Effects of Different Protein Levels on Growth Performance and Stress Parameters in Beef Calves Under Heat Stress

Won Seob Kim

Konkuk University

Jalil Ghassemi Nejad

Konkuk University

Dong Qiao Peng

Konkuk University

Yong Ho Jo

Konkuk University

Jong Kyoo Kim

Michigan State University

Hong Gu Lee (✉ hglee66@konkuk.ac.kr)

Konkuk University <https://orcid.org/0000-0002-0679-5663>

Research

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

Posted Date: June 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-631186/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

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

Authors

Won Seob Kim¹, Jalil Ghassemi Nejad¹, Dong Qiao Peng¹, Yong Ho Jo¹, Jong Kyoo Kim²
and Hong Gu Lee^{1*}

¹Department of Animal Science and Technology, Sanghuh College of Life Sciences, Konkuk University, Seoul 05029, Korea

²Animal Science and Food Science and Human Nutrition, Michigan State University, 474 S Shaw Ln #2209D, East Lansing, MI 48824, USA

*Corresponding author: Hong Gu Lee,

Tel: +82-2-450-0523, Fax: +82-2-455-1044, E-mail: hglee66@konkuk.ac.kr

ORCID

Won Seob Kim <https://orcid.org/0000-0002-0234-5665>

Jalil Ghassemi Nejad <https://orcid.org/0000-0001-6578-8829>

Dong Qiao Peng <https://orcid.org/0000-0002-0408-0381>

Yong Ho Jo <https://orcid.org/0000-0002-9842-5765>

Jong Kyoo Kim <https://orcid.org/0000-0002-4827-5762>

Hong Gu Lee <https://orcid.org/0000-0002-0679-5663>

Abstract

Background: This study investigated the effects of dietary protein levels under various heat stress (HS) levels on the growth performance and stress parameters in Korean native beef calves. Forty calves (body weight of 202.2 ± 3.31 kg) were randomly assigned to climatic-controlled chambers with 3×3 factorial arrangements. Treatment groups were 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.

Results: 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.

Conclusions: 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

Background

The drastic change in ambient temperature and humidity can create multiple problems 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 sufficiently [3]. Heat stress induces behavioral and metabolic changes in cattle that are intended to maintain homeothermy, often at the expense of decreased productivity and profitability [4]. 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, which is presumably an evolutionary strategy to reduce the “heat increment” of feeding [5, 6]. This leads to dystrophia and is thought to be responsible for reduced production in growing cattle [7].

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 have been developed to compensate for the effects of HS. Overall intake usually reduces during hot weather, and it may lead to reverse the growth of animals. Reformulation to account for reduced DMI, greater nutrient requirements during HS, dietary heat increment, and avoiding nutrient excesses can help to reduce the adverse effects of HS [11]. In calf growth, protein is important to utilize essential nutrients for promote

skeletal muscle growth [12]. In calves in a hot environment, protein supplementation levels need to be increased to compensate for reduced feed intake [12].

Limited information regarding the dietary protein level of beef calves during HS is available. The Nutrient Requirements of Beef Cattle (2016) stated guidance of specification managements under HS; however, this lacks details. With that, a thorough investigation of dietary protein levels to compensate HS is needed. Therefore, the objective of the current study was to demonstrate the different dietary protein levels under various HS conditions on growth performance and stress parameters of Korean native beef calves. The study can also provide foundational data to establish dietary protein level guidelines under the HS condition of Korean native beef calves.

Methods

Animals and climatic chamber details

Forty male Korean native beef 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 genotyping [13] in order to homogenize heat tolerance characteristics. Animals were assigned to 1 of 4 groups ($n=4/\text{group}$); 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 $\times$ 2.5 m $\times$ 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 was adjusted by using the regulators to maintain THI index for each experiment from 0900 to 1900 h and to recover THI similar to outside environment from 1900 to 0900. 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. One extra group was performed as a control (threshold) at THI level of 68 to 70 together with dietary CP level of 12.5%. Calves were subjected to ambient temperature of 22°C for 7 days (Thermoneutral: TN) including the adaptation period for one week prior to the beginning of the experiment, after which 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 levels. 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, Laubisruestrasse 508712 Staefa ZH, Switzerland). The THI was calculated using the dry bulb temperature (T_{db} , °C) and RH using the following formula: $THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$ according to a previous study [14]. 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. The residues of both feed and water were recorded on the next day after offering on daily basis (0900 h). The chemical composition of the feed is shown in Table 2. Body weight (BW) of each individual was measured at d0 and d28 for each experimental group.

