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Thermal Conditioning Can Improve Thermoregulation of Young Chicks During Exposure to Low Temperatures

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The risk of climate change is increasing year by year and changing environmental temperatures will increasingly have effects on productivity in the poultry industry. Thermal conditioning is a method of improving thermotolerance and productivity in chickens (*Gallus gallus domesticus*) that experience high ambient temperatures. Thermal conditioning involves exposure of chickens to high temperatures at an early age. This conditioning treatment can affect tolerance to other type of stress. However, the effect of thermal conditioning on tolerance of low temperatures has not been investigated. Therefore, in this study we investigated the effect of thermal conditioning in chickens on thermoregulation during exposure to low temperatures. Three day-old female broiler chicks were exposed to high ambient temperatures (40°C for 12 h) as a thermal conditioning treatment. A control group of chicks was kept at 30°C. At 7 days-old, both groups of chicks were exposed to low temperatures (16 ± 0.5°C) for 3 h. Thermal conditioning treatment reduced the decrease in rectal temperature during cold exposure that occurred in control chicks. In addition, hypothalamic mRNA expression of brain derived neurotrophic factor, thyrotropin-releasing hormone and arginine vasotocin genes was higher in thermal conditioning treated chicks than control chicks. The mRNA expression of avian uncoupling protein in the liver was also higher in thermal conditioning chicks. These results suggest that thermal conditioning treatment can improve thermoregulatory mechanisms of chicks under low temperature environments.

Keywords: thermal conditioning, low temperature exposure, tolerance, thermoregulation, poultry

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INTRODUCTION

The external environment, especially environmental temperature, has a great impact on the health and productivity of livestock animals. Currently, global warming is progressing due to the emission of warming gases associated with the use of fossil fuels. The increase in heat stress for livestock caused by global warming has led to a decrease in productivity and an increase in health risk (Lu et al., 2007;

Kilic and Simsek, 2013; Lara and Rostagno, 2013; Zaboli et al., 2018; Vandana et al., 2021). On the other hand, global warming brings not only increasing summer temperatures but also climate change such as heavy precipitation, high temperature extremes (Fischer and Knutti, 2015). Due to this climate change, sudden low temperatures in winter, that is, the invasion of cold waves, is also a problem. For example, the major cold wave that hit East Asia and North America during the 2017-2018 winter season has been attributed to the meandering of the jet stream due to the reduction of sea ice in the Alaskan Sea caused by global warming (Tachibana et al., 2019). Environmental temperatures below the lower limit of the thermoneutral zone have a significant impact on livestock productivity. The effect of low environmental temperatures is generally referred to as cold stress. In poultry, cold stress can cause various effects such as increased feed intake, decreased digestibility, and decreased egg production (Arad and Marder, 1982; Sahin and Sahin, 2001; Blahova et al., 2007). And more, in broiler chicken (*Gallus gallus domesticus*), it is reported that low environment temperature increases the risk of ascites (Shlosberg et al., 1992: Yahav et al., 1997; Ipek and Sahan, 2006). As mentioned above, chickens are constantly at high risk of being stressed by environmental temperatures. Therefore, there is an urgent need to improve their ability to adapt to environmental temperature, that is, their ability to thermoregulate.

Thermal conditioning treatment in which young chicks are exposed to high environmental temperatures is one of the methods for improvement of thermoregulation in chickens under high ambient temperature environment, namely it improves heat tolerance in chickens (Arjona et al., 1988; Yahav and Hurwitz, 1996; Yahav and McMurtry, 2001; Tanizawa et al., 2014; Ouchi et al., 2021). The improvement of thermoregulation by thermal conditioning treatment is due to improvement of physiological and behavioral responses under high temperature environment. It is speculated that the thermal conditioning treatment changes the response to environmental temperature under heat environment by causing a modification of central thermoregulatory mechanisms (Tanizawa et al., 2014; Ouchi et al., 2020). Although the effects of thermal conditioning treatment on thermoregulation at high temperatures have been studied, there are no reports on the effects of the thermal conditioning treatment on thermoregulation at low temperatures.

Adult chickens have a wide temperature range of 13 to 28°C as a thermoneutral zone. However, young chicks are extremely sensitive to environmental temperature, and the thermoneutral zone is around 30°C until 2 weeks of age (Meltzer, 1983). Therefore, young chicks are vulnerable to cold stress. Various adaptive reactions occur in chickens under cold environments. For example, there is shivering to produce heat, or the release of thyroid hormone into the blood and increases the production of heat in peripheral organs.

