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Effects of different protein levels on growth performance and stress parameters in beef calves under heat stress

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Abstract

This study investigated the effects of dietary protein levels under various heat stress (HS) conditions on the growth performance and stress parameters in Korean native beef calves. Male calves ($n = 40$; initial BW = 202.2 ± 3.31 kg) were randomly assigned to climatic-controlled chambers with 3×3 factorial arrangements. Calves were assigned into three dietary protein levels (low protein; LP = 12.5%, medium protein; MP = 15%, and high protein; HP = 17.5%) and three stress levels (mild: temperature-humidity index (THI) = 74 to 76, moderate: THI = 81 to 83, and severe: THI = 89 to 91) with control (threshold: THI = 70 to 73 and dietary protein level 12.5%). The calves were subjected to ambient temperature (22 °C) for 7 days and subsequently to the temperature and humidity corresponding to the target THI level for 21 days. As a result, average daily gain (ADG) was decreased ($P < 0.05$) under severe HS level compared to the mild and moderate HS stress levels. However, HP increased ADG ($P < 0.05$) than moderate levels (LP) and severe levels (LP and MP). Under different HS levels (mild, moderate, and severe), HR, RT, and blood cortisol were increased compared to a threshold level, but no differences were observed in the parameters among various protein levels. Varied HS levels decreased the levels of blood glucose, NEFA, and amino acids (AAs) (lysine and glutamic acid) compared to a threshold ($P < 0.05$). But, the HP group resulted in increased levels of blood glucose, NEFA, and AAs (lysine and glutamic acid) compared to LP and MP groups under severe HS stress. The expression level of the HSP70 gene ($P < 0.05$) in PBMC and hair follicles was increased following an increase in moderate and severe HS levels. Also, HSP70 gene expression in the HP group was decreased compared with LP and MP groups under intense HS level. Overall, HS in Korean native beef calves exhibited negative effects on ADG, blood

glucose, NEFA, and AA profile. However, 17.5% of dietary protein (HP) could compensate for the growth of heat-exposed Korean native beef calves through the regulation of homeostasis by protein and energy metabolism. Also, it was evident that adequate protein (HP) is used as a major nutrient for HSP70 synthesis in PBMC and hair follicles causing, a boost in the immune system of heat-exposed Korean native beef calves.

Keywords: Temperature-humidity index, Heat stress, Growth performance, Protein levels, Korean native beef calves, Stress parameters

Introduction

The drastic ambient temperature and humidity increase can create multiple issues in animal agriculture. Temperatures above the thermal neutral zone may increase the probability of heat stress (HS) occurrence and influence animals' physiology^{1,2}. HS can be detrimental to lactation, growth, and reproduction in most livestock animals; however, beef cattle can be more vulnerable to coping with HS because they lack the ability to dissipate heat sufficiently³. Heat stress induces behavioral and metabolic changes in cattle that are intended to maintain homeothermy, often at the expense of decreased productivity and profitability⁴. Various responses to HS have been reported, including reduced dry matter intake (DMI), activity, productivity, and metabolic rate. Among these HS feedback, reduced feed intake is the first noticeable sign of HS, presumably an evolutionary strategy to reduce the "heat increment" of feeding^{5,6}. This leads to dystrophia and is known to reduce production in growing cattle⁷.

Previous studies suggested management strategies to reduce the unfavorable outcomes from HS, such as physical and genetic modification and improved nutritional management^{4,8}. Genetical improvement against HS can be achieved by introducing thermostable breeds. *Bos indicus* possesses physiological and cellular thermotolerance compared to *Bos taurus*^{9,10}. Nutritional modifications also have suggested compensating for the adverse effects of HS. Overall intake usually reduces during hot weather, and it may lead to reverse growth. In order to offset the reduced feed intake and increased nutrient requirements during HS, diet reformulation is required¹¹. In growing calves, protein is an essential nutrient to maintain skeletal muscle growth¹². In calves in a hot environment, protein supplementation levels need to be increased to compensate for reduced feed intake¹².

Limited information is available regarding the dietary protein level of beef calves during the HS situation. Despite that NASEM¹² states guidance of specification managements under HS, it lacks details. Therefore, the objective of the current study was to 1) demonstrate the

different dietary protein levels under various HS conditions on growth performance and stress parameters 2) to provide a guide line for dietary protein supplementation under the HS condition in Korean native beef calves.

Material and methods

Animals and climatic chamber details

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University (Approval No: KU18178), and animal care was performed according to the committee's guidelines and as per ARRIVE Guidelines/Checklist. Forty male calves (body weight of 202.2 ± 3.31 kg) were used for ten experiments (four calves per experiment). Calves were selected based on heat shock protein 70 (HSP70) expression via single nucleotide polymorphism genotyping¹³ in order to homogenize heat tolerance characteristics. Animals were assigned to 1 of 4 groups (n=4/group); threshold, mild, moderate, and severe stress group. Two animals were housed in each chamber. Animals were offered feed and water individually. The size of each climatic chamber was 2.5 m × 2.5 m × 3 m (length, width, and height, respectively). The chambers were designed and programmed to operate over a temperature range of 18 to 34°C and a humidity range of 60 to 100% RH. Each chamber was fitted with controllable temperature and RH regulators to maintain the desired temperature and RH from 0900 to 1900 h.

Management conditions and treatment

The temperature and RH in a chamber were controlled by regulators to maintain THI for each treatment from 0900 to 1900 h. During the night-time (from 1900 to 0900 h), THI was maintained under 68, which does not influence the physiological parameters of the animals¹⁴. A completely randomized design with a 3×3 factorial arrangements with 3 dietary protein levels

(low protein; LP = 12.5%, medium protein; MP = 15%, high protein; HP = 17.5%) and 3 stress levels (mild: temperature-humidity index (THI) = 74 to 76, moderate: THI = 81 to 83, severe: THI = 89 to 91) was conducted. A control group (threshold) was assigned at a THI level of 68 to 70 with a dietary CP level of 12.5%. Calves were exposed to the ambient temperature of 22°C for 7 days (Thermoneutral: TN), including one week of the adaptation period prior to the experiment initiated. Following that, the temperature and RH in the chambers were raised to each THI level for 21 days (HS). Four THI treatments were categorized as threshold (22 to 24°C, 60% RH: THI = 70 to 73), mild (26 to 28°C, 60% RH: THI = 74 to 76), moderate (29 to 31°C, 80% RH: THI = 81 to 83), and severe (32 to 34°C, 80% RH: THI = 89 to 91) stress levels¹⁵. The study included four different THI groups and was designed to consist of four levels of dry bulb temperature (22 to 24°C, 26 to 28°C, 29 to 31°C, 32 to 34°C) and two levels of RH (60 and 80%). Temperature and RH inside the chamber were recorded at intervals of 1 s using two sensors (SHT7x, Sensirion AG, Staefa, Switzerland). The THI was calculated using the dry bulb temperature (T_{db} , °C) and RH using the following formula¹⁶:

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$$

The diets used in this study were composed of 40% roughage (*Phleum pratense* L.) and 60% concentrate. The feed was weighed and offered twice a day at 0900 and 1700 h (Table 1). Feed and water intake were measured daily basis (0900 h). The chemical composition of the feed is shown in Table 2. The BW of each animal was measured at d0 and d28 after trial initiation when before the morning feed delivery (0800 h).

Chemical analysis

Dry matter (DM; method 930.15), crude protein (CP; method 984.13), crude ash (method 942.05), crude fat (method 920.39), ADF (method 973.18) were analyzed according to AOAC¹⁷. The NDF content was determined according to the procedure of Van Soest, et al.¹⁸. The

level of Ca and P were measured inductively coupled plasma spectroscopy (method 945.46) ¹⁷. While DM was determined by drying ground diets in a vacuum oven at 100°C overnight, crude ash content was measured by incineration at 550°C overnight in a muffle furnace (KMF-500, Lab Corporation, Seoul, South Korea). In order to measure CP contents, total nitrogen content in each diet was measured using the Kjeltec™ System (Kjeltec™ 2400, FOSS, Denmark), and the final CP content was calculated as nitrogen × 6.25. Crude fat content was analyzed using the ether extraction system (ANKOMXT15 Extractor, ANKOM Technology, Macedon, NY). Mineral content was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo-Fisher Scientific, Waltham, MA). Amino acid (AA) composition in each diet was determined using the AA analyzer (Beckman 6300, Beckman, Fullerton, CA) (Table 3).