Chemical analysis

Each experimental diet was ground and the approximate composition of 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 analysed according to AOAC [15]. The NDF content was determined according to the procedure of Van Soest et al. [16]. Ca and P were analysed inductively coupled plasma spectroscopy (method 945.46) [15]. 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, the Republic of Korea). To measure CP contents, total nitrogen content in each diet was measured using the Kjeltex™ System (Kjeltex™ 2400, FOSS, Denmark) and the final CP content was calculated as nitrogen $\times$ 6.25. Crude fat content was analysed using the ether extraction system (ANKOMXT15 Extractor, ANKOM Technology, Macedon, NY) and 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 analyser (Beckman 6300, Beckman, Fullerton, CA).

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). The HR, expressed in beats per minute (BPM), was obtained using a stethoscope (TS-DIA01002, Tenso Medical Instrument Co., Zhejiang, China) that was 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 from each calf were collected every three days (d1, d4, d7, d10, d13, d16, d19, d22, d25, d28) at 1400 h from jugular venepuncture into 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) for each experiment. Serum samples were obtained from blood after centrifugation at $2,700 \times g$ at 4°C for 15 minutes. Serum was transferred to 1.5 mL tube (Eppendorf AG, Hamburg, Germany) and kept at -80°C until further analysis. The samples of blood serum were analysed for glucose (Fuji Dri-Chem Slide Glu-PIII, Fujifilm Corp., Tokyo, Japan) and blood urea nitrogen (BUN, Fuji Dri-Chem Slide BUN-PIII, Fujifilm Corp., Tokyo, Japan) using DRI CHEM 7000i biochemistry analyser (Fuji Film, Tokyo, Japan). The non-esterified fatty acids (NEFA) was analysed using Roche Free Fatty Acids kits (Roche, Mannheim, Germany) with HITACHI 7600 chemistry autoanalyser (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 analyser (Sykam S433, Sykam GmbH, Germany).

Sampling and isolation of peripheral blood mononuclear cells

Immediately after blood collection, the samples were stored at room temperature and transferred to the laboratory for blood separation and further analyses. For isolation of PBMCs, the blood samples were processed 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 × 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 manufacturer's instructions.

Hair follicle collection and storage

The hair follicle samplings were conducted as previously reported by Kim et al. (2020) [17]. Hair follicles were collected from the tails of each calf every three days at 1400 h. Tail hairs (25 to 30 hairs) were pulled from the Korean native beef calves. Individual hairs were grasped as close to the skin as possible and then rapidly pulled out. The hair follicles were washed using DEPC-treated water. After washing, the hair follicles were placed into a 5 mL specimen jar filled with RNAlater™ (Ambion, Austin, TX) following by storing for total RNA analysis.

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. 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 follicle samples from each calf were removed from the RNAlater™. Then, the bottom centimeter of each treatment of hair, containing the hair follicle, was cut and placed directly into a 2 ml microcentrifuge tube that contained 1 ml of TRIzol™. Specifically, total RNA was isolated from hair follicles using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was 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 Bioanalyser 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. First-strand cDNA was synthesized using RNA (1 µg) and an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The expression of genes in PBMCs, including HSP70, was analysed by real-time quantitative PCR (RT qPCR) amplification with SYBR- Green® as described previously [18]. All reactions were performed in triplicate in order to improve the reliability of assessment and in 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, the Republic of 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) [19], 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- $\Delta\Delta$ CT method [20]. The coefficient of variation of the housekeeping gene was checked prior to the calculation 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. Hair follicles were cut off 5-10 mm of 20 hairpieces and placed into a 1.5 mL tube. The 300 µL 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 µL proteinase K was added (20 mg/mL, G-spin Total DNA extraction Kit, iNtRON Biotechnology, Seongnam, the Republic of Korea), followed by incubation at 56°C overnight. Next, 300 µL protein precipitation solution was added (7 M Ammonium acetate, Tech & Innovation, Seongnam, the Republic of Korea) and centrifuged at 13,000 rpm 20°C for 15 minutes. After this, 600 µL of supernatant was transferred to a new 1.5 mL tube and incubated at 65°C for 1 hour after 10 µL RNase solution was added (10 mg/mL, G-spin Total DNA extraction Kit, iNtRON Biotechnology, the Republic of Korea). Subsequently, 600 µL 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, the Republic of 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, the Republic of Korea) followed by using the T100 96-well Thermal Cycler (Bio-Rad Laboratories Inc, Seoul, the Republic of Korea): 95°C for 2 minutes, 35 cycles of 20 seconds at 95°C, 40 seconds at 56°C, 1 minutes 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 C allele was cut into 895-bp and 1128-bp fragments [13]. 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 type were expressed as lanes 1 to 15 except lane 9 (TC type).

