

Effect of Short-Term Thermal Conditioning on Physiological and Behavioral Responses to Subsequent Acute Heat Exposure in Chicks

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It is known that thermal conditioning at an early age results in improved heat tolerance, and reduces mortality when re-exposed to heat in later life in chickens. However, the mechanism of thermal conditioning is not fully understood. The objective of this study was to investigate the effect of early thermal conditioning on physiological and behavioral responses in acute heat-exposed chicks. Six-day-old chicks (White Plymouth Rock) were exposed to high temperature at 40°C for 3 h while control chicks were kept at 30°C. Four days after treatment, both groups were challenged to high temperature at 40°C for 15 min. We found that the initiation times for behavioral responses (panting and wing-droop posture) in experienced chicks were later than those in control. At the end of heat-exposure treatment, the rectal temperature in experienced chicks was lower than that in control while there was no difference in respiration rate between the groups. Compared with control, experienced chicks had a lower level of plasma corticosterone. Gene expression levels of brain-derived neurotrophic factor, thyrotropin-releasing hormone, interleukin-6 and lipopolysaccharide-induced tumor necrosis factor were significantly lower in the brain of experienced chicks than in the control chicks. These results suggest that thermal conditioning may change response to subsequent heat exposure by altering the central thermoregulation system, resulting in an alleviation of heat stress.

Key words: chick, thermal challenge, thermal conditioning, thermotolerance

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Introduction

Summer heat with the progress of the global warming is one of the major concerns in the modern poultry industry because chickens generally have a low tolerance to heat. Various countermeasures were reported to reduce the negative effect of heat stress on chickens (Yahav *et al.*, 1995; Sahin *et al.*, 2003), but the problem has not yet been solved. One strategy might be the screening of chicken breeds that have a high tolerance to heat (Gowe and Fairfull, 2008). Another strategy is a treatment that brings out the potential ability of chickens. Under high environmental temperatures, chickens are known to acclimatize to heat by modulating their behavioral and/or physiological responses for thermoregulation (Yahav, 2000). Taking advantage of the immaturity of thermoregulation in young chicks (Dunnington and

Siegel, 1984; Modrey and Nichelmann, 1992), thermal conditioning is a process in which chicks are exposed to high environmental temperature for 24 h during the first week of life (Arjona *et al.*, 1988; Yahav *et al.*, 1997; Yahav, 2000). The treatment results in significantly increased feed intake and body weight gain, and decreased mortality with heat tolerance (Yahav and Hurwitz, 1996; Yahav *et al.*, 1997; Yahav and Plavnik, 1999). However, such treatments are difficult to use in practice, and impose a heavy stress load on chicks. To reduce the load in thermal conditioning, it is necessary to consider the quality and quantity of the load, such as duration and temperature. In addition, it will help the breeding of chickens to investigate the details of treatment using various breeds and lines of chicken.

The objective of this study, therefore, was to investigate the effect of short, early thermal conditioning on the physiological and behavioral responses in acute heat-exposed chicks (White Plymouth Rock).

Materials and Methods

All experiments were conducted in accordance with the

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regulations of the Animal Experiment Committee of Hiroshima University.

Animals

Fertilized White Plymouth Rock eggs were collected from chickens reared at our institute, stored at 15°C for 10 days, and incubated in a Murai MIC-14C incubator (Murai Incubator Co., Nagoya, Japan), the temperature and relative humidity of which were $37.7 \pm 0.2^\circ\text{C}$ and $65 \pm 5\%$, respectively. Newly hatched chicks were housed in wooden cages with a wire-mesh floor ($18 \times 25 \times 20$ cm) at a population density of 3 chicks per cage. The birds were maintained in a room with 24-h lighting and at a temperature of 30°C. They were given free access to a commercial starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan) and water until the end of the experiment.

