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Effects of Cyclic High Ambient Temperature on Muscle Imidazole Dipeptide Content in Broiler Chickens

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Imidazole dipeptides possess important bioregulatory properties in animals. This study aimed to evaluate the effect of high ambient temperature on muscle imidazole dipeptides (carnosine, anserine, and balenine) in broiler chickens. Sixteen 14-day-old male broiler chickens were divided into two groups, which were reared under thermoneutral (25 ± 1 °C) or cyclic high ambient temperature (35 ± 1 °C for 8 h/day) for 4 weeks. Chickens exposed to cyclic high ambient temperatures displayed lower skeletal muscle anserine and carnosine content than control chickens. Balenine could not be detected in the pectoral muscle of either group. The pectoral muscles of broiler chickens kept under cyclic high-temperature exhibited significantly lower mRNA expression of *carnosine synthase 1*, which synthesizes carnosine and anserine; but a significantly higher mRNA expression of *carnosinase 2*, which degrades carnosine and anserine. Our results suggest that heat exposure decreases pectoral imidazole dipeptide content in broiler chickens. This may be attributed to a lower expression of imidazole dipeptide-synthesizing genes, but higher levels of genes involved in their degradation.

Key words: anserine, broiler chicken, carnosinase, carnosine, carnosine synthase, imidazole dipeptide

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Introduction

Imidazole dipeptides (IDPs), such as carnosine (β-alanyl-L-histidine), anserine (β-alanyl-N^{π}-methyl-L-histidine), and balenine (β-alanyl-N^{τ}-methyl-L-histidine), are abundant in the skeletal muscle of vertebrates[1–3]. These IDPs exert metal chelation, antioxidant, antiglycation, and antifatigue activity[4].

Consumers tend to believe that food contributes to their health[5]. Goddard and Muringai[6] reported that consumers were willing to pay more for pork with enhanced carnosine levels. Chicken meat contains more anserine than other meats, such as beef and pork[7], making it a good source of IDPs. The Consumer Affairs Agency of Japan launched the "Foods with Function Claims (FFC)" system, which allowed businesses to independently evaluate the safety and scientific claims of their food products, as well as label their functionality[8]. Chicken and pork rich in IDPs have been registered as FFC foods[8].

Attempts to increase muscle IDP content in chickens have focused on changes to the birds' diet[9–13], such as enrichment in either histidine or β-alanine. In skeletal muscles, IDP synthesis is mediated by carnosine synthase 1[14,15], whereas IDPs are degraded by carnosinase 2[16–18]. In chickens, muscle carnosine content correlates with mRNA expression of *carnosine synthase 1*[19].

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Livestock animals are normally reared under high ambient temperatures (HT), which are above their thermoneutral zone. These conditions alter the animals' physiology, metabolism, and meat quality[20]. In pigs, one of the consequences is decreased carnosine and *carnosine synthase 1* mRNA expression in skeletal muscles[21,22]. This finding raised the possibility that muscle IDPs content varied seasonally, with summer values lower than the expected FFC content. However, no reliable quantitative variation in these IDPs in meat from chickens reared under HT conditions has been reported.

Thus, the present study sought to investigate the effect of prolonged heat exposure on muscle IDP (carnosine, anserine, and balenine) content in broiler chickens. To mimic a realistic "extremely hot day", defined by the Japan Meteorological Agency as daily maximum temperatures above 35 °C[23], a cyclic HT environment (35 ± 1°C for 8 h/day) was used.

Materials and Methods

Animal experiment

The experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of Kagoshima University (approval number: A22013). Sixteen newly hatched male Ross308 broiler chicks (*Gallus gallus domesticus*) were supplied by a commercial hatchery (Kumiai Hina Centre, Kagoshima, Japan). The chicks were housed in an electrically heated battery brooder, and provided with water and a starter diet until they were 11 days old. On day 11, the chicks were housed individually in wire-bottomed aluminum cages (50 × 40 × 60 cm) and fed a growth diet. On day 14, chicks were randomly divided into two groups. One group of chicks was exposed to cyclic HT (35 ± 1 °C for 8 h every day), while another group was kept in a thermoneutral (TN) environment at 25 ± 1 °C. On day 25, the chicks were fed a finisher diet. The formulations and nutrient compositions of the diets are reported in Table 1. On day 42, all chickens were sacrificed by cervical dislocation following carbon dioxide anesthesia, after measuring their body temperature and body weight. Following dissection, the right half of the pectoralis major muscle was used to determine drip loss; whereas a portion of the left half was snap-frozen in liquid nitrogen and stored at -80 °C for subsequent determination of free amino acid content and gene expression.