Statistical analysis

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

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

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 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 and was included in the model when appropriate but was removed from the model when not significant. 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.

Results

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

Dietary protein levels in each HS treatment increased protein intake ($P < 0.01$), BW gain ($P < 0.01$), and ADG ($P < 0.01$) in all experimental groups compared with that in the basal diet 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

During the TN period, there was no difference ($P > 0.05$) caused by dietary protein levels and HS levels in HR and RT. However, during the HS period, dietary protein levels in each HS treatment increased HR ($P < 0.05$) and RT ($P < 0.01$) in all experimental groups compared with that in the basal diet group (Table 6). There was no difference ($P > 0.05$) as a result of

increased dietary protein levels in HR and RT (Table 6). HS levels increased HR ($P < 0.01$) and RT ($P < 0.01$) (Table 6).

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

During the TN period, there was no difference ($P > 0.05$) of dietary protein levels and HS levels on 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 enhanced glucose ($P < 0.01$) and NEFA ($P < 0.01$) (Table 7). HS levels increased cortisol ($P < 0.01$), BUN ($P < 0.05$) and decreased glucose ($P < 0.01$) and NEFA ($P < 0.01$) (Table 7).

During the TN period, dietary protein levels and HS levels did not affect ($P > 0.05$) blood AA profiles (Not shown in the Table). Nevertheless, during the HS period, Dietary protein levels in each treatment 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 (Table 8). Increased dietary protein levels enhanced lysine ($P < 0.05$), and glutamic acid ($P < 0.05$). HS levels 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 was evaluated by time course over the total period (28 days: Figure 2). 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, $P < 0.05$).

The HSP70 gene expression in hair follicles was evaluated by time course over the total period (28 days: Figure 3). There was no difference among LP, MP, and HP groups on HSP70 gene expression in mild HS stress. In moderate HS stress, HSP70 gene expression was increased in LP, MP, and HP groups 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

Protein is required daily for the calf to maintain biological processes, as well as to repair tissues and to form blood. In calf growth, protein is important to utilize essential nutrients for muscle growth and development.

Recent data showed that high-yield cows started to reduce milk yield at a THI of approximately 68 [21]. In another study, dairy cows were exposed to conditions well above this threshold (mean THI = 82.4), which induced hyperthermia, tachypnea, and a significant reduction in DMI [22]. In previous studies, heat-stressed calves showed reduced DMI, BW gain, and ADG [23, 24]. In the current study, the beef calves showed decreased in DMI, BW gain, and ADG after exposure to HS conditions (Table 5). 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. A common strategy is to increase the energy and nutrient density of the formula (reduced fiber, increased

concentrates and supplemental fat, supplemental AAs), as feed intake is significantly reduced during HS [25]. Dietary protein levels may need to be increased during HS in growing calves. In heat-stressed calves, protein supplementation levels need to be increased to compensate for reduced feed intake [12]. The lack of energy sources available for calf growth caused by HS was compensated through appropriate dietary protein supplementation. However, discrepancies have been reported within the literatures regarding the benefits and negative consequences of increased protein and altered protein solubility [26, 27]. Baumgard LH and Rhoads Jr RP [28] reported that appropriate dietary protein improved growth performance in calves, but the dietary excess protein had negative effect on energetic cost. Excess N above requirements reduces ME by 7.2 kcal/g of N [29]. When 19 and 23 % CP diets were fed, milk yield was reduced by over 1.4 kg [30], and the energy cost associated with synthesizing and excreting urea accounted for the reduced milk yield [31]. 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 [25]. 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 [32]. 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 [33, 34]. 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 [29]. 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 [35].