Experimental Design

Birds (6 days old) were distributed into experimental groups based on their body weight so that the average body weight was as uniform as possible for each treatment (control: 108.7 ± 2.3 g, treatment: 110.8 ± 2.3 g). The age for the thermal conditioning applied in this study was referred to the previous reports (De Basilio *et al.*, 2001, 2003). Thereafter, treatment chicks were exposed to high temperature at 40°C for 3 h while control chicks were kept at 30°C. Body weight and feed intake were measured every day until the chicks were 10 days old. After the last measurement of body weight and feed intake, both groups were challenged to high temperature at 40°C for 15 min without feed and water (Figure 1). The condition of acute heat load applied in this study was based on that shown to be effective in the previous report (Yanagita *et al.*, 2011). Rectal temperature and respiration rate were also measured before and after the heat exposure test. The respiration rate was recorded by measuring the number of respiratory flank movements for the last 15 sec. Throughout the thermal challenge, the initiation times of dissipation behaviors (panting and wing-droop: Etches *et al.*, 2008) in chicks were monitored visually and recorded. At the end of the test, all chicks were bled by cardiac puncture

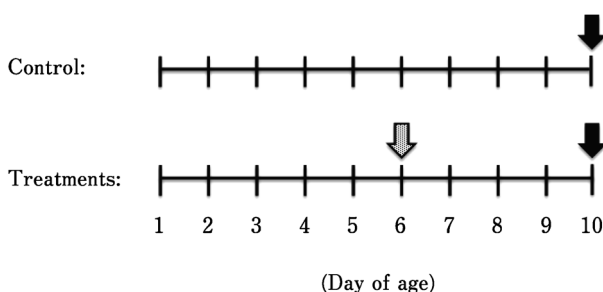


Fig. 1. **Schedule of experimental procedure.** Treatment chicks were exposed to high temperature at 40°C for 3 h (short-term thermal conditioning) at 6 days old, and both groups were received to high temperature at 40°C for 15 min (subsequent acute heat exposure) at 10 days old.

⇩, short-term thermal conditioning; ↓, subsequent acute heat exposure.

and blood was collected into heparinized tubes and centrifuged for 15 min. Immediately after blood collection, they were decapitated, and their diencephalons including the hypothalamus were also collected. Harvested plasma was stored at -20°C until assayed. Hypothalamic tissue samples were collected and snap frozen in liquid nitrogen and stored at -80°C prior to RNA isolation.

Plasma Levels of Glucose, Free Fatty Acid (FFA) and Corticosterone (CORT)

The plasma concentrations of glucose and FFA were measured using a commercial kit (Glucose C II-Test Wako and NEFA C-Test Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan).

Plasma CORT concentrations were measured by enzyme immunoassay, modifying the method used in a previous study (Isobe *et al.*, 2007). In brief, plasma was extracted with dichloromethane, and the organic phase was decanted to a glass tube. After drying the tubes with the extracted organic phase, borate buffer was added and used for the assay. The reconstituted samples were applied into wells of microtitre plates coated with goat anti-rabbit IgG antibody followed by the addition of anti-corticosterone antibody (COSMO BIO Co., Tokyo, Japan) and HRP-conjugated corticosterone (COSMO BIO Co., Tokyo, Japan). After 2 h, incubation plates were washed and 3,3'-5,5'-tetramethylbenzidine solutions were applied to the substrate. Then, optical density was calculated at the 450 nm wavelength using an Ultramark Microplate Reader (BIO-RAD Laboratories, Tokyo, Japan). All samples were run in the same assay to avoid inter-assay variations. Intra-assay coefficient of variation was 7.3%.