Table 1. **Composition of experimental diets.**

	Starter	Grower	Finisher
Ingredients (g/100 g)			
Corn meal	48.85	53.10	58.15
Soybean meal	42.60	37.78	32.70
Corn oil	4.45	5.20	5.80
$CaCO_3$	1.00	1.00	0.90
$CaHPO_4$	1.50	1.30	1.15
NaCl	0.50	0.50	0.50
Methionine	0.25	0.32	0.20
Lysine-HCl	0.20	0.15	
Threonine	0.10	0.10	0.10
Valine	0.05	0.05	
Mineral and vitamin premix[a)]	0.50	0.50	0.50
Calculated analysis			
Crude protein (%)	23.30	21.50	19.50
Metabolizable energy (MJ/kg)	13.01	13.46	13.87

[a)]Content per kg of the vitamin and mineral premix: vitamin A, 90 mg; vitamin D3, 1 mg; DL-alpha-tocopherol acetate, 2000 mg; vitamin K3, 229 mg; thiamin nitrate, 444 mg; riboflavin, 720 mg; calcium d-pantothenate, 2174 mg; nicotinamide, 7000 mg; pyridoxine hydrochloride, 700 mg; biotin, 30 mg; folic acid, 110 mg; cyanocobalamin, 2 mg; calcium iodinate, 108 mg; MgO, 198,991 mg; $MnSO_4$, 32,985 mg; $ZnSO_4$, 19,753 mg; $FeSO_4$, 43,523 mg; $CuSO_4$, 4,019 mg; and choline chloride, 299,608 mg.

Determination of muscle malondialdehyde (MDA) concentration

To evaluate lipid peroxidation in the skeletal muscles of chickens at slaughter, MDA content was determined colorimetrically following reaction with 2-thiobarbituric acid, as described by Ohkawa et al.[24]. Briefly, 0.3 g of the pectoralis major muscle was homogenized in 1 mL of 1.15% KCl and centrifuged at 20,000 × *g* for 5 min. Then, 80 µL of supernatant was mixed with 80 µL of 8.1% sodium dodecyl sulfate, 220 µL of 20% acetic acid (pH 3.5), and 300 µL of 0.8% 2-thiobarbituric acid. After vortexing, the samples were incubated at 95 °C for 1 h, transferred to ice, mixed with 1 mL butanol-pyridine 15:1 (v/v), vortexed again, and finally centrifuged at 20,000 × *g* for 5 min. Absorbance of the supernatant, which contained the butanol-pyridine layer, was measured at excitation and emission wavelengths of 535 and 585 nm, respectively.

Determination of skeletal muscle drip loss

Drip loss was measured using the method described by Berri et al.[25]. The right half of the pectoralis major muscle was weighed immediately after dissection, placed in a plastic bag, and stored for 48 h in a low-temperature incubator (MIR 153; Sanyo Electric Co., Osaka, Japan) set at 4 °C, then wiped and weighed again. The difference in mass corresponded to drip loss, which was expressed as a percentage of the initial muscle mass.

Determination of free amino acid and imidazole dipeptide concentrations

Free amino acids were quantified by pre-column high-performance liquid chromatography (HPLC) as detailed previously[26,27]. One gram of frozen pectoralis major muscle was weighed and homogenized in 10 mL ice-cold 0.1 M HCl containing 100 µM d-norvaline (FUJIFILM Wako Chemicals, Osaka, Japan) as internal standard. Hexane (10 mL) was added, mixed by vortexing for 1 min, and centrifuged at 22,000 × *g* for 5 min. Next, 400 µL of the lower layer was mixed with 1,200 µL water-acetonitrile (1:2 v/v) by vortexing for 1 min and centrifuged at 22,000 × *g* for 5 min. The resulting supernatant was filtered using a 0.2-µm pore size filter.

The supernatant was analyzed by a NexeraX2 HPLC system (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a Kinetex 2.6

μm column (EVO C18; 100×3.0 mm; Phenomenex, Torrance, CA, USA). The amino acids were separated following the Shimadzu Corporation (2019) manual based on gradient elution with two mobile phases. Mobile phase A consisted of 17 mM potassium dihydrogen phosphate and 3 mM dipotassium hydrogen phosphate; whereas mobile phase B contained a mixture of deionized water:acetonitrile:methanol (15:45:40). Pre-column derivatization was performed by the HPLC system using 45 μL mercaptopropionic acid, 22 μL ortho-phthalaldehyde, and 7.5 μL samples. The mixture was incubated for 2 min. Then, 5 μL of fluorenylmethyl chloroformate was mixed and let stand for 2 min. Finally, 1 μL of the mixture was injected into the column. Flow rate was set to 0.85 mL/min and column temperature to 35 °C. An RF-20Axs high-sensitivity fluorescence detector (Shimadzu Co., Ltd.) was set to Ch1 excitation/emission of 350/450 nm and Ch2 excitation/emission of 266/305 nm.