Heat stress increased the blood level of fight or flight lipophilic steroid called cortisol throughout the trial. Generally, HS and blood cortisol levels are correlated [36, 37]. Consistently, in addition to the present study, our previous studies also found that cattle kept under HS showed higher blood cortisol levels [24, 36].

Blood glucose and NEFA decreased in HS groups. This may be related to negative energy balance due to the decreased DMI. The decline in blood glucose under HS was previously observed in rat [38], chicken [39], sheep [40], and cows [41]. 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 a another reason for decreased blood glucose [29].

One characteristic response allows for adipose lipolysis and NEFA mobilization to reduce insulin sensitivity and circulating insulin [42]. Increased circulating NEFA are typical in restricted-fed animals for utilized of energy sources. The enhanced fatty acid oxidation during nutrient limitation is a classic strategy to maintain glucose [28]. Post-absorbable carbohydrate metabolism is also altered by reduced insulin action during dietary restriction, reducing glucose absorption by adipose tissue [23]. 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 [43] that can occur due to HS as a consequence of decreased feed intake.

Increased circulating NEFA are typical observed in under-fed animals in order to utilize energy efficiently. However, previous studies showed that basal plasma NEFA levels are reduced under heat-stressed conditions in rodents [44], pigs [45], sheep [46], and cattle [22, 47] while overall DMI was reduced. It has been suggested that the acceleration of ketone

production from NEFA reduces blood NEFA under HS. The blood ketone levels decreased while urine ketone levels remained unchanged as ambient temperature increased [48].

Moreover, HS increased lipoprotein lipase in adipose tissue [44], which suggests that adipose tissue of hyperthermia animals has an increased capacity to absorb and store intestinal and hepatic-derived triglycerides [28].

In the current study, heat-stressed animals given 17.5 % of dietary protein levels (HP) tended to increase blood glucose and NEFA. This indicates that the HP was supplied by energy source from glucose and NEFA converted to energy that could maintain growth under HS [28]. A required for understanding metabolic adaptations that occur during HS is an understanding of the physiological and metabolic adjustments that growing animals initiate during malnutrition [28], which can be a consequence result of diminished DMI due to HS.

Changes in postabsorptive nutrient partitioning occur to support a controlling physiological state, and one well-described strategy is the glucose-sparing effect that growing animals utilized when on a lowered plane of nutrition or in negative balance. Inadequate nutrient consumption during HS conditions is associated with a variety of metabolic changes implemented to support the synthesis of high-priority tissues like skeletal muscle [49].

Under heat stress, 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 cows [50]. Additionally, we assumed that reduced blood flow might also be suppressed the absorption of AAs. Heat stress alters the nutrient partitioning and it lead to reduce the delivery of protein precursors to the adipose tissue or muscle [29]. 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 utilization [22]. Previous studies revealed that contribution of AAs to the circulating glucose pool is

increased during HS situation [22, 51]. 78% of plasma AAs reduction was due to the decline in gluconeogenic AAs [22]. This study suggests that the plasma AAs decline during HS may be the result of increased AAs utilization for gluconeogenesis. In the current study, we noted that HP increased lysine and glutamic acid in heat-stressed Korean native beef calves.

In the 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 muscle [52]. Lower lysine and glutamic acid concentration could be related 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 finding, the supplement of higher level of protein is suggested during heat exposure in calves. Increased protein uptake will be utilized for glucogenic and ketogenic synthesis and will diminish the negative effects of HS.

The HSP genes result in the expression of proteins with chaperone roles that act in protecting cells from HS and in the removal of damaged proteins [53, 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 temperature [55]. The transcription of HSP genes and, consequently, the synthesis of HSPs are enhanced upon exposure of cells to HS [56]. The hyperthermia resulting from HS can also disturb the cellular functions and result in physiological changes that cause a negative impact on animal production [57]. 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 [58]. The expression level of HSP70 was reported to increase at the cellular level under HS conditions [59]. 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 for non-invasive method to demonstrate the level of HSP in HS animals.

Conclusions

Heat stress had direct effects on protein metabolism and growth, including decreased BW gain and ADG in heat-stressed Korean native beef calves. The decline in BW gain and ADG cannot be completely defined by the decreased DMI. The decrease in blood glucose, NEFA and blood AAs profiles and increase in BUN and HSP70 gene expression during heat stress affect growth through energy and protein metabolism. In conclusion, 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.