The aim of this study was to examine the effect of thermal conditioning treatment on thermoregulatory responses during low temperature exposure in chicks. Thus, we exposed chicks to cold temperatures after thermal conditioning treatment. In addition, the body temperature of chicks during cold exposure, the expression of thermoregulatory genes in the hypothalamus and liver, and blood biochemical parameters were investigated in this study.

MATERIALS AND METHODS

The handling of birds was performed in accordance with the regulations of the Animal Experiment Committee of Hiroshima University (authorization No. C19-15) and complied with Law No. 105 and Notification No. 6 of the Japanese government.

Animals

Newly hatched female chicks (Chunky) were obtained from a local farm (Fukuda Hatchery, Okayama, Japan). The chicks were maintained in a room at 30 ± 0.2°C under continuous light. Birds were housed in polypropylene boxes (36 × 40 × 30 cm) with sawdust litter at a density of 6 chicks per box during the experiment period. A commercial starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan) and water were supplied *ad libitum* until the end of experiment.

Experimental Design

The experiment design of this study is shown in **Figure 1**. A total of 32 chicks were used in Experiment 1 and 2. At 3 days of age, the chicks were divided into two groups (n = 8 per group), with the mean body weight of each group as similar as possible (92 ± 5.3g and 93 ± 5.1g). Chicks in a thermal conditioning treatment group were exposed to high temperature environment (40 ± 0.5°C) as described elsewhere (Ouchi et al., 2021). A commercially available heat chamber (Type P-008B, Showa Furanki, Saitama, Japan), which can maintain the chamber at any desired temperature by means of a thermostat was used for thermal conditioning treatment. The chicks of thermal conditioning treatment group were promptly placed in the heat chamber pre-set at 40°C. They were exposed to treatment for 12 hours after entering the chamber. During thermal conditioning treatment they had free access to feed and water. Chicks in the thermal conditioning treatment group experienced one thermal conditioning treatment at 3 days of age and were reared in thermoneutral zone as in the control group until low temperature exposure. The chicks of control group were kept at thermoneutral zone until low temperature exposure.

Experiment 1: Effect of Thermal Conditioning on Rectal Temperature During Cold Exposure

At 7 days of age, both control and thermal conditioning group of chicks were exposed to low ambient temperature (16 ± 0.5°C) for 180 min. The 7-day-old period was selected because a previous report indicated that the thermal conditioning treatment suppressed the increase in rectal temperature due to heat exposure at the 7-day-old period (Ouchi et al., 2021). Low temperature exposure was performed using a climate control room, which was pre-regulated to 16°C. The chicks of both groups were promptly moved from the thermos neutral zone to

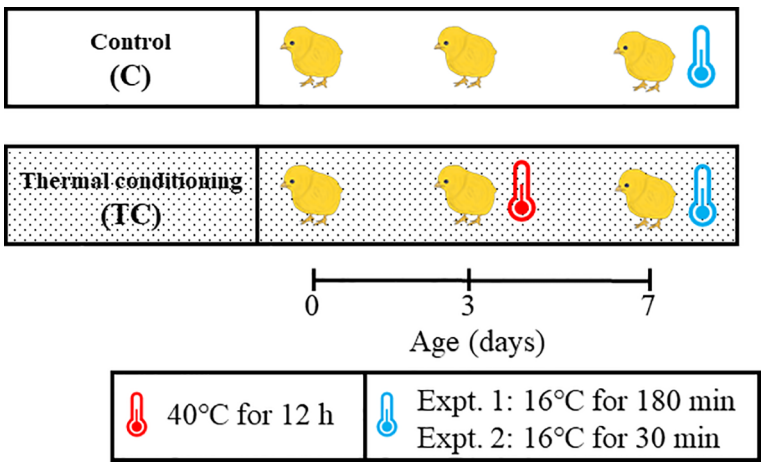

FIGURE 1 | Experiment design for this study. A thermal conditioning group (TC) was exposed to 40°C for 12 hours at 3 days of age. At 7 days of age, both TC and control groups (C) were exposed to a low temperature environment (16°C) for 180 minutes in experiment and for 30 minutes in experiment 2. Control chicks were kept in their thermoneutral zone until the low temperature exposure.

a prepared climate control room for low temperature exposure. Rectal temperature was measured every 30 min during low temperature exposure. The number of chicks was 8 per group.