A total of 24 compounds were measured: alanine, anserine, arginine, asparagine, aspartic acid, balenine, β-alanine, carnosine, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The concentrations of these compounds in the pectoral muscle are expressed as mg/100 g of tissue.

RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction (PCR)

Skeletal muscle tissue was homogenized in ISOGEN II (Nippon Gene, Tokyo, Japan) and 60 ng of purified total RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (RR036A; TaKaRa Bio, Shiga, Japan). Real-time PCR was performed as described previously[28], with the following primers: carnosine synthase 1, 5′- GATGCCCCTCACCATAGACC-3′ and 5′- CCCAGTACGCACACAGTCAT-3′; carnosinase 2, 5′-GGCGCAACAACATTCTGGTC-3′ and 5′-TGGATTGCTACCCACTCAGC-3′; carnosine N-methyltransferase 1, 5′-CGGTGACAGAGATCCGCC-3′ and 5′-CTCCTGATCCTGCTCCTCCT-3′; and 18S ribosomal RNA, 5′-AAACGGCTACCACATCCAAG-3′ and 5′-CCTCCAATGGATCCTCGTTA-3′. The resulting mRNA levels were expressed relative to the values for control chickens.

Statistical analysis

Data are presented as the mean ± standard error of the mean. Student's *t*-test was conducted to compare growth performance, concentration of free amino acids and IDPs, as well as gene expression between the HT and TN groups. Pearson's correlation and regression analyses were used to compare drip loss and IDPs (anserine and carnosine). Statistical analyses were conducted in R version 4.3.2[29]. The 't.test' function was utilized for Student's *t*-test, the 'cor' function for Pearson's correlation coefficients, and the 'lm' function for regression analysis. Statistical significance was set at $P < 0.05$.

Results and Discussion

When exposed to HT, chickens exhibit slower growth and poor feed efficiency, along with decreased meat yield[30,31]. In this study, we confirmed that cyclic HT conditions lowered final body weight, body weight gain, and feed intake in broiler chickens; while significantly increasing body temperature (Table 2). Muscle, liver, and heart weight was also lower in chickens maintained under cyclic HT (Table 2). In contrast, HT conditions have been shown to increase MDA levels and muscle drip loss in chicken meat[20]. The pectoral muscles of chickens kept under cyclic HT conditions showed higher lipid peroxidation and drip loss than those of control chickens (Table 3). These results suggest that the cyclic HT environment used in this study realistically triggered the negative effects commonly observed in broiler chickens maintained in HT-inducing environments.

Chronic heat stress enhances systemic amino acid catabolism in chickens[32]. Here, the levels of most free amino acids measured in chickens kept under cyclic HT conditions were lower than those of control animals (Table 4), with significant differences in muscle histidine and tyrosine contents. Cyclic HT conditions resulted in significantly decreased carnosine and anserine

Table 2. **Effect of a cyclic high ambient temperature on growth performance parameters of broiler chickens.**

	Thermo-neutral temperature (25 ± 1 °C)			Heat ambient temperature (35 ± 1 °C for 8 h/day)		
Final body weight (g)	2391.69	±	153.30	1768.59	±	151.92[a)]
Body weight gain (g)	2015.82	±	149.31	1383.24	±	149.18[a)]
Feed intake (g)	3171.12	±	151.04	2476.11	±	140.34[a)]
Feed conversion ratio	1.60	±	0.09	1.87	±	0.13
Body temperature (°C)	40.78	±	0.09	41.39	±	0.15[a)]
Pectoral major muscle (g)	436.45	±	19.72	323.06	±	28.48[a)]
Pectoral minor muscle (g)	88.05	±	4.09	61.54	±	4.56[a)]
Leg muscles (g)	467.21	±	33.24	362.79	±	28.57[a)]
Liver (g)	35.44	±	2.27	26.73	±	1.92[a)]
Heart (g)	8.39	±	0.65	6.36	±	0.65[a)]
Abdominal fat tissue (g)	17.5	±	2.59	16.89	±	2.90

Results are expressed as mean ± standard error of the mean (n = 8). [a)]$P < 0.05$ (vs. control).