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:

477 Deoxyribonucleic acid; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; RPS15A:
478 Ribosomal protein S15a; B2M: Beta 2 microglobulin; EDTA: Ethylenediaminetetraacetic
479 acid; GLM: Generalized linear model; HSD: Honest significant difference; ME:
480 Metabolizable energy; RUP: Rumen undegradable protein; MUN: Milk urea nitrogen; UUN:
481 Urine urea nitrogen; MCP: Microbial crude protein;

482

483 **Acknowledgements and funding**

484 This study was supported by grants from Cooperative Research Program for Agriculture
485 Science and Technology Development (Project Co. PJ015025022021) funded by Rural
486 Development Administration, Republic of Korea. The authors would like to thank J. H. Jo, J.
487 S. Lee, J. E. Kim, K. Y. Park for their valuable assistance during the experiment (labotory of
488 Animal Nutrition, Physiology & Proteomics, Konkuk University, Republic of Korea).

489

490 **Availability of data and materials**

491 Not applicable

492

493 **Authors information**

494 Affliations

495 ¹Department of Animal Science and Technology, Sanghuh College of Life Sciences, Konkuk
496 University, Seoul 05029, Korea

497 Won Seob Kim, Jalil Ghassemi Nejad, Dong Qiao Peng, Yong Ho Jo, and Hong Gu Lee

498 ²Animal Science and Food Sciences and Human Nutrition, Michigan State University, East
499 Lansing, 48824, USA

500 Jong kyoo Kim

501

502

503 Contributions

504 WSK and HGL, Conception, design, and drafting of the manuscript. WSK, JGN, DQP, YHJ,

505 JKK and HGL, Drafting the article or revising it critically for valuable inteelectual content.

506 WSK, Acquisition of data. WSK, Analysis and interpretation of data. The author(s) read and

507 approved the final manuscript.

508

509 Corresponding author

510 Correspondence to Hong Gu Lee

511 Tel: +82-2-450-0523, Fax: +82-2-455-1044, E-mail: hglee66@konkuk.ac.kr

512

513 **Ethics approval section and consent to participate**

514 All procedures involving animals were approved by the Institutional Animal Care and Use

515 Committee (IACUC) of Konkuk University (Approval No: KU18178).

516

517 **Consent for publication**

518 Not applicable

519

520 **Competing interests**

521 We cerify that there is no conflict of interest with any financial orgnization regarding the

522 material discussed in the manuscript.

523

References

1. Rhoads M, Rhoads R, VanBaale M, Collier RJ, Sanders S, Weber WJ, 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.* 2009, 92:1986-1997.
2. Kim KH, KIM DH, OH YK, LEE SS, LEE HJ, KIM DW, et al. Productivity and energy partition of late lactation dairy cows during heat exposure. *Anim. Sci. J.* 2010, 81:58-62.
3. Farooq U, Samad H, Shehzad F, Qayyum A. Physiological responses of cattle to heat stress. *World Appl Sci J* 2010, 8:38-43.
4. West JW. Interactions of Energy and Bovine Somatotropin with Heat Stress. *J. Dairy Sci.* 1994, 77:2091-2102.
5. National Research Council. Nutrient Requirements of Dairy Cattle: Seventh Revised Edition. Washington, DC: The National Academies Press. 2001.
6. O'brien M, Rhoads R, Sanders S, Duff G, Baumgard L. Metabolic adaptations to heat stress in growing cattle. *Domest. Anim. Endocrinol.* 2010, 38:86-94.
7. Morrison S: Ruminant heat stress: effect on production and means of alleviation. *J. Anim. Sci* 1983, 57:1594-1600.
8. Beede D, Collier R. Potential nutritional strategies for intensively managed cattle during thermal stress. *J. Anim. Sci* 1986, 62:543-554.
9. Paula-Lopes F, Chase C, Al-Katanani Y, Krininger C, Rivera R, Tekin S, 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. *Reprod.* 2003, 125:285-294.
10. Hansen P. Physiological and cellular adaptations of zebu cattle to thermal stress. *Anim. Reprod. Sci.* 2004, 82:349-360.

