-Leu–appear to be useful as biomarkers of heat stress because their concentrations are affected by heat stress, and administration of these amino acids has been found to afford thermotolerance in chicks. NPY has been considered as a food-intake regulator; however, our studies have revealed a novel role for NPY in reducing stress and body temperature in chicks under heat stress. As heat stress is a serious concern for the present and future poultry industry, our and other studies (Furukawa *et al*., 2015; Nanto *et al*., 2015; El-Deep *et al*., 2016; Mahmoudi *et al*., 2018) will contribute to overcome this serious, global challenge.

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