Physiological parameters under heat stress

The physiological parameters of heat-stressed calves, including heart rate (HR) and rectal temperature (RT), were measured every three days (1400 h) ¹⁹. The HR, expressed in beats per minute (BPM), was obtained using a stethoscope (TS-DIA01002, Tenso Medical Instrument Co., Zhejiang, China) placed directly onto the left thoracic region under one of the auscultation foci for one minute. The RT was measured after checked HR with a large animal clinical thermometer (TES-1300 Thermometer, E&E PROCESS Instrument Co., Vaughan, ON, Canada) that was inserted to a depth of 3 cm into the animal's rectum and held in contact with the mucosa for one minute.

Blood parameters under heat stress

Blood samples were collected every three days (d1, 4, 7, 10, 13, 16, 19, 22, 25, and 28) at 1400 h from jugular venepuncture using non-heparinized vacutainers (20 mL; Becton-

Dickinson, Belliver Industrial Estate, PL6 7BP, Plymouth, UK) and ethylenediaminetetraacetic acid-treated vacutainers (4 mL; Becton-Dickinson, Franklin Lakes, NJ, USA)²⁰. Serum samples were obtained from blood after centrifugation at $2,700 \times g$ at 4°C for 15 minutes. Serum was transferred to a 1.5 mL tube (Eppendorf AG, Hamburg, Germany) and kept at –80°C until further analysis. Serum was analyzed for glucose (Fuji Dri-Chem Slide Glu-PIII, Fuji Film Corp., Tokyo, Japan) and blood urea nitrogen (BUN, Fuji Dri-Chem Slide BUN-PIII, Fuji Film Corp., Tokyo, Japan) using DRI CHEM 7000i biochemistry analyzer (Fuji Film, Tokyo, Japan). Non-esterified fatty acids (NEFA) was analyzed using Roche Free Fatty Acids kits (Roche, Mannheim, Germany) with HITACHI 7600 chemistry–autoanalyzer (Hitachi, Tokyo, Japan). Blood cortisol was determined using a commercial Bovine ELISA test kit (Life Diagnostics, Inc, West Chester, PA, USA). Plasma was deproteinized with 10% sulfosalicylic acid for analysis of AA profiles using the AA analyzer (Sykam S433, Sykam GmbH, Germany).

Sampling and isolation of peripheral blood mononuclear cells

The PBMC isolation from collected blood was conducted within 8 hours of the sample collection. Density gradient centrifugation was used to separate PBMCs from the whole blood. The whole blood was diluted 1:1 with $1 \times$ PBS (Hyclone, Laboratories, INC., Logan, UT, USA) and layered gently over Histopaque-1077 (Sigma-Aldrich Inc., St. Louis, MO, USA). All the PBMC isolation steps were performed at room temperature, following the manufacturer's instructions²¹.

Hair follicle collection and storage

The hair follicle samplings were conducted as previously reported by Kim, et al.¹⁵. Hair follicles were collected from the tails of each calf every three days at 1400 h. Approximately 25 to 30 strands of hair were pulled from the tail head of calves. Hairs were grasped as close to

the skin as possible and then rapidly pulled out. The hair follicles were washed using DEPC-treated water. The hair follicles were then placed into a 5 mL specimen jar filled with RNAlater™ (Ambion, Austin, TX) for total RNA extraction.

Total RNA extraction and real-time PCR analysis

Total RNA was extracted from PBMCs using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions ²¹. Total RNA was extracted from the hair follicles on the day of collection and stored at room temperature for 14 days in RNAlater™ (Ambion, Austin, TX, USA). The hair segments, containing the hair follicle (1 cm from bottom), were cut, and transferred into a 2 ml microcentrifuge tube with 1 ml of TRIzol™. The RNA quality and quantity of isolated were measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260/280 ratios of all RNA samples were greater concentration than 1.8. The RNA quality was assessed using an RNA 6000 Nano Lab Chip kit (Agilent, Palo Alto, CA, USA). The RNA integrity number (RIN) was confirmed in a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) to determine whether the purified total RNA could be used in real-time PCR. The average RIN of PBMCs was 8.4 (7.8 to 9.4). The RNA samples were stored at -70°C until analysis. The first-strand cDNA was synthesized using RNA (1 µg) and an iScript cDNA synthesis kit (Bio Rad, Hercules, CA, USA). The expression of genes in PBMCs, including HSP70, was analyzed by real-time quantitative PCR (RT qPCR) amplification with SYBR- Green® as described previously ²². All reactions were performed in triplicate and a total reaction volume of 20 µL per well in a 96-well plate using a Chromo4™ four-color real-time detector (MJ Research, Waltham, MA, USA). The reaction mixture contained 100 ng of cDNA, 10 µL of 2× SYBR Green PCR master mix (Bio-Rad) and 0.6 µL of primers at 10 µM (Bioneer, Daejeon, South Korea) in autoclaved water. The thermal cycling conditions were as follows: initial incubation at 95°C for 3 minutes followed by 40 cycles of

denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, after which the samples were heated at 95°C for 10 seconds, cooled to 65°C for 5 seconds and then heated to 95°C at a rate of 0.5 °C/s. The results were monitored using post-PCR melt curve analysis of amplification reactions (in triplicate from all samples) and sequencing amplification products. Primers were designed using the National Center for Biotechnology Information Primer-BLAST (Table 4). The threshold cycles for each sample were normalized to housekeeping genes (GAPDH, RPS15A, and B2M) ²³, and the relative expression of the target gene was quantified as the fold change of expression of the target gene relative to the expression of the thermoneutral control according to the 2- $\Delta\Delta$ rCT method ²⁴. The coefficient of variation of the housekeeping gene was checked before calculating of the results to ensure that it did not exceed 5%.

DNA extraction from hair follicle for SNP genotype of HSP70

The DNA was extracted from the hair follicle using the manual method ²⁵. Hair follicles were cut off 5-10 mm of 20 hairpieces and placed into a 1.5 mL tube. The 300 μ L tissue lysis (TL) buffer contained 1 M Tris-Cl pH 8.0, 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0, 5 M NaCl, 10% sodium dodecyl sulfate, and distilled water were added and mixed by inverting. Then, 5 μ L proteinase K was added (20 mg/mL, G-spin Total DNA extraction Kit, iNtRON Biotechnology, Seongnam, South Korea), followed by incubation at 56°C overnight. Following that, 300 μ L protein precipitation solution was added (7 M Ammonium acetate, Tech & Innovation, Seongnam, South Korea) and centrifuged at 13,000 rpm 20°C for 15 minutes. After this, 600 μ L of supernatant was transferred to a new 1.5 mL tube and incubated at 65°C for 1 hour after 10 μ L RNase solution was added (10 mg/mL, G-spin Total DNA extraction Kit, iNtRON Biotechnology, South Korea). Subsequently, 600 μ L was added into the tube and centrifuged again (13,000 rpm, 20°C for 15 minutes). The solution was divided

into two other 1.5 mL tubes, with each tube for 600 µL, and this was followed by adding 600 µL isopropanol to each tube, inverting the sample, and incubating at –20°C for 1 hour. After that, it was centrifuged at 13,000 rpm, 4°C for 10 minutes, then the supernatant was discarded, and 300 µL 70% EtOH was added to wash the DNA extraction. Finally, the sample was centrifuged at 13,000 rpm, 4°C for 5 minutes, and the supernatant was discarded. The pellet was dried at room temperature for 1 hour, then 50 µL Tris-EDTA (TE) buffer was added (1× TE buffer, pH 8.0, Tech & Innovation, South Korea) for storage.