Experiment 2: Effect of Thermal Conditioning on Plasma Biochemical Parameter, and Diencephalic, and Hepatic Gene Expression During Cold Exposure

At 7 days of age, chicks in control and thermal conditioning groups were exposed to a cold environment (16 ± 0.5°C) for 30 min. Chicks were handled as in Experiment 1. The number of chicks was 8 per group. Immediately after cold exposure, chicks were anesthetized by isoflurane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and decapitated. Blood, diencephalic tissues and hepatic tissues were harvested. From the time of anesthesia to the completion of blood and all tissue collection and freezing took less than 3 minutes. The blood was centrifuged, and then plasma was stored at -20°C until analysis. The diencephalic samples were collected by referring to the atlas (Kuenzel and Masson, 1988). The diencephalic and hepatic tissue were stored at -80°C until RNA isolation.

Measurements of Plasma Biochemical Parameters

The concentration of plasma glucose, free fatty acids (FFA) and corticosterone (CORT) were measured. Plasma glucose and FFA concentrations were measured using a commercially available assay kit and according to the instructions (Glucose CII-Test Wako and NEFA C-Test Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan). Plasma CORT levels were measured by enzyme immunoassay, as used in previous studies (Tanizawa et al., 2014; Ouchi et al., 2021). For both assays, optical density was measured using an Ultramark Microplate Reader (BIO-RAD Laboratories, Tokyo, Japan).

Measurements of Diencephalic and Hepatic Gene Expression

Trizol reagent (Invitrogen, CA, USA) was used for isolation of total RNA from the collected tissues. The isolation analysis was performed according to the manufacturer's instructions. The total RNA concentration was measured by the spectrophotometer (NanoDrop-ONE, Thermo Scientific, Inc) at 260 nm. Thereafter, the RNA samples were treated with DNase I using a DNA-free kit (Ambion, Austin, USA). cDNAs were synthesized from DNase treated total RNA samples using a PrimeScript RT reagent kit (TaKaRa Bio Inc., Shiga, Japan). Gene expression was measured using a light cycler system (Light cycler Nano: Roche Applied Science, IN, USA). Target genes in diencephalic tissue were brain derived neurotrophic factor (BDNF), thyrotropin-releasing hormone (TRH), neuropeptide Y (NPY), corticotropin-releasing hormone (CRH), arginine vasotocin (AVT), pro-opiomelanocortin (POMC). Target genes in hepatic tissue were avian uncoupling protein (av-UCP), carnitine palmitoyltransferase 1 (CPT1), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC). The sequences of primers are shown in **Table 1**. The cycling conditions of PCR was: initialization at 95°C for 5 minutes, amplification of 45 cycles with 95°C for 10 seconds and 60°C for 20 seconds. The total volume of PCR was 20 µl, containing 2 µl of cDNA, 1.5 µl of each 0.2 µM primers, 10 µl of 2 × FastStart Essential DNA Green Master (Roche Life Science, Basel, Switzerland) and 5 µl of PCR grade water. Ribosomal protein S17 (RPS17) was used for geometrical mean of internal control after confirming that there was no significant difference in ct values between the control and thermal conditioning groups, and $2^{-\triangle\triangle Ct}$ methods were used for normalization of data. The ct values of RPS 17 in diencephalon are control= 20.41 ± 0.351 and TC= 20.24 ± 0.378. And those in

TABLE 1 | Primer sequences for real time PCR.

Gene name	Forward (5' → 3')	Reverse (5' → 3')	Accession no.	Product Size (bp)
RPS17	AAGCTGCAGGAGGAGGAGAGG	GGTTGGACAGGCTGCCGAAGT	NM_204217	136
BDNF	CAGCTTGGCTTACCCAGGTC	GTGTTCAAAAGTGTCCGCCA	NM_001031616	106
TRH	AGACAGCATCCAGGCAGAAG	AGATGGCAGACTGCTGAAGG	NM_001030383	184
NPY	GGCACTACATCAACCTCATC	CTGTGCTTTCCCTCAACAA	NM_205473	93
CRH	CGATTTCTTCCCTCAGCAG	GGAAGTACTCCTCTCCCATGC	NM_001123031	80
AVT	TGAGGAGGACTACATGCCTTC	ACTGCAGCAGACACCATTG	NM_205185	91
POMC	AACAGCAAGTGCCAGGACC	ATCACGTACTTGCGGATGCT	NM_001031098	146
UCP	ACCAACACGGTGGAGTACC	TGGAGGCGAAGCTCATC	NM_204107	124
CPT-1	TCAGACACCACAGCAACACA	ATCAGCCACAGGTCCAAATC	AY675193	82
FAS	AGTGATGGGATTGCTGCC	CATAAACCACAGGCACCG	J04485	122
ACC	GCCATCTCTCTCTTTGGTCG	CATGTGCTCAAATACCACCG	J03541	105