Hypothalamic Gene Expression

RNA was isolated from the dissected tissue using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. To rule out the possibility that PCR products would result from the amplification of genomic DNA contaminating the RNA sample, RNA samples were treated with DNase I using the DNA-free kit (Ambion, Austin, USA). Total RNA (500 ng) was reverse transcribed at 42°C for 15 min in 10 μl of 1 $\times$ Prime Script RT Enzyme Mix I (Takara, Tokyo, Japan). The reaction product was subjected to real-time PCR performed according to the user instructions for the Light Cycler system (Roche Applied Science, IN, USA). In brief, following a denaturation step at 95°C for 10 s, PCR was carried out with a thermal protocol consisting of 95°C for 5 s and 60°C for 20 s in a 20 μl buffer containing 1 $\times$ SYBR Premix EX Taq (Takara, Tokyo, Japan) and 0.2 μM of each primer. Primers used for real-time PCR (ribosomal protein S17 (RPS17), corticotropin-releasing hormone (CRH), arginine vasotocin (AVT), brain-derived neurotrophic factor (BDNF), thyrotropin-releasing hormone (TRH), interleukin-6 (IL-6), lipopolysaccharide-induced tumor necrosis factor (LITAF)) are shown in Table 1. To normalize the data, ΔC_T was calculated for each sample by subtracting C_T of RPS17 from C_T of the gene of interest. For relative quantitation, ΔC_T for the defined control group was subtracted from the ΔC_T of each experimental sample to generate $\Delta\Delta C_T$. The $\Delta\Delta C_T$ was then used to cal-

Table 1. Oligonucleotide primer sequences for real-time PCR

Primer	Forward (5' -3')	Reverse (5' -3')	Accession No.
RPS17	AAGCTGCAGGAGGAGGAGAGG	GGTTGGACAGGCTGCCGAAGT	NM_204217
CRH	CGATTTCTTCCCTCAGCAG	GGAAGTACTCCTCTCCCATGC	NM_001123031
AVT	TGAGGAGGACTACATGCCTTC	ACTGCAGCAGACACCATTG	NM_205185
BDNF	CAGCTTGGCTTACCCAGGTC	GTGTTCAAAAGTGTCGCCCA	NM_001031616
TRH	AGACAGCATCCAGGCAGAAG	AGATGGCAGACTGCTGAAGG	NM_001030383
IL-6	CTCCTCGCCAATCTGAAGTC	CCCTCACGGTCTTCTCCATA	HM367074
LITAF	TGTGTATGTGCAGCAACCCGTAGT	GGCATTGCAATTTGGACAGAAGT	NM_204267

RPS17, ribosomal protein S17; CRH, corticotropin-releasing hormone; AVT, arginine vasotocin; BDNF, brain-derived neurotrophic factor; TRH, thyrotropin-releasing hormone; IL-6, interleukin-6; LITAF, lipopolysaccharide-induced tumor necrosis factor.

culate the approximate fold difference, $2^{-\Delta\Delta CT}$. The results were expressed as the gene of interest mRNA/RPS17 mRNA ratio.

Statistical Analysis

The data were analyzed using the commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998). All data were evaluated by Student-*t* test. Statistical significance was set at $P < 0.05$. Data were expressed as means $\pm$ SEM.

Results

Effect of Early Thermal Conditioning on Feed Intake and Body Weight Gain during Post-treatment

During 6–10 days old, no significant differences between control and treatment groups were detected in cumulative feed intake (control: 104.2 ± 4.5 g, treatment: 97.0 ± 3.4 g) and body weight gain (control: 63.4 ± 4.9 g, treatment: 58.1 ± 4.3 g).

Effect of Early Thermal Conditioning on Body Temperature and Respiration Rate after Thermal Challenge

The effect of early heat exposure on rectal temperature and respiration rate in chicks is shown in Figure 2. Before the acute heat challenge, rectal temperature in control and treated chicks was 41.6 ± 0.1 and $41.7 \pm 0.1^\circ\text{C}$, respectively. The temperatures of both groups were elevated by acute heat stress but that of the experienced chicks was significantly lower than control chicks ($p < 0.05$; control: $43.09 \pm 0.08^\circ\text{C}$, treatment: $42.87 \pm 0.06^\circ\text{C}$). Although respiration rates in both groups also increased, those in treated and control chicks did not differ significantly before and after the 15-min heat stress (0 min; control: 97.5 ± 3.5 , treatment: 92.5 ± 3.3 , 15 min; control: 116.8 ± 3.6 , treatment: 108.2 ± 5.6).