Table 3. **Effects of a cyclic high ambient temperature on MDA concentration and drip loss in the skeletal muscle of broiler chickens.**

	Thermo-neutral temperature (25 ± 1 °C)	Heat ambient temperature (35 ± 1 °C for 8 h/day)
Muscle MDA concentration (nmol MDA equivalents /g tissue)	193.02 ± 57.15	252.81 ± 17.49[a)]
Drip loss (%)	3.25 ± 0.67	4.65 ± 0.87[a)]

Results are expressed as mean ± standard error of the mean (n = 8). [a)]$P < 0.05$ (vs. control). MDA, malondialdehyde

Table 4. **Effect of a cyclic high ambient temperature on muscle free amino acids and imidazole dipeptide concentration in broiler chickens.**

	Thermo-neutral temperature (25 ± 1 °C)	Heat ambient temperature (35 ± 1 °C for 8 h/day)
Alanine	1.67 ± 0.22	1.63 ± 0.13
Arginine	4.58 ± 0.92	2.88 ± 0.62
Asparagine	4.30 ± 0.71	3.07 ± 0.38
Aspartic Acid	4.26 ± 0.57	4.14 ± 0.92
β-Alanine	2.53 ± 0.78	2.88 ± 0.52
Cystine	41.32 ± 4.55	36.15 ± 5.64
Glutamic Acid	15.95 ± 3.47	12.80 ± 2.57
Glutamine	25.63 ± 5.82	24.13 ± 4.36
Glycine	24.56 ± 6.14	22.00 ± 5.14
Histidine	0.77 ± 0.08	0.53 ± 0.09[a)]
Isoleucine	0.75 ± 0.10	0.58 ± 0.10
Leucine	2.23 ± 0.27	1.69 ± 0.27
Lysine	2.60 ± 0.21	3.14 ± 0.47
Methionine	0.88 ± 0.15	0.66 ± 0.14
Phenylalanine	1.80 ± 0.28	1.33 ± 0.22
Proline	0.73 ± 0.03	0.66 ± 0.05
Serine	12.34 ± 1.14	14.84 ± 1.41
Threonine	6.10 ± 1.40	3.78 ± 0.80
Tryptophane	0.61 ± 0.12	0.58 ± 0.10
Tyrosine	4.81 ± 0.80	2.03 ± 0.20[a)]
Valine	2.09 ± 0.22	1.92 ± 0.21
Anserine	1035.17 ± 63.97	705.66 ± 69.27[a)]
Carnosine	226.90 ± 23.11	168.63 ± 21.84[a)]

Results are expressed as mean ± standard error of the mean (n = 8). [a)]$P < 0.05$ (vs. control).

levels in the pectoral muscles of chickens at slaughter (Table 4); whereas balenine could not be detected in either group. The amount of anserine and carnosine was more than 30% lower in the pectoral muscle of chickens kept under cyclic HT conditions (muscle IDP content: 1262.07 ± 67.32 and 874.29 ± 75.03 mg/100 g, control group and HT group, respectively). A decreased muscle carnosine content has been observed in pigs kept under HT conditions[21,22]. Moreover, pigs raised in a constant HT environment (30 °C for 7 days) were reported to have low carnosine in the longissimus dorsi muscle[21]. HT conditions favor the generation of reactive oxygen species (ROS) in various tissues as the heat load increases[33,34]. Because IDPs and their derivatives are involved in antioxidant activity[35–40], IDPs may act as reducing agents that neutralize heat stress-induced ROS.

The decrease in muscle IDPs content may be explained by the lower muscle free histidine level observed in this study. Muscle histidine content is generally high relative to the Km of carnosine synthase 1[41]. In contrast, even though muscle β-alanine contributes to carnosine formation[42], its content did not differ between the two groups (Table 4). Another possible explanation for the decrease in muscle IDPs may be the suppression of *de novo* IDP synthesis, which is mediated by carnosine synthase[14,15]. In chickens, there is a correlation between muscle carnosine content and mRNA levels of *carnosine synthase 1*,