Polymerase chain reaction and restricted fragment length polymorphisms

Primer sequences for the target gene HSP70 were 5'-GCCAGGAAACCAGAGACAGA-3' (forward), 3'-CCTACGCAGGAGTAG-3' (reverse), which was referenced from GenBank (accession number M98823.1). HSP70 gene was amplified by 2× Taq polymerase chain reaction (PCR) premix kit (Solgent Co. Ltd, Daejeon, South Korea) followed by using the T100 96-well Thermal Cycler (Bio-Rad Laboratories Inc, Seoul, South Korea): 95°C for 2 minutes, 35 cycles of 20 seconds at 95°C, 40 seconds at 56°C, 1 minute for 72°C, and finally 5 minutes at 72°C. The product of PCR was digested by BslI (New England Biolabs, Pickering, ON, Canada) for 3 hours, in which the C allele was cut into 895-bp and 1128-bp fragments¹³. Then, the type of HSP70 was shown by the separation of electrophoresis according to a 2% agarose gel. The standard of the HSP70 genotype was shown in Figure 1, in which CC types were expressed as lanes 1 to 24 except lane 9 (TC type).

Statistical analysis

THI data, growth performance, physiological parameters, blood parameters, and HSP gene expression were analyzed using repeated-measures analysis and the GLM procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The model was as follows¹⁵:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma(\alpha)_{ik} + \varepsilon_{ijk}$$

where Y_{ijk} is the observation of calf k at sampling time j for the given treatment i , μ is the overall mean, α_i is the fixed effect of treatment i (threshold, mild, moderate, and severe stress level), β_j is the fixed effect of sampling time j (every 3 days), $\gamma(\alpha)_{ik}$ is the random effect of calf k nested in treatment i and ε_{ijk} is the residual effect. Calf identity was included in the model as a random effect. The subject of the REPEATED statement was the effect of the calf. Preplanned orthogonal contrasts were conducted to test the effects of dietary protein (Control vs. 9 experimental diets), dietary protein levels (12.5, 15, and 17 %), HS levels (mild, moderate, and severe), and the interaction between dietary protein level and HS level. A Tukey's honest significant difference (HSD) test was performed for mean comparisons. The covariance structures (autoregressive order 1, unstructured and compound symmetry) for the repeated measures model were tested and the structure that best fit the model was chosen based on the smallest value of Schwarz's Bayesian information criterion. The first day of sampling in each THI group was included as a covariate to correct the means. The covariate factor was included in the model when appropriate but was removed from the model when it was insignificant. Data are presented as least square means and associated standard errors. Differences were considered statistically significant if the P -value was less than 0.05. Means with p -values between 0.05 and 0.10 reflected a tendency to differ.

263

264 **Results**

265 **The effects of dietary protein levels on DMI, protein intake, and growth performance under** 266 **heat stress.**

267 Dietary protein levels in each HS treatment increased protein intake ($P < 0.01$), BW gain
268 ($P < 0.01$), and ADG ($P < 0.01$) in all experimental groups compared with that in the basal diet
269 group (Table 5). However, the supplemental dietary protein decreased DMI ($P < 0.01$) in all

experimental groups compared with that in the control (Table 5). Increased dietary protein levels enhanced protein intake ($P < 0.01$), BW gain ($P < 0.01$), and ADG ($P < 0.01$) without affecting the DMI (Table 5). HS levels decreased DMI ($P < 0.01$), protein intake ($P < 0.01$), BW gain ($P < 0.05$), and ADG ($P < 0.05$) (Table 5).

The effects of dietary protein levels on physiological parameters under heat stress

Heart rates were not changed ($P > 0.05$) by the dietary protein or HS levels in HR and RT during the TN period. However, during the HS period, dietary protein levels in HS treatments increased HR ($P < 0.05$) and RT ($P < 0.01$) compared with that in the basal diet group (Table 6). Increased dietary protein levels did not affect ($P > 0.05$) HR and RT (Table 6). HR ($P < 0.01$) and RT ($P < 0.01$) were increased as HS level increases (Table 6).

The effects of dietary protein levels on blood parameters under heat stress

During the TN period, no difference ($P > 0.05$) was found in blood cortisol, BUN, glucose, and NEFA (Table 7). However, during the HS period, dietary protein levels in each HS treatment increased cortisol ($P < 0.01$) and BUN ($P < 0.01$) and decreased glucose ($P < 0.01$) and NEFA ($P < 0.01$) in all experimental groups compared with that in the basal diet group (Table 7). Increased dietary protein levels rose glucose ($P < 0.01$) and NEFA ($P < 0.01$) (Table 7). Blood cortisol ($P < 0.01$), BUN ($P < 0.05$) increased as HS level increases but decreased glucose ($P < 0.01$) and NEFA ($P < 0.01$) (Table 7).

Supplement of different dietary protein levels decreased lysine ($P < 0.05$), glutamic acid ($P < 0.05$), valine ($P < 0.05$), and histidine ($P < 0.05$) in all experimental groups compared with that in the basal diet group during HS (Table 8). Increased dietary protein levels enhanced lysine ($P < 0.05$), and glutamic acid ($P < 0.05$). Increased level of HS decreased lysine ($P < 0.05$), glutamic acid ($P < 0.05$), valine ($P < 0.01$), and histidine ($P < 0.05$) (Table 8).

The effects of dietary protein levels on HSP70 mRNA expression in PBMCs and hair follicles under heat stress

The HSP70 gene expression in PBMCs and hair follicles were evaluated by time course over the total period (28 days: Figure 2 and 3). In mild HS stress, HSP70 gene expression was increased in LP, MP, and HP groups after rapid exposure to HS conditions (LP, $P < 0.01$; MP, $P < 0.05$; HP, $P < 0.05$). In moderate HS stress, HSP70 gene expression was increased in LP, MP, and HP groups after rapid exposure to HS conditions (LP, $P < 0.05$; MP, $P < 0.05$; HP, $P < 0.05$). In severe HS stress, HSP70 gene expression was increased in LP, MP, and HP groups after rapid exposure to HS conditions (LP, $P < 0.01$; MP, $P < 0.01$; HP, $P < 0.05$).

In hair follicles, protein supplements did not alter the expression of HSP70 gene in mild HS stress (Figure 3). In moderate HS stress, HSP70 gene expression was increased by LP, MP, and HP treatments after rapid exposure to HS conditions (LP, MP, and HP; $P < 0.05$). In severe HS stress, HSP70 gene expression was increased in LP, MP, and HP groups after rapid exposure to HS conditions (LP, $P < 0.01$; MP, $P < 0.01$; HP, $P < 0.05$).

Discussion

Climate change causes increased Earth's average yearly surface temperature, and it is inextricably linked with animal agriculture. It has been reported that a drastic rise in temperature and humidity may affect animal's physiology and productivity in a direct or indirect manner. Among others, the number of studies conducted in cattle reported the effects of HS.

In previous dairy cow studies, high-yield cows started to reduce milk yield at a THI of approximately 68¹⁴. Dairy cows exposed to climate above the threshold THI (mean THI = 82.4) showed hyperthermia, tachypnea, and a significant reduction in DMI²⁶. In calves, HS also reduced DMI and ADG^{20,27}. Similar to the findings of previous studies, our current results

showed that the beef calves exposed to HS decreased DMI and ADG. Reduced feed intake is a conserved response among animals that received HS and is likely an attempt to reduce metabolic heat production ^{8,11}.

However, we observed that 17.5 % (HP) of dietary protein level did not affect DMI but increased protein intake, BW gain, and ADG under HS conditions (Table 5). There are several nutritional strategies to consider in cattle while under HS.

Feeding energy and nutrient-dense diet, e.g., reduced fiber, increased supplemental fat, and AA, is commonly used to compensate for the reduced feed intake under HS ²⁸. Primarily, protein is one of the most critical nutrients offered during HS conditions to calves ¹². Protein is required daily for young animals to maintain biological processes, such as repairing tissues and producing blood. Especially in growing calves, protein is one of the most critical nutrients for muscle growth and development ^{6,12}.