RPS17, ribosomal protein S17; BDNF, brain-derived neurotrophic factor; TRH, thyrotropin-releasing hormone; NPY, neuropeptide Y; CRH; corticotropin-releasing hormone; AVT, arginine vasotocin; POMC, proopiomelanocortin; UCP, uncoupling protein; CPT-1, carnitine palmitoytlansferase-1; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase.

liver are control= 22.31 ± 0.441 and TC= 22.84 ± 0.521. These date are presented as mean ± SEM. Since both RPS17 ct values showed a normal distribution, a t-test was performed (in diencephalon: P= 0.662, in liver: P= 0.475).

Statistical Analysis

StatView (Version 5, SAS Institute, Cary, USA, 1998) was used for stastical analysis. Rectal temperature data f were evaluated by two-way repeated ANOVA. Other data were evaluated by t-test after confirming that they were normally distributed to determine the effect of thermal conditioning treatment. $P < 0.05$ was used to indicate significant differences. All data were expressed as means ± standard error of the mean (SEM).

RESULTS

Effect of Thermal Conditioning on Rectal Temperature During Cold Exposure

Figure 2 shows the changes in rectal temperature in thermal conditioning treated and control chicks during cold exposure. There was a significant effect of thermal conditioning on rectal temperature (P = 0.012), with rectal temperatues of thermal conditioning treated chicks higher than those of control chicks. In addition, there was a significant (P < 0.001) effect of time. Rectal temperatures of both groups of chicks declined 30 min after cold exposure, then increased from 60 min onwards after cold exposure. The interaction between effect of time and thermal conditioning was not observed (P = 0.855).

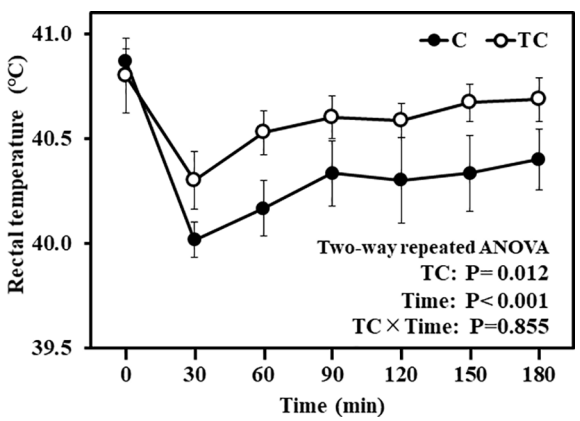

FIGURE 2 | Changes in rectal temperature during and after low temperature exposure in experiment 1. The number of chicks in each group was n = 8. C, control; TC, thermal conditioning.

Effect of Thermal Conditioning on Plasma Parameters After Cold Exposure

The plasma glucose concentration of thermal conditioning treated group tended to be lower than that of control (P = 0.061). There were no significant differences between control and thermal conditioning chicks in plasma FFA (P = 0.428) and corticosterone (P = 0.517) concentrations (**Figure 3**). FFA concentrations in both groups were around 200 nEq/dL, and corticosterone concentrations were around 4 ng/mL.

Effect of Thermal Conditioning on Diencephalic Gene Expression After Cold Exposure

Figure 4 shows the expression of thermoregulation and stress related genes in diencephalic tissue. Expression of BDNF and TRH genes was higher in thermal conditioning treated chicks than in control chicks (**Figures 4A, B**; P = 0.011, P = 0.020). In the gene expression of AVT, the level of thermal conditioning treated chicks was tendency higher than that of control (**Figure 4E**; P = 0.086). However, there was no significant difference between thermal conditioning treated chicks and control chicks on the gene expression of NPY, CRH and POMC (**Figures 4C–E**; P = 0.192, P = 0.314, P = 0.356).