Effect of Early Thermal Conditioning on Behavioral Responses during Thermal Challenge

Table 2 shows the result of the effect of early heat exposure on the initiation times of panting and wing-droop during the 15-min test. The experienced chicks tended to start panting later than control did ($p = 0.054$), and the initiation time of wing-droop in the treatment group was significantly later than in control ($p < 0.05$).

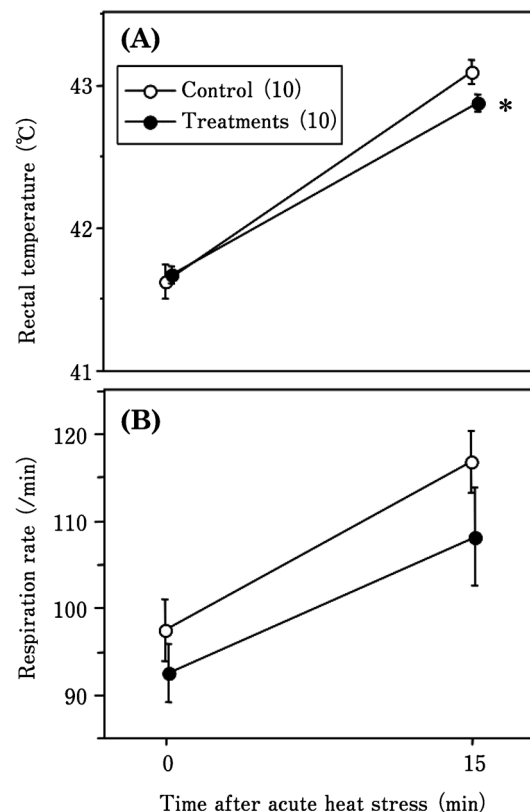


Fig. 2. Rectal temperature and respiration rate before and after the heat exposure test in control and thermal conditioning chicks. Values are means $\pm$ SEM of the number of chicks in parentheses. * $P < 0.05$, compared with control at each point.

Effect of Early Thermal Conditioning on Blood Parameters after Thermal Challenge

The effect of early heat exposure on blood parameters after acute heat stress is shown in Table 3. No significant differences were detected in plasma glucose and FFA (Table 3; $p > 0.1$) between the groups while the level of CORT in treated chicks was significantly lower than in control (Table 3; $p < 0.05$).

Table 2. Effect of early-age thermal conditioning on initiation time of behavioral responses to acute heat exposure in chicks

	Control (10)	Treatment (10)	P-value
Panting (min)	5.4±0.6	7.4±0.8	0.054
Wing-droop (min)	5.4±0.9	8.5±0.9	0.022

Values are means±SEM of the number of chicks in parentheses.

Table 3. Effect of early-age thermal conditioning on plasma glucose, free fatty acid (FFA) and corticosterone (CORT) in acute heat exposed chicks

	Control (10)	Treatment (10)	P-value
Glucose (mg/dl)	328.66±43.32	364.03±66.08	0.660
FFA (mEq/l)	227.23±24.79	245.69±23.41	0.595
CORT (ng/ml)	44.69±6.58	22.81±5.90	0.024

Values are means±SEM of the number of chicks in parentheses.