In previous studies, the effects of supplying protein and improving protein solubility are controversial ^{29,30}. Baumgard and Rhoads Jr ³¹ stated that appropriate dietary protein improved growth performance in calves, but the excess dietary protein harmed energy cost. Excess N above requirements reduces ME by 7.2 kcal/g of N ³². When 19 and 23 % of CP diets were fed, milk yield was reduced by over 1.4 kg ³³. The energy cost associated with synthesizing and excreting urea accounted for the reduced milk yield ³⁴. Dietary protein degradability may be especially important under HS conditions. A possible reason why highly degradable protein diets may be harmful during HS was reduced rumen motility and passage rate ²⁸. This allows for a longer residence time and thus more extensive protein degradation.

Also, diets with low (31.2% of CP) and high (39.2% of CP) RUP fed during HS had no effect on DMI; however, milk yield increased by 2.4 kg/d and BUN reduced from 17.5 to 13.3 mg/100 mL for the diet containing higher RUP ³⁵. In the current study, heat-stressed calves increased BUN levels. The mechanism of BUN increment is unclear, but this might be due to

exceeding rumen ammonia production or protein degradation from skeletal muscle ^{36,37}. Exceeding ammonia production can be harmful to ruminants and requires extra energy to synthesize urea or egest (7.2 kcal/g of nitrogen; and this increases heat production ³². However, the protein supplements in this study did not affect the BUN level of calves throughout the trial. Our result is consistent with previous report that revealed additional protein (17.5%) supplements under high ambient temperature can be beneficial to maintain skeletal muscle growth without adverse effect ³⁸.

It has been previously reported that the blood parameters can be affected by HS. Foremost, HS increased the blood level of fight or flight lipophilic steroid called cortisol throughout the trial. In multiple studies, the correlation between HS and blood cortisol levels were observed ^{19,27,39}.

In the current study, blood glucose and NEFA decreased in HS groups. This might be related to the negative energy balance resulting from the decreased DMI. This was agreed with previous findings in the rat ⁴⁰, chicken ⁴¹, sheep ⁴², and cows ⁴³. A possible scenario that could explain decreased blood glucose level under HS is that of increased glucose utilization as an anti-stress response. Insufficient glucose supplements because of reduced DMI and increased cell glucose uptake could be another reason for decreased blood glucose ³².

HS condition also appears to influence the lipogenic activity in the animal body. Increased blood NEFA level is typically observed in restricted-fed animals when using lipid as an energy source. A rise in the concentration of circulating NEFAs elevates fatty acid oxidation, thereby maintaining the animal body's glucose level ³¹. Post-absorbable carbohydrate metabolism is also altered by reduced insulin action during the dietary restriction, reducing glucose absorption by adipose tissue ⁶. The reduced absorption of nutrients coupled with the prolonged net release of NEFA by adipose tissue is a key homeostatic mechanism implemented by malnutrition animals ⁴⁴ that can occur due to HS as a consequence of decreased feed intake.

Despite the fact that the blood NEFA level is regulated by energy availability, multiple studies previously noted reduced plasma NEFA level under heat-stressed conditions in rodents⁴⁵, pigs⁴⁶, sheep⁴⁷, and cattle^{26,48} while overall DMI was reduced. This might be related to ketone metabolism. In cattle, high-temperature conditions adversely affect lipogenesis that mobilizes adipose tissue and reduce ketone body synthesis through NEFA, which results in increased reliance on glucose utilization in the body⁴⁹. Also, Baumgard and Rhoads Jr³¹ suggested that this is a natural mechanism for withstanding heat loads, as β -oxidation of NEFA can generate more metabolic heat than that of carbohydrates.

Moreover, HS increased lipoprotein lipase in adipose tissue⁴⁵, suggesting that adipose tissue of hyperthermia animals has an increased capacity to absorb and store intestinal and hepatic-derived triglycerides³¹.

In the current study, heat-stressed animals given 17.5 % of dietary protein levels (HP) tended to increase blood glucose and NEFA. This may indicate that the HP was used as an energy source to synthesize from glucose and NEFA³¹.

Changes in postabsorptive nutrient partitioning support a controlling physiological state. A well-described mechanism is a glucose-sparing effect is that growing animals utilize fat or protein source when on a lowered plane of nutrition or in a negative balance. Inadequate nutrient consumption during HS conditions is associated with various metabolic changes implemented to support the synthesis of high-priority tissues like a skeletal muscle⁵⁰.

We also noted that the blood contents of lysine, glutamic acid, valine, and histidine are decreased as HS is severe. When plasma glucose level is low, AA utilization from gluconeogenesis is observed in heat-stressed cows⁵¹. Additionally, we assumed that reduced blood flow might also be suppressed the absorption of AAs. Heat stress alters the nutrient partitioning, leading to reducing the delivery of protein precursors to the adipose tissue or muscle³². In the same context, cows under HS reduced plasma free AA, and increased BUN,

MUN, and UUN, which combined with similar microbial crude protein synthesis and decreased milk protein output, suggesting increased whole-body AAs utilization²⁶. Previous studies revealed that the contribution of AAs to the circulating glucose pool is increased during the HS situation^{26,52}. 78% of plasma AAs reduction was due to the decline in gluconeogenic AAs²⁶. This study suggests that the plasma AAs decline during HS may result from increased AAs utilization for gluconeogenesis. We found that HP increased lysine and glutamic acid in heat-stressed Korean native beef calves in the current study.

In cattle, lysine is inadequately synthesized in the body; therefore, AA needs to be provided by the diet to fulfill the requirement. In addition, glutamic acid is one of the semi-essential AAs in skeletal muscle⁵³. Lower lysine and glutamic acid concentration could be related to DMI reduction due to HS. Increased dietary protein led to improved lysine and glutamic acid utilization in animals under HS. It also led to help calves to sustain growth.

Additionally, other amino acids including lysine, methionine, glutamic acid, valine, isoleucine, and leucine were increased in the HP group. Based on our findings, the supplement of a higher protein level is suggested during heat exposure in calves. Additional protein supplements can cause increased glucogenic and ketogenic activity, which can diminish the adverse effects of HS.

The HSP genes result in the expression of proteins with chaperone roles that act in protecting cells from HS and removing damaged proteins^{21,54}. In the current study, the HSP70 gene expression in PBMCs and hair follicles was increased in HS groups. One of the signals for HSP70 synthesis is protein denaturation in response to elevated temperature⁵⁵. The transcription of HSP genes and, consequently, the synthesis of HSPs are enhanced upon exposure of cells to HS⁵⁶. The hyperthermia resulting from HS can also disturb the cellular functions and result in physiological changes that cause a negative impact on animal production (Hansen, 2004). In cattle, HSP72 is absent or expressed at a low level under non-stressed

conditions and is referred to as the inducible member of the HSP70 family ⁵⁷. The expression level of HSP70 was reported to increase at the cellular level under HS conditions ⁵⁸. The current study showed that HSP70 gene expression in hair follicles increased in all dietary protein level groups (LP, MP, and HP). This can be interpreted as an appropriate dietary protein level (17.5%) that affects the synthesis of HSP70 and enhances immune function. In addition, our current study suggested that the hair follicles can be used as a non-invasive method to demonstrate the level of HSP in HS animals.

Conclusion

Heat stress directly affected protein metabolism and growth, including decreased BW gain and ADG in heat-stressed Korean native beef calves. The decreased DMI cannot wholly define the decline in BW gain and ADG. The decrease in blood glucose, NEFA, and blood AAs profiles and increased BUN and HSP70 gene expression during heat stress affect growth through energy and protein metabolism. In conclusion, the appropriate dietary protein level was considered to be 17.5% due to improved growth performance by energy and protein metabolism, utilization of AAs and the synthesis of HSP70, and enhanced immune function (Figure 4).