Effect of Thermal Conditioning on Hepatic Gene Expression After Cold Exposure

Figure 5 shows gene expression in liver tissue after cold exposure. The expression level of av-UCP was higher in thermal conditioning treated chicks than control chicks (**Figure 5A**; P = 0.048). There were no differences between control and thermal conditioning treated chicks in the expression levels of CPT-1, FAS and ACC (**Figures 5B–D**; P = 0.2674, P = 0.550 and P = 0.450).

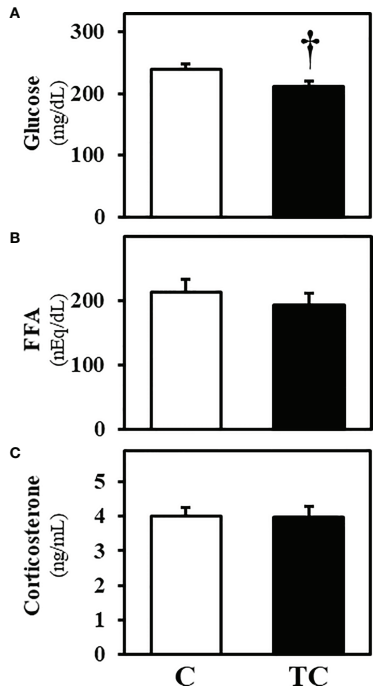

FIGURE 3 | Plasma metabolite concentrations after low temperature exposure in experiment 2 (mean ± SEM). **(A)** shows plasma glucose concentrations, **(B)** shows plasma free fatty acid (FFA) concentrations and **(C)** shows plasma corticosterone concentrations. The number of chicks in each group was n = 8. C, control; TC, thermal conditioning. † means a differential trend between the two groups.

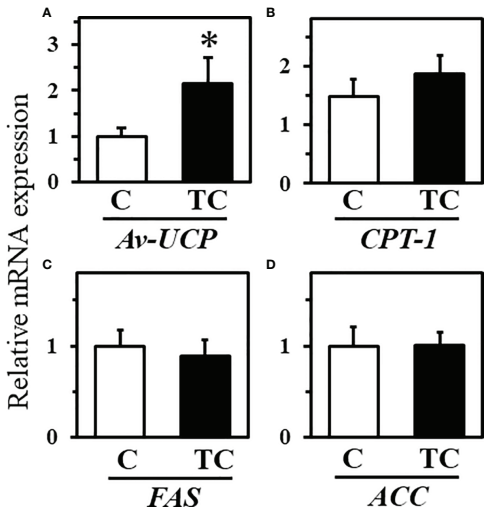

FIGURE 5 | Gene expression levels in liver tissue after low temperature exposure in experiment 2 (mean ± SEM). **(A)** shows gene expression of uncoupling protein (UCP), **(B)** shows gene expression of carnitine palmitoytransferase 1 (CPT-1), **(C)** shows gene expression of fatty acid synthase (FAS), **(D)** shows gene expression of acetyl-CoA carboxylase (ACC). The number of chicks in each group was n = 8. * indicates a significant difference between groups.