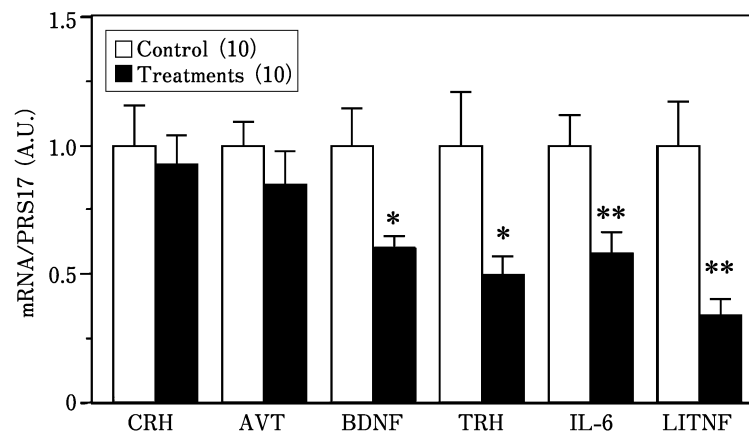


Fig. 3. Relative quantity of hypothalamic gene expressions immediately after the heat exposure test in control and thermal conditioning chicks. Values are means±SEM of the number of chicks in parentheses. * $P < 0.05$, ** $P < 0.01$, compared with control.

Effect of Early Thermal Conditioning on Hypothalamic Gene Expressions after Thermal Challenge

Figure 3 illustrates the effect of early heat exposure on hypothalamic gene expressions after the 15-min heat stress. Early heat exposure did not influence CRH and AVT mRNA levels but the gene expression levels of BDNF and TRH in treated chicks were lower than those in control chicks ($p < 0.05$). Also, the levels of IL-6 and ILTAF mRNA in the experienced chicks were significantly lower than those in the control animals ($p < 0.01$).

Discussion

Experience of early thermal conditioning in chicks is known to result in greater feed intake and higher body weight at marketing age (Yahav and Hurwitz, 1996; Yahav *et al.*,

1997; Yahav and Plavnik, 1999), but we could not detect similar results in the short-term. One reason for the disagreement might be the difference in loading time of thermal conditioning because chicks were conditioned by exposure to heat stress for 24 h in the previous studies. However, the 3-h thermal conditioning in the present study was enough to modulate the responses to thermal exposure later on: lower levels of body temperature, plasma CORT and central gene expression of thermoregulation related peptides by subsequent thermal challenge. From the retardation of heat dissipation behaviors (panting and wing-droop), it is easy to conclude that early thermal conditioning may delay physiological and behavioral responses to subsequent heat load. Zhou *et al.* (1997) suggested that the heat exposure could make chicks cope with high environmental temperatures

without panic because the lying time and the frequency of standing-lying in inexperienced chickens were significantly higher than in experienced chickens at a later age. Because posture to disturb heat loss (lying) and high-frequency posture change raises body temperature, they concluded that the experience might be useful for hyperthermic prevention. In other words, early heat exposed chickens might be only accustomed to high environmental temperatures. However, we found that repeated exposure to thermal conditioning from 1 and 6 d of age failed to attenuate hyperthermia (unpublished data). Thus, the interpretation that early thermal conditioning produced thermotolerance with changed metabolism, but chicks do not become accustomed to high environmental temperatures, appears correct.

It is well known that TRH or CORT plays an important role in thermoregulation, as central or peripheral injection of TRH or CORT increased rectal temperature in chicks (Takahashi *et al.*, 2005). From the present results, attenuation of elevated levels of plasma CORT and TRH gene expression would be a cause of suppressed febrile reaction in early heat exposed chicks. Arjona *et al.* (1990) implied that thermogenesis might be reduced by thermal conditioning due to reduced thyroid activity. Reduction in circulating triiodothyronine (T_3) after early thermal conditioning has been reported (Yahav and Hurwitz, 1996; Yahav and Plavnik, 1999). Moreover, uncoupling protein (UCP) is a molecular determinant for the regulation of resting metabolic rate by thyroid hormone (de Lange *et al.*, 2001), and expression of avUCP mRNA was significantly lower in thermal conditioned chicks than in control chicks. UCP mRNA expression in pectoral muscle and body temperature are quickly adjusted in chicks after early thermal conditioning (Taouis *et al.*, 2002). Although we did not investigate effect of thermal conditioning on avUCP in muscle, these results suggest that reduced T_3 may be part of the mechanism associated with improved thermotolerance by early age heat conditioning.