Abbreviations

HS: Heat stress; LP: Low protein; MP: Medium protein; HP: High protein; THI: Temperature-Humidity Index; ADG: Average daily gain; HR: Heart rate; RT: Rectal temperature; NEFA: Non-esterified fatty acids; AAs: Amino acids; PBMC: Peripheral blood mononuclear cell; HSP: Heat shock protein; DMI: Dry matter intake; NRC: National Research Council; SNP: Single nucleotide polymorphism; RH: Relative humidity; CP: Crude protein; TN: Thermoneutral; BW: Body weight; DM: Dry matter; NDF: neutral detergent fiber; ADF: Acid

detergent fiber; AOAC: Association of official analytical chemists; BPM: Beat per minute; BUN: Blood urea nitrogen; PBS: Phosphate buffered saline; DEPC: Diethyl pyrocarbonate; RNA: Ribonucleic acid; PCR: Polymerase chain reaction; DNA: Deoxyribonucleic acid; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; RPS15A: Ribosomal protein S15a; B2M: Beta 2 microglobulin; EDTA: Ethylenediaminetetraacetic acid; GLM: Generalized linear model; HSD: Honest significant difference; ME: Metabolizable energy; RUP: Rumen undegradable protein; MUN: Milk urea nitrogen; UUN: Urine urea nitrogen; MCP: Microbial crude protein;

Declaration of competing interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Author statement

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References

- 1 Rhoads, M. et al. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. *J. Dairy Sci.* 92, 1986-1997 (2009).
- 2 Kim, K. H. et al. Productivity and energy partition of late lactation dairy cows during heat exposure. *Anim. Sci. J.* 81, 58-62 (2010).
- 3 Farooq, U., Samad, H., Shehzad, F. & Qayyum, A. Physiological responses of cattle to heat stress. *World Appl. Sci. J* 8, 38-43 (2010).
- 4 West, J. W. Interactions of Energy and Bovine Somatotropin with Heat Stress. *J. Dairy Sci.* 77, 2091-2102 (1994).
- 5 National Research Council. Nutrient requirements of dairy cattle: 7th edition. Washington, DC (2001).
- 6 O'brien, M., Rhoads, R., Sanders, S., Duff, G. & Baumgard, L. Metabolic adaptations to heat stress in growing cattle. *Domest. Anim. Endocrinol* 38, 86-94 (2010).
- 7 Morrison, S. Ruminant heat stress: effect on production and means of alleviation. *J. Anim. Sci.* 57, 1594-1600 (1983).
- 8 Beede, D. & Collier, R. Potential nutritional strategies for intensively managed cattle during thermal stress. *J. Anim. Sci.* 62, 543-554 (1986).
- 9 Paula-Lopes, F. et al. Genetic divergence in cellular resistance to heat shock in cattle: differences between breeds developed in temperate versus hot climates in responses of preimplantation embryos, reproductive tract tissues and lymphocytes to increased culture temperatures. *Reproduction* 125, 285-294 (2003).
- 10 Hansen, P. Physiological and cellular adaptations of zebu cattle to thermal stress. *Anim. Reprod. Sci.* 82, 349-360 (2004).
- 11 West, J. W. Effects of heat-stress on production in dairy cattle. *J. Dairy Sci.* 86, 2131-2144 (2003).
- 12 National Academies of Sciences, E., Medicine. Nutrient Requirements of Beef Cattle: Eighth Revised Edition. Washington, DC: The National Academies Press (2016).
- 13 Basiricò, L. et al. Cellular thermotolerance is associated with heat shock protein 70.1 genetic polymorphisms in Holstein lactating cows. *Cell Stress Chaperones* 16, 441-448 (2011).
- 14 Zimbelman, R. et al. A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high producing dairy cows. *Proceedings of the Southwest Nutrition Conference* (ed. RJ Collier). 158-169 (2009).
- 15 Kim, W. S. et al. Identification of heat shock protein gene expression in hair follicles as a novel indicator of heat stress in beef calves. *Animal* 14, 1502-1509 (2020).
- 16 National Research Council. A guide to environmental research on animals. *Natl. Acad. Sci.* Washington, DC(1971).

507 17 AOAC. Official methods of analysis, 14th edn, Washington, DC. Assoc Anal Chem (1990).

508 18 Van Soest, P. v., Robertson, J. & Lewis, B. Methods for dietary fiber, neutral detergent fiber, and
509 nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583-3597 (1991).

510 19 Kim, W. S. et al. Correlation between blood, physiological and behavioral parameters in beef calves
511 under heat stress. *Asian-Australas J Anim Sci* 31, 919-925 (2018).

512 20 Kim, W.-S., Peng, D.-Q., Jo, Y.-H., Nejad, J. G. & Lee, H.-G. Responses of beef calves to long-
513 term heat stress exposure by evaluating growth performance, physiological, blood and behavioral
514 parameters. *J. Therm. Biol.* 100, 103033 (2021).

515 21 Kim, W.-S., Ghassemi Nejad, J., Roh, S.-G. & Lee, H.-G. Heat-Shock Proteins Gene Expression in
516 Peripheral Blood Mononuclear Cells as an Indicator of Heat Stress in Beef Calves. *Animals* 10, 895
517 (2020).

518 22 Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative CT method.
519 *Nat. Protoc.* 3, 1101-1108 (2008).

520 23 Kishore, A. et al. Selection of stable reference genes in heat stressed peripheral blood mononuclear
521 cells of tropically adapted Indian cattle and buffaloes. *Mol. Cell. Probes* 27, 140-144 (2013).

522 24 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time
523 quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods (San Diego, Calif.)* 25, 402-408
524 (2001).

525 25 Peng, D. Q. et al. Effect of alcohol dehydrogenase 1C (ADH1C) genotype on vitamin A restriction
526 and marbling in Korean native steers. *Asian-Australas J Anim Sci* 30, 1099-1104,
527 doi:10.5713/ajas.16.0708 (2017).

528 26 Gao, S. et al. The effects of heat stress on protein metabolism in lactating Holstein cows. *J. Dairy*
529 *Sci.* 100, 5040-5049 (2017).

530 27 Tao, S., Monteiro, A. P. A., Thompson, I. M., Hayen, M. J. & Dahl, G. E. Effect of late-gestation
531 maternal heat stress on growth and immune function of dairy calves. *J. Dairy Sci.* 95, 7128-7136
532 (2012).

533 28 Baumgard, L. et al. Feeding and managing cows to minimize heat stress, Proc. 23rd Tri-State Dairy
534 Nutrition Conference, Fort Wayne, Indiana, Ohio State University, Ames, 61-74 (2014).

535 29 Huber, J. et al. Heat stress interactions with protein supplemental Fat, and fungal cultures. *J. Dairy*
536 *Sci.* 77, 2080-2090 (1994).

537 30 Huber, J., Wu, Z., Chan, S. & Chen, K. Feeding for high production during heat stress. Western
538 Large Herd Management Conf. Las Vegas, NV, 183-192 (1993).

539 31 Baumgard, L. H. & Rhoads Jr, R. P. Effects of heat stress on postabsorptive metabolism and
540 energetics. *Annu. Rev. Anim. Biosci.* 1, 311-337 (2013).

541 32 Tyrrell, H. Influence of excess protein intake on energy metabolism of the dairy cow. Fifth Symp.
542 Energy Metab. Farm Anim. 69-71 (1970).

543 33 Danfaer, A., Thysen, I. & Østergaard, V. Effect of the level of dietary protein on milk production. I.
544 Milk yield, liveweight gain and health. Research Report no. 492, Copenhagen, Beretning fra
545 Stratens Husdyrbrugs forsog (1980).

546 34 Oldham, J. Protein-energy interrelationships in dairy cows. *J. Dairy Sci.* 67, 1090-1114 (1984).

547 35 Belibasakis, N., Ambatzidis, P., Aktsali, P. & Tsirgogianni, D. Effects of degradability of dietary
548 protein on milk production and blood components of dairy cows in hot weather. *World Rev. Anim.*
549 *Prod.* 30, 21-26 (1995).