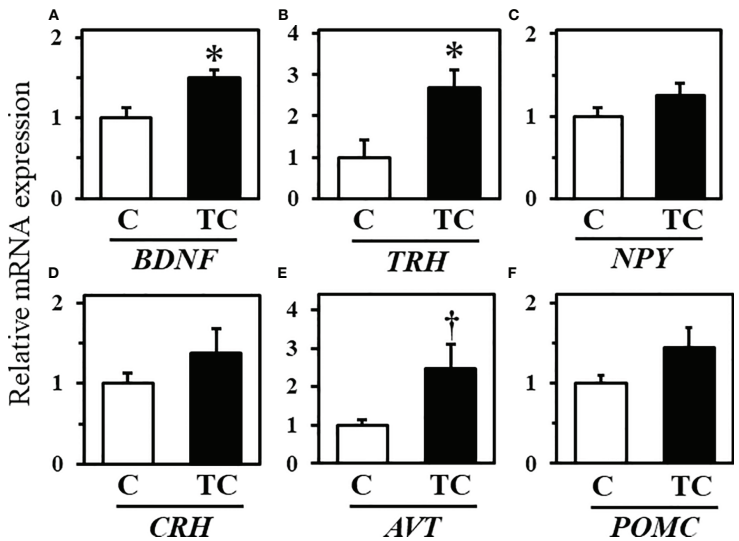

FIGURE 4 | Gene expression levels in hypothalamic tissue after exposure to low temperature in experiment 2 (mean ± SEM). **(A)** shows gene expression of brain-derived neurotrophic factor (BDNF), **(B)** shows gene expression of thyrotropin-releasing hormone (TRH), **(C)** shows gene expression of neuropeptide Y (NPY), **(D)** shows gene expression of corticotropin-releasing hormone (CRH), **(E)** shows gene expression of arginine vasotocin (AVT), and **(F)** shows gene expression of proopiomelanocortin (POMC). The number of chicks in each group was n = 8.* Indicates a significant difference between groups.

DISCUSSION

Thermal conditioning treatment of chicks is known to alter thermoregulatory mechanisms of chickens under high ambient temperatures (Yahav and McMurtry, 2001; Ouchi et al., 2021). This study shows that thermal conditioning at early age affects the decrease in rectal temperature due to exposure to low temperature environment. In addition, it was found that thermal conditioning affects the expression of several important genes related to thermoregulation in the diencephalon and liver after 30 minutes of low temperature exposure.

Thermal conditioning significantly reduced the magnitude of the decline of rectal temparture during and after cold exposure, suggesting a recovery of rectal temperature after the 30 minute cold esposure was similar in control and conditioned chicks. In a previous study, it was reported that thermal conditioning treatment supressed the rise in body temperature during heat exposure at y days of age, especially the rise in rectal temperature 30 min after exposure (Ouchi et al., 2021). Thus, thermal conditioning at an early age may improve responsiveness to both high and low environmental temperatures.

Homeothermic animals can maximize activities necessary for life support by keeping their body temperature constant. They can respond and make adjustments when ambiet temperatures are outside the thermoneutral zone. These adaptive responses include behavioral adaptation and physiological adaptation (Etches et al., 2008; Yahav, 2014). Physiological adaptation is to maintain body temperature homeostasis by actions of autonomic nervous system and the endocrine system. The hypothalamic-pituitary-thyroid (HPT) axis is involved in thermoregulation through the actions of thyroid hormones (Mullur et al., 2014). TRH is secreted from the hypothalamic neurons and acts on thyrotropin-producing cells in the anterior pituitary gland to control the synthesis and secretion of thyroid stimulating hormone (TSH), thus playing an important role as the starting point of the HPT axis. It has been reported that a lot of TRH neurons and perikaryal are present in hypothalamus of chickens (Geris et al., 1999; De Groef et al., 2005). In the present study, TRH gene expression was higher in thermal conditioning treated chicks than in control chicks. Therefore, it is suggested that HPT axis in thermal conditioning treated chicks was more active than that in untreated chicks during low temperature exposure. The HPT axis is one of the mechanisms that controls heat production in peripheral tissues. This is because thyroid hormones released from the thyroid gland by activation of the HPT axis stimulate heat production and metabolism by peripheral tissues such as liver and muscle (Williamson and Davison, 1985; Lam et al., 1986). UCP is one of the proteins activated in peripheral tissues by thyroid hormone. UCP plays a role in thermogenesis by uncoupling mitchondria (Cannon and Nedergaard, 1985; Himms-Hagen and Harper, 2001). Av-UCP is present in chickens and has high homology with mammalian UCP3 (Raimbault et al., 2001; Toyomizu et al., 2002). Moreover, it is reported that cold stimulation increases the expression of av-UCP in chickens (Toyomizu et al., 2002; Dridi et al., 2008). In this study, hepatic av-UCP gene expression after cold exposure in thermal conditioning treated chicks was higher than that of control chicks. Thus, in thermal conditioning treated chicks, the decrease in body temperature caused by exposure to low temperatures may have been activated av-UCP, together with activation of the HPT axis generating heat compared to the control chicks.