A recent report revealed that central injection of proinflammatory cytokines, such as IL-6, induced febrile reaction in birds, and these cytokines are known as the central component of avian fever (Marais *et al.*, 2011). From the present results, lower gene expression levels of endogenous pyrogen, IL-6 and LITAF (Turrin and Plate-Salamán, 2000) would also contribute to attenuation of increased rectal temperature in chicks exposed to thermal conditioning. Because there is no report about the relationship between thermal condition and endogenous pyrogens, further research is needed to investigate the effect of thermal conditioning on production of central cytokines in chicks.

The elevated level of BDNF gene expression by acute heat stress seemed to be alleviated by thermal conditioning (Figure 3). Previous studies revealed that central administration of exogenous BDNF reduced energy intake and increased body temperature and expression of UCP1 in brown adipose tissue in mice (Wang *et al.*, 2007a,b). Similar to TRH, IL-6 and LITAF, reduced BDNF may be part of the mechanism associated with improved thermotolerance by early age heat conditioning (Figure 3). Moreover, thermal conditioning is

related to the plasticity of the brain with BDNF (Katz and Meiri, 2006; Kisliouk and Meiri, 2009). Sensory development during critical periods is thought to depend on alterations in neural network organization (Knudsen, 2004). This restructuring is mediated by growth-signal transduction, which is activated by the neurotrophic factor BDNF (Katz and Meiri, 2006) and carried by a series of phosphorylation steps (Mansuy and Shenolikar, 2006). Meiri (2008) demonstrated a correlation between the expression of both the mRNA and protein of 14-3-3 ϵ in the frontal hypothalamus and thermal control establishment. Furthermore, it has been shown that there is a dissociation between the biochemical pathways underlying neuronal plasticity in different brain structures. Although, as mentioned above, the possibility that early heat exposed chicks are accustomed to high environmental temperatures was refuted, it is very interesting that BDNF is related to both memory development (Tokuyama *et al.*, 2000) and metabolic control (Wang *et al.*, 2007a, b), and plays a key role in early thermal conditioning in chicks (Katz and Meiri, 2006; Kisliouk and Meiri, 2009). Elucidation of the molecular mechanism behind the interplay between hippocampal BDNF and hypothalamic neurons for thermoregulation needs further investigation.

Improving the acquisition of thermotolerance in poultry species is possible by exposing them to high ambient temperatures during critical periods (first week of life: Yahav and Hurwitz, 1996; De Basilio *et al.*, 2001; Yahav and McMurtry, 2001). Improved thermotolerance acquisition would be caused not only by the aforementioned brain plasticity but also the development of central thermosensitive neurons. The center for the regulation of body temperature control in both avians and mammals is neuroanatomically located in the preoptic/anterior hypothalamus (Basta *et al.*, 1997; Pierau *et al.*, 1998). This area probably plays a dual function, monitoring local temperature changes and integrating temperature information from the periphery. Until day 5 post-hatching, the hypothalamic neuronal thermosensitivity is characterized by a high cold sensitivity (up to 30%). Between days 5 and 10 post-hatching hypothalamic neuronal cold sensitivity decreases significantly from 30 to 14% while warm sensitivity increases from 5 to 14% (Tzschenke and Basta, 2000).

In conclusion, early age thermal conditioning at 40°C for 3 h attenuates elevated body temperature and gene expressions of thermoregulation-related peptides by subsequent thermal challenge, and retards heat dissipation behaviors in White Plymouth Rock chicks. The fact that thermotolerance develops by thermal conditioning during the first week of life may make it a useful tool for prevention of heat death as a practical technique for broiler breeders, because White Plymouth Rock is often used for broiler breeding. Further work on the breed/line-specific effect of thermal conditioning is necessary for the selection of animals for both welfare and productivity.

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