550 36 Getahun, D., Getabalew, M., Zewdie, D., Alemneh, T. & Akebereg, D. Urea Metabolism and
551 Recycling in Ruminants. *BJSTR* 20, 14790-14796 (2019).

552 37 Klusmeyer, T. H., McCarthy, R. D., Jr., Clark, J. H. & Nelson, D. R. Effects of source and amount
553 of protein on ruminal fermentation and passage of nutrients to the small intestine of lactating cows.
554 *J. Dairy Sci.* 73, 3526-3537 (1990).

555 38 Bunting, L. D. et al. Seasonal Effects of Supplemental Fat or Undegradable Protein on the Growth
556 and Metabolism of Holstein Calves. *J. Dairy Sci.* 79, 1611-1620 (1996).

557 39 Ghassemi Nejad, J. et al. Wool cortisol is a better indicator of stress than blood cortisol in ewes
558 exposed to heat stress and water restriction. *Animal* 8, 128-132 (2014).

559 40 Mitev, S., Dinevska-Kovkarovska, S. & Miova, B. Effect of the acclimation to high environmental
560 temperature on the activity of hepatic glycogen phosphorylase (a+ b and a), liver glycogen content
561 and blood glucose level in rats. *J. Therm. Biol.* 30, 563-568 (2005).

562 41 Rahimi, G. Effect of heat shock at early growth phase on glucose and calcium regulating axis in
563 broiler chickens. *Int. J. Poult. Sci* 4, 790-794 (2005).

564 42 Achmadi, J., Yanagisawa, T., Sano, H. & Terashima, Y. Pancreatic insulin secretory response and
565 insulin action in heat-exposed sheep given a concentrate or roughage diet. *Domest. Anim.*
566 *Endocrinol.* 10, 279-287 (1993).

567 43 Itoh, F., Obara, Y., Rose, M. T., Fuse, H. & Hashimoto, H. Insulin and glucagon secretion in
568 lactating cows during heat exposure. *J. Anim. Sci.* 76, 2182-2189 (1998).

569 44 Vernon, R. G. Effects of diet on lipolysis and its regulation. *Proceedings of the Nutrition Society*
570 51, 397-408 (1992).

571 45 Sanders, S. R., Cole, L. C., Flann, K. L., Baumgard, L. H. & Rhoads, R. P. Effects of acute heat
572 stress on skeletal muscle gene expression associated with energy metabolism in rats. *FASEB*
573 *Journal* 23, 598 (2009).

574 46 Pearce, S. C. et al. Effects of heat stress on energetic metabolism in growing pigs. *FASEB Journal*
575 25, 1052 (2011).

576 47 Sano, H., Takahashi, K., Ambo, K. & Tsuda, T. Turnover and oxidation rates of blood glucose and
577 heat production in sheep exposed to heat. *J. Dairy Sci.* 66, 856-861 (1983).

578 48 Shwartz, G., Rhoads, M., VanBaale, M., Rhoads, R. & Baumgard, L. Effects of a supplemental
579 yeast culture on heat-stressed lactating Holstein cows. *J. Dairy Sci.* 92, 935-942 (2009).

- 49 Wheelock, J., Rhoads, R., VanBaale, M., Sanders, S. & Baumgard, L. Effects of heat stress on energetic metabolism in lactating Holstein cows. *J. Dairy Sci.* 93, 644-655 (2010).
- 50 Van Milgen, J. & Noblet, J. Partitioning of energy intake to heat, protein, and fat in growing pigs. *J. Anim. Sci.* 81, E86-E93 (2003).
- 51 Cowley, F., Barber, D., Houlihan, A. & Poppi, D. Immediate and residual effects of heat stress and restricted intake on milk protein and casein composition and energy metabolism. *J. Dairy Sci.* 98, 2356-2368 (2015).
- 52 Colditz, P. & Kellaway, R. The effect of diet and heat stress on feed intake, growth, and nitrogen metabolism in Friesian, F1 Brahman× Friesian, and Brahman heifers. *Aust. J. Agric. Res* 23, 717-725 (1972).
- 53 Kim, Y.-S. et al. Effect of glutamine on heat-shock protein beta 1 (HSPB1) expression during myogenic differentiation in bovine embryonic fibroblast cells. *Food Sci Biotechnol* 27, 829-835 (2018).
- 54 Parsell, a. & Lindquist, S. The Function of Heat-Shock Proteins in Stress Tolerance: Degradation and Reactivation of Damaged Proteins. *Annu. Rev. Genet* 27, 437-496 (1993).
- 55 Ananthan, J., Goldberg, A. L. & Voellmy, R. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* 232, 522-524 (1986).
- 56 Zou, J., Guo, Y., Guettouche, T., Smith, D. F. & Voellmy, R. Repression of Heat Shock Transcription Factor HSF1 Activation by HSP90 (HSP90 Complex) that Forms a Stress-Sensitive Complex with HSF1. *Cell* 94, 471-480 (1998).
- 57 Kristensen, T. N., Løvendahl, P., Berg, P. & Loeschcke, V. Hsp72 is present in plasma from Holstein-Friesian dairy cattle, and the concentration level is repeatable across days and age classes. *Cell Stress Chaperones* 9, 143-149 (2004).
- 58 Lacetera, N. et al. Heat Stress Elicits Different Responses in Peripheral Blood Mononuclear Cells from Brown Swiss and Holstein Cows. *J. Dairy Sci.* 89, 4606-4612 (2006).

605 **Table 1. Ingredient composition of experimental diets.**

	Threshold		Mild		Moderate			Severe		
	Bas	LP	MP	HP	LP	MP	HP	LP	MP	HP
Ingredient, %										
of DM										
Corn Grain,										
Steamed flaked	21.1	28.5	28.3	28.8	29.8	29.5	28.7	29.9	29.2	29.8
Soybean Meal	8.9	7.9	14.5	27.5	10.1	18.2	27.2	10.1	18.3	27.1
Wheat bran	21.9	14.1	3.7	1.5	9.9	2.8	0.3	9.8	2.8	0.2
Corn germ meal	6.1	7.5	11.5	0.2	8.2	7.5	1.8	8.2	7.7	0.9
Timothy hay	40	40	40	40	40	40	40	40	40	40
Limestone	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
Vit & mineral										
premix*	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

606 * The premix contained (% as-is basis): trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix, 37.2; selenium premix, 0.07; and
607 dry corn distillers grains with soluble, 45.7%; Ca, 12.1%; P, 0.41%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 383 mg/kg; Zn, 884 mg/kg; Fe,
608 216 mg/kg, vitamin A, 300,000 IU/kg, vitamin D, 85,000 IU/kg and vitamin E, 2,300 IU/kg.
609 THI = Temperature humidity index, $THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$.
610 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.
611 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

612 **Table 2. The chemical composition of experimental diets provided to the Korean native beef calves.**

	Threshold		Mild		Moderate			Severe		
	Bas	LP	MP	HP	LP	MP	HP	LP	MP	HP
Analyzed										
composition, %										
of DM										
CP	12.48	12.47	14.89	17.39	12.52	15.04	17.54	12.59	15.10	17.45
RDP ¹	5.96	5.46	6.18	7.04	5.30	6.13	7.06	5.32	6.17	6.95
RUP ¹	6.39	7.01	8.71	10.35	7.22	8.91	10.48	7.27	8.93	10.50
TDN ¹	70.44	72.17	72.88	73.20	72.68	73.17	73.27	72.71	73.12	73.45
TDN ¹ (kg)	3.63	3.06	3.09	3.10	3.03	3.05	3.06	2.78	2.79	2.81
NDF	40.62	38.48	37.37	35.02	37.69	36.37	35.09	37.66	36.45	34.78
ADF	20.70	19.96	19.73	19.48	19.75	19.56	19.47	19.74	19.59	19.38
Fat	3.83	3.81	4.36	4.05	3.93	4.20	4.16	3.93	4.22	4.08
Ash	5.71	5.37	5.40	5.65	5.33	5.44	5.64	5.33	5.45	5.60

Ca	0.94	0.93	0.94	0.98	0.93	0.95	0.97	0.93	0.95	0.97
P	0.49	0.44	0.42	0.36	0.42	0.39	0.37	0.42	0.39	0.36
ME ¹ (Mcal/kg)	2.55	2.61	2.63	2.65	2.63	2.64	2.65	2.63	2.64	2.65
NEm ¹ (Mcal/kg)	1.64	1.69	1.72	1.73	1.71	1.73	1.73	1.71	1.72	1.73
NEg ¹ (Mcal/kg)	1.04	1.08	1.10	1.11	1.09	1.11	1.11	1.09	1.11	1.11

613 ¹Values were estimated using NRC (2016).