There was an indication that AVT gene expression may have been higher in thermal conditioning treated chicks than control chciks. Administration of AVT induced hypothermia, decreased heart rate, respiratory rate and oxygen consumption, but did not induce quivering behavior in birds (John and George, 1992). Additionally, AVT can increase plasma thyroxine (T4) and decrease triiodothyronine (T3) levels (John and George, 1992; Ruuskanen et al., 2021). Thyroid hormones, including T3 and T4, play important roles in avian heat production and thermoregulation (Klandorf et al., 1981; Decuypere et al., 2005), and plasma AVT concentrations have been negatively correlated with body surface temperature (Giloh et al., 2012). Therefore, AVT may have a relationship with thermogenesis in birds. However, it is not clear if gene expression of AVT during cold exposure was increased by thermal conditioning treatment. AVT is associated with heat production as well as water balance in the body. It has been reported that plasma AVT concentrations were increased due to water deficiency in the body (Koike et al., 1977; Arad et al., 1985; Saito and Grossmann, 1998). It is known that water in the body plays an important role in body heat production and body temperature maintenance. Although water is closely related to body temperature, it is not known if thermal conditioning treatment affects body water. This needs to be considered in future experiments.

There was no difference between thermal conditioning treated chicks and control chicks in CRH and POMC gene expression and no difference in plasma corticosterone levels after low temperature exposure. The genes mentioned above are closely associated with stress responses (Herman and Cullinan, 1997; Bonfiglio et al., 2011; Herman et al., 2016) and stress responses involve the hypothalamo-pituitary-adrenal (HPA) axis. Thermal conditioning treatment had no effect on the HPA axis after low temperature exposure. The HPA axis can be activated within several minutes after a stimulus is detected by an animal as a stressor (Cockrem, 2007). While activation of the HPA axis can raise blood glucose levels (Munck et al., 1984; Wingfield et al., 1998; Valeria et al., 2012), blood glucose levels in chicks after cold exposure were not affected by thermal conditioning treatment. However, there could have been effects of the treatment before the first sampling point 30 min after the beginning of the cold exposure, so further investigation will be worthwhile in the future.

It has been reported that BDNF plays a role in feeding regulation and energy metabolism in animals (Nakagawa et al., 2000; Xu et al., 2003). Gene expression of BDNF was higher in thermal conditioning treated chicks than control chicks in the current study. It is probable that the treated group required more energy because it maintained a higher body temperature than the control group. Increased BDNF expression in the diencephalon may have been associated with increased energy metabolism. It was reported that thermal conditioning treatment at early age induced DNA methylation and

demethylation at hypothalamus in chicks (Yossifoff et al., 2008; Kisliouk and Meiri, 2009; Ouchi et al., 2021). DNA methylation is one of the epigenetic modifications that regulate gene expression without changing the gene sequence (Kass et al., 1997; Crider et al., 2012). It has been reported that thermal conditioning treatment alters DNA methylation levels in the BDNF promoter region (Yossifoff et al., 2008; Ouchi et al., 2021). It can be suggested that the increase in BDNF gene expression in thermal conditioning treated group was due to DNA methylation reorganization in the central BDNF gene promoter region due to thermal conditioning treatment.

Prior to this study, there were many reports that thermal conditioning treatment affets physiological and behavioral responses in high temperature environment (Yahav and Hurwitz, 1996; Yahav and McMurtry, 2001; Ouchi et al., 2021). In this study, as a new finding, it was shown that thermal conditioning treatment affects the physiological responses in a low temperature environment. Such a phenomenon is recognized as cross-resistance, that is, the acquisition of tolerance to other stresses by stress treatment (Li and Gong, 2011). Poultry are exposed to many kinds of environmental stressors (Janczak et al., 2007; Zhang et al., 2011; Tsiouris et al., 2018). Although it may be possible to acquire tolerance to various environmental stressors by thermal conditioning treatment, further research is needed to investigate the mechanism for acquiring cross tolerance. And more, it is reported that thermal conditioning treatment at 3 days of age improve thermotorelance in 42-day-old broiler chickens (Yahav and McMurtry, 2001). Therefore, it is likely that the effects of thermal conditioning treatment in ealry life period on physiological responses to the low temperature environment will be confirmed in older chickens that had a long period of time since the thermal conditioning treatment. However, futher pursuit is needed because the thermoregulation of chickens changes as they grow.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiment Committee of Hiroshima University (authorization No. C19-15).

AUTHOR CONTRIBUTIONS

YO collected the data used in this study, analyzed the data, and drafted the manuscript. TB designed the study and analyzed the partial data. JC and VC contributed to the development and writing. YO and TB interpreted the data, and reviewed and improved the manuscript. All authors contributed to the article and approved the submitted version.

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