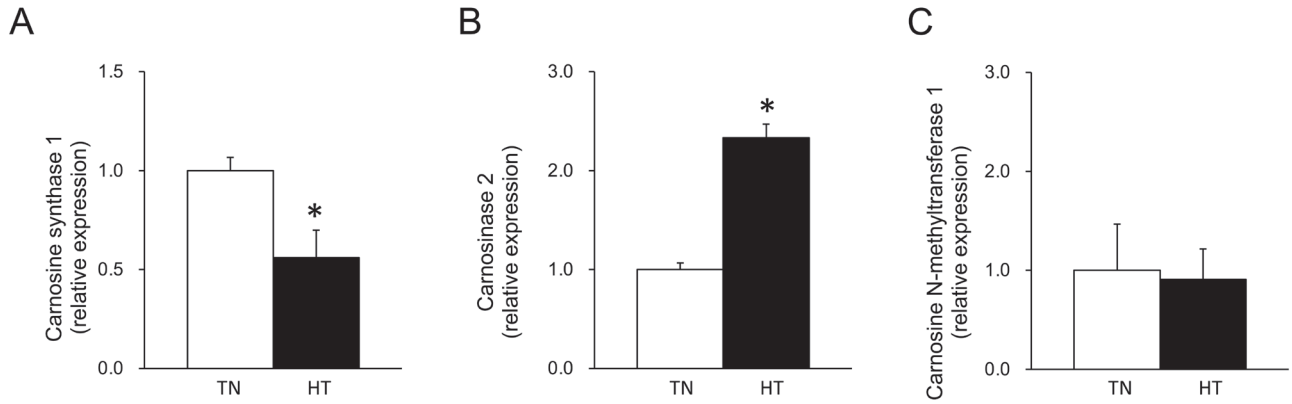

Fig. 1. **Effect of a cyclic high ambient temperature on the genes encoding for carnosine synthase 1 (A), carnosinase (B), and carnosine N-methyltransferase (C) in the pectoralis major muscle of broiler chickens.** Quantitative real-time PCR results are normalized to the levels of 18S mRNA and expressed relative to the mean value for control chickens. Values are expressed as the mean ± the standard error (n = 8 per treatment) *, $P < 0.05$ (vs. control chickens).

which is responsible for IDP synthesis in skeletal muscle[19]. In pigs, *carnosine synthase 1* mRNA expression was found to be significantly decreased in the longissimus dorsi muscle 3 weeks after heat exposure[22]. In accordance with these studies, we found that cyclic HT resulted in decreased *carnosine synthase 1* mRNA expression in the pectoral muscles of broiler chickens (Figure 1). It has been theorized that prolonged heat stress reduces energy generation efficiency and ATP synthesis in birds[43]. Because carnosine synthase requires ATP to work[44], it seems reasonable that broiler chickens kept under cyclic HT conditions would suppress *de novo* synthesis of carnosine and anserine to reduce ATP consumption in skeletal muscles. In addition, in the present study, we found significantly higher mRNA expression of *carnosinase 2*, which degrades carnosine and anserine[16–18], in the pectoral muscles of broiler chickens kept under cyclic HT conditions compared to that in control chickens (Figure 1). Instead, we observed no difference in the expression of *carnosine N-methyltransferase 1*, which is responsible for carnosine methylation. These results suggest that cyclic HT exposure results in decreased muscle IDP content via both reduced synthesis and increased degradation of anserine and carnosine in the pectoral muscle of chickens. However, owing to the lack of reports on the transcriptional regulation of *carnosine synthase 1* or *carnosinase 2*, the mechanism by which cyclic HT affects the expression of these genes requires further investigation.

Dietary supplementation with carnosine has been reported to decrease drip loss, cooking loss, shear force, and hardness of broiler chicken meat[13]. Furthermore, muscle carnosine levels correlate with drip loss in broiler chicken meat[45]. In agreement with the above evidence, we found that muscle IDP content correlated negatively with muscle drip loss in the pectoralis muscle of broiler chickens ($r = -0.605$, Pearson's correlation). Similarly, regression analysis suggested that muscle IDP concentration might influence muscle drip loss ($R^2 = 0.3205$, $P < 0.05$). These results point to the importance of muscle IDPs for the water-holding capacity of chicken meat during storage. Further studies are required to determine whether regulating muscle IDP content in chickens maintains or improves the water-holding capacity of chicken meat.

In conclusion, raising broiler chickens under cyclic HT conditions resulted in significantly decreased skeletal muscle IDP content and increased muscle drip loss, which was accompanied by changes in the mRNA levels of *carnosine synthase 1* and *carnosinase 2* in the pectoral muscles of these animals.

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Author Contributions

Conceptualization: D. K., Y. O., A. O., and D. I.; methodology: D. I. and A. O.; validation: A. K., S. S., and D. I.; formal analysis: A. K., M. K., and S. G.; investigation: A. K., M. K., and S. G.; statistical analysis: A. K. and S. I.; original draft preparation: A. K. and D. I.; manuscript review and editing: S. S., D. K., Y. O., S. I., A. O., and D. I.; supervision: A. O.; project administration: D. I. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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