614 THI = Temperature humidity index, $THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$.

615 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.

616 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

617 **Table 3. Amino acids (AA) concentration (%) of experimental diets.**

	Threshold		Mild		Moderate			Severe			SEM	<i>P</i> -value
	Bas	LP	MP	HP	LP	MP	HP	LP	MP	HP		
Analyzed												
composition, %												
of DM												
Lysine	0.34 ^b	0.31 ^b	0.67 ^{ab}	1.42 ^a	0.27 ^b	0.81 ^{ab}	1.67 ^a	0.22 ^b	0.52 ^{ab}	1.52 ^a	0.058	< 0.01
Methionine	0.21 ^b	0.24 ^b	0.31 ^{ab}	0.52 ^a	0.22 ^b	0.44 ^a	0.51 ^a	0.19 ^b	0.42 ^a	0.41 ^a	0.067	< 0.01
Aspartic acid	0.62	0.77	0.81	0.72	0.81	0.71	0.68	0.62	0.68	0.61	0.041	0.13
Serine	0.42	0.57	0.62	0.45	0.42	0.62	0.54	0.52	0.58	0.47	0.028	0.65
Glutamic acid	2.14 ^c	2.28 ^c	2.81 ^b	3.51 ^a	2.12 ^c	2.77 ^b	3.62 ^a	2.12 ^c	2.92 ^b	3.42 ^a	0.072	< 0.01
Glycine	0.72	0.67	0.71	0.82	0.55	0.62	0.77	0.57	0.62	0.85	0.048	0.72
Alanine	1.13	0.92	1.12	1.14	0.81	1.09	1.02	0.74	1.11	1.41	0.082	0.69
Valine	0.82 ^b	0.85 ^b	0.91 ^b	1.32 ^a	0.71 ^b	0.82 ^b	1.21 ^a	0.92 ^b	0.82 ^b	1.42 ^a	0.032	< 0.01
Isoleucine	0.57 ^b	0.62 ^b	0.91 ^a	0.82 ^a	0.55 ^b	0.92 ^a	0.71 ^{ab}	0.52 ^b	0.97 ^a	0.97 ^a	0.057	<0.01
Leucine	1.92 ^b	1.82 ^b	1.89 ^b	2.42 ^a	1.71 ^b	1.67 ^b	2.62 ^a	1.87 ^b	1.97 ^b	2.32 ^a	0.081	<0.01

Tyrosine	0.42	0.32	0.47	0.54	0.42	0.55	0.31	0.52	0.37	0.33	0.074	0.82
Phenylalanine	0.65	0.72	0.62	0.67	0.55	0.71	0.77	0.91	0.71	0.58	0.092	0.51
Histidine	0.45	0.42	0.57	0.55	0.42	0.52	0.42	0.37	0.52	0.47	0.074	0.84
Arginine	0.92	0.82	1.12	0.92	0.91	1.09	0.81	0.97	0.88	0.82	0.078	0.64
Tryptophan	0.14	0.11	0.17	0.15	0.21	0.07	0.09	0.11	0.21	0.09	0.001	0.67
Cystine	0.24	0.32	0.33	0.42	0.44	0.42	0.37	0.42	0.41	0.38	0.081	0.75
Proline	0.92	0.52	0.87	0.71	0.62	0.51	0.62	0.41	0.62	0.59	0.031	0.82

618 THI = Temperature humidity index, $THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$.

619 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.

620 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

621 ^{a, b}Values with different superscripts in the same row differ significantly at $P < 0.05$.

622 **Table 4. Primer sequences, lengths, and accession numbers in bovine.**

Gene	Accession number	Sequence	Length (bp)
HSP70	U09861	F: TACGTGGCCTTCACCGATAC R: GTCGTTGATGACGCGGAAAG	171
GAPDH	NM_001034034.2	F: GGCAAGGTCATCCCTGAG R: GCAGGTCAGATCCACAACAG	166
RPS15A	NM_001037443.2	F: CCGTGCTCCAAAGTCATCGT R: GGGAGCAGGTTATTCTGCCA	200
B2M	NM_173893.3	F: GACACCCACCAGAAGATGGA R: CAGGTCTGACTGCTCCGATT	125

623 HSP = Heat shock protein; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; RPS15A =

624 ribosomal protein S15a; B2M = beta-2-microglobulin

625 **Table 5. Effects of dietary protein levels on DMI, protein intake, and growth performance in Korean native beef calves under heat stress.**

Item	Threshold	Mild			Moderate			Severe			P-values for contrast			
	Bas	LP	MP	HP	LP	MP	HP	LP	MP	HP	vs Bas	Protein	THI	Protein*THI
DMI, kg/d	53±04	43±02	44±01	45±04	42±02	41±01	41±02	37±02	38±02	39±03	<001	084	<001	089
Protein intake, g/d	6354±1214	5190±94	6245±121	7236±199	5171±125	6176±235	7096±238	4841±116	5791±266	6580±207	<001	<001	<001	095
Initial BW, kg	205.1±185	2006±137	2006±125	2040±112	1952±103	1913±82	1962±101	2071±89	2096±52	2120±98	095	090	021	099
Final BW, kg	227.7±174	221.1±149	2228±108	2268±114	2099±112	2123±70	2178±107	2198±116	2253±72	2328±92	094	060	032	099
BW Gain, kg	226±13	205±15	222±34	228±19	146±16	210±15	216±07	126±31	156±24	208±08	<001	<001	<005	052
ADG, kg	081±005	073±006	079±012	082±007	052±006	075±005	077±003	045±011	056±009	074±003	<0.01	<001	<005	053

626 vs Bas = Control (with 12.5% dietary protein levels, THI 68 to 70) vs 9 experimental diets (with 3 dietary protein levels, with 3 THI levels).

627 THI = Temperature humidity index, $THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$.

628 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.

629 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

630 **Table 6. Effects of dietary protein levels on heart rate and rectal temperature in Korean native beef calves under heat stress.**

	Threshold	Mild			Moderate			Severe			<i>P</i> -values for contrast			
	Bas	LP	MP	HP	LP	MP	HP	LP	MP	HP	vs Bas	Protein	THI	Protein*THI
HR														
TN	66.3 ± 2.9	66.8 ± 2.3	70.5 ± 3.2	66.8 ± 2.3	65.0 ± 3.0	64.8 ± 3.3	69.3 ± 7.5	64.8 ± 2.7	68.0 ± 3.2	63.3 ± 3.7	0.79	0.63	0.53	0.54
HS	69.5 ± 3.4	74.0 ± 0.8	74.3 ± 2.7	72.0 ± 4.4	77.8 ± 3.1	80.8 ± 3.3	77.3 ± 4.7	87.5 ± 2.9	87.3 ± 4.4	84.3 ± 4.8	< 0.05	0.62	< 0.01	0.99
RT														
TN	39.0 ± 0.1	38.8 ± 0.1	39.0 ± 0.1	38.9 ± 0.1	39.0 ± 0.1	38.9 ± 0.2	38.8 ± 0.1	38.9 ± 0.1	38.9 ± 0.1	39.0 ± 0.1	0.83	0.76	0.91	0.53
HS	39.0 ± 0.1	39.3 ± 0.1	39.3 ± 0.1	39.3 ± 0.1	39.6 ± 0.1	39.7 ± 0.1	39.7 ± 0.0	39.8 ± 0.1	40.0 ± 0.1	40.0 ± 0.1	< 0.01	0.26	< 0.01	0.56

631 vs Bas = Control (with 12.5% dietary protein levels, THI 68 to 70) vs 9 experimental diets (with 3 dietary protein levels, with 3 THI levels).

632 THI = Temperature humidity index, $THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$.

633 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.

634 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

635 **Table 7. Effects of dietary protein levels on blood parameters in Korean native beef calves under heat stress.**

	Threshold	Mild			Moderate			Severe			<i>P</i> -values for contrast			
	Bas	LP	MP	HP	LP	MP	HP	LP	MP	HP	vs Bas	Protein	THI	Protein*THI
Cortisol														
(ng/mL)														
TN	82±10	11.1±13	88±17	86±14	92±18	87±21	87±27	101±16	86±22	97±16	098	062	089	097
HS	92±13	92±11	98±23	98±18	101±16	84±22	93±18	168±16	223±21	168±44	<001	064	<001	052
BUN														
(µg/mL)														
TN	167±12	148±09	142±07	135±08	128±08	132±07	142±09	151±09	147±08	138±12	090	092	091	094
HS	172±08	139±08	157±14	142±09	181±07	188±08	179±08	218±07	224±05	225±07	<001	093	<005	084
Glucose														
(µg/mL)														
TN	665±22	653±21	648±26	688±44	638±23	650±31	640±29	663±30	643±64	650±31	099	082	042	087
HS	685±17	665±22	638±26	653±32	590±26	535±43	648±30	383±47	408±64	595±40	<001	<001	<001	007
NEFA														
(uEq/L)														
TN	2042±352	227±181	1767±287	1895±321	1576±421	2104±447	1821±241	1627±182	1917±181	2211±181	098	091	079	094
HS	2205±287	2005±175	1687±208	1783±192	1654±187	1856±243	1898±70	1253±198	162±178	1565±200	<001	<001	<001	019

636 vs Bas = Control (with 12.5% dietary protein levels, THI 68 to 70) vs 9 experimental diets (with 3 dietary protein levels, with 3 THI levels).

637 THI = Temperature humidity index, $THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$.

638 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.

639 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

640 **Table 8. Effects of dietary protein levels on blood amino acid profiles in Korean native beef calves under heat stress.**

	Threshold	Mild			Moderate			Severe			<i>P</i> -values for contrast			
	Bas	LP	MP	HP	LP	MP	HP	LP	MP	HP	vs Bas	Protein	THI	Protein*THI
A.A in plasma														
(nmol)														
Lysine	84.3±8.2	80.9±6.5	75.5±6.2	82.5±7.5	78.4±4.2	74.4±2.8	85.5±1.8	67.5±4.7	72.5±7.1	83.5±7.2	<0.05	<0.05	<0.05	<0.05
Methionine	92.4±10.5	90.8±4.2	85.5±7.2	83.5±7.2	72.5±4.2	74.5±10.1	77.4±7.2	68.5±7.1	65.5±7.2	64.7±4.2	0.09	0.17	<0.05	0.48
Aspartic acid	22.7±8.2	19.8±4.2	12.5±7.1	22.4±4.1	19.2±7.1	22.4±3.1	19.2±3.2	24.5±3.7	22.4±4.2	25.7±2.2	0.92	0.12	0.62	0.52
Serine	109.1±15.3	102.5±4.1	108.2±9.2	121.1±5.6	122.4±7.2	125.1±7.1	122.1±3.2	125.4±7.1	129.1±7.2	122.1±7.2	0.62	0.84	0.62	0.83
Glutamic acid	242.5±28.4	228.4±7.2	220.4±8.2	246.7±15.8	200.4±7.2	250.4±7.1	252.3±6.2	192.4±7.1	202.4±2.7	220.4±7.1	<0.05	<0.05	<0.05	0.71
Glycine	298.1±19.5	252.4±18.4	262.7±7.2	272.4±3.5	267.4±7.2	292.7±6.2	301.4±23.8	261.5±7.2	292.7±6.3	322.7±6.8	0.42	0.32	0.43	0.71
Alanine	288.4±17.6	252.7±6.2	262.7±9.2	277.4±6.2	222.4±9.2	262.4±7.2	267.4±7.7	222.7±6.2	228.2±9.2	242.7±9.2	0.85	0.97	0.52	0.52
Valine	16.8±2.1	14.2±3.4	13.8±4.6	15.7±6.2	10.2±5.4	13.8±7.1	12.2±6.2	8.6±4.3	10.8±4.1	14.6±6.2	<0.05	0.07	<0.01	0.08
Isoleucine	25.2±2.9	23.5±4.9	25.7±9.2	30.4±6.2	29.7±6.2	28.8±6.7	28.7±10.2	34.7±6.2	32.7±1.2	42.7±4.3	0.87	0.76	0.68	0.85
Leucine	32.5±4.2	27.5±6.2	32.4±7.1	33.4±7.2	29.2±6.2	35.7±6.8	42.4±5.5	28.4±6.2	32.7±8.1	42.4±6.2	0.08	0.06	<0.02	0.32
Tyrosine	21.5±7.2	20.9±6.2	18.7±6.2	15.4±6.2	22.8±6.7	21.9±6.4	18.4±2.5	19.4±3.2	15.4±6.1	12.7±6.2	0.71	0.82	0.90	0.82
Phenylalanine	90.8±10.8	82.4±6.2	84.5±9.1	89.4±4.2	85.4±6.8	92.4±7.1	95.7±6.2	82.7±6.2	92.7±7.4	105.4±6.2	0.81	0.82	0.46	0.83
Histidine	58.5±4.6	48.7±6.2	52.5±6.7	56.8±6.2	45.7±5.2	58.8±1.7	55.7±4.8	42.7±8.4	45.7±6.2	45.2±2.2	<0.05	0.09	<0.04	0.73

Arginine	100.8±7.2	95.7±16.4	88.7±6.4	98.1±6.8	92.4±6.8	98.7±6.8	92.4±13.5	90.5±6.2	101.4±8.4	108.7±6.8	0.82	0.85	0.62	0.82
Tryptophan	125±1.1	118±6.2	97±6.2	85±0.9	109±6.4	148±6.2	127±6.2	145±6.2	11.4±2.9	128±6.2	0.24	0.46	0.62	0.66
Cystine	39.4±8.2	32.7±6.1	29.7±6.4	28.4±6.2	32.7±2.8	29.8±4.2	25.7±2.5	28.7±6.1	26.4±1.4	22.4±4.2	0.57	0.21	0.69	0.67

641 vs Bas = Control (with 12.5% dietary protein levels, THI 68 to 70) vs 9 experimental diets (with 3 dietary protein levels, with 3 THI levels).

642 THI = Temperature humidity index.

643 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.

644 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

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Figure 1. Agarose gel electrophoresis of HSP70 gene. Lane 1 to 24 are standard for CC type except lane 9. Lane 9 is standard for TC type.

Figure 2. Effects of dietary protein levels on the mRNA expression of HSP70 in the PBMCs of calves during the climate chamber experiment. The data are presented as the means $\pm$ standards error ($n = 4$). Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91. LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

Figure 3. Effects of dietary protein levels on the mRNA expression of HSP70 in hair follicles of claws during the climate chamber experiment. The data are presented as the means $\pm$ standards error ($n = 4$). Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91. LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

Figure 4. Effects of dietary protein levels on productivity in Korean native beef calves.

657 This manuscript has undergone English language editing by HARRISCO. The text has been checked for correct use of grammar and common
658 technical terms, and edited to a level suitable for reporting research in a scholarly journal.
659 HARRISCO uses experienced, native English speaking editors. Full details of the editing service can be found at
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