11. West JW. Effects of heat-stress on production in dairy cattle. *J. Dairy Sci.* 2003, 86:2131-2144.
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, Morera P, Primi V, Lacetera N, Nardone A, Bernabucci U. Cellular thermotolerance is associated with heat shock protein 70.1 genetic polymorphisms in Holstein lactating cows. *CELL STRESS CHAPERON* 2011, 16:441-448.
14. National Research Council (U. S.). Committee on Physiological Effects of Environmental Factors on Animals. Guide to environmental research on animals. Washington, DC: The National Academy of Sciences. 1971.
15. AOAC. Official methods of analysis. Assoc Anal Chem 1990.
16. Van Soest Pv, Robertson J, Lewis B: Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 1991, 74:3583-3597.
17. Kim WS, Ghassemi Nejad J, Peng DQ, Jung US, Kim MJ, Jo YH, et al. Identification of heat shock protein gene expression in hair follicles as a novel indicator of heat stress in beef calves. *Animal* 2020:1-8.
18. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc* 2008, 3:1101-1108.
19. Kishore A, Sodhi M, Khate K, Kapila N, Kumari P, Mukesh M. Selection of stable reference genes in heat stressed peripheral blood mononuclear cells of tropically adapted Indian cattle and buffaloes. *Mol. Cell. Probes* 2013, 27:140-144.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001, 25:402-408.
21. Zimbelman R, Rhoads R, Rhoads M, Duff G, Baumgard L, Collier R. A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk

- 573 production in high producing dairy cows. In Proceedings of the Southwest Nutrition
574 Conference (ed RJ Collier). 2009: 158-169.
- 575 22. Gao S, Guo J, Quan S, Nan X, Fernandez MS, Baumgard L, et al. The effects of heat stress
576 on protein metabolism in lactating Holstein cows. *J. Dairy Sci.* 2017, 100:5040-5049.
- 577 23. O'Brien MD, Rhoads RP, Sanders SR, Duff GC, Baumgard LH. Metabolic adaptations to
578 heat stress in growing cattle. *Domest Anim Endocrinol* 2010, 38:86-94.
- 579 24. Tao S, Monteiro APA, Thompson IM, Hayen MJ, Dahl GE. Effect of late-gestation maternal
580 heat stress on growth and immune function of dairy calves. *J. Dairy Sci.* 2012, 95:7128-7136.
- 581 25. Baumgard L, Abuajamieh M, Stoakes S, Sanz-Fernandez M, Johnson J, Rhoads R, et al.
582 Feeding and managing cows to minimize heat stress. In Proceedings of 23rd Tri-State Dairy
583 Nutrition Conference. 2014: 61.
- 584 26. Huber J, Higginbotham G, Gomez-Alarcon R, Taylor R, Chen K, Chan S, et al. Heat stress
585 interactions with protein supplemental Fat, and fungal cultures. *J. Dairy Sci.* 1994, 77:2080-
586 2090.
- 587 27. Huber J, Wu Z, Chan S, Chen K. Feeding for high production during heat stress. In Western
588 Large Herd Management Conf Las Vegas, NV. 1993: 183-192.
- 589 28. Baumgard LH, Rhoads Jr RP. Effects of heat stress on postabsorptive metabolism and
590 energetics. *Annu Rev Anim Biosci* 2013, 1:311-337.
- 591 29. Tyrrell H. Influence of excess protein intake on energy metabolism of the dairy cow. In
592 Proceedings of the 5th Symposium on Energy Metabolism, 1970.
- 593 30. Danfaer A, Thyssen I, Østergaard V. Effect of the level of dietary protein on milk production.
594 I. Milk yield, liveweight gain and health. *CAB Direct* 1980, 165.
- 595 31. Oldham J. Protein-energy interrelationships in dairy cows. *J. Dairy Sci.* 1984, 67:1090-1114.

32. Belibasakis N, Ambatzidis P, Aktsali P, Tsirgogianni D. Effects of degradability of dietary protein on milk production and blood components of dairy cows in hot weather. *World Rev Anim Prod.* 1995, 30:21-26.
33. Getahun D, Getabalew M, Zewdie D, Alemneh T, Akeberegn D. Urea Metabolism and Recycling in Ruminants. *BJSTR* 2019, 20:14790-14796.
34. Klusmeyer TH, McCarthy RD, Jr., Clark JH, Nelson DR. Effects of source and amount of protein on ruminal fermentation and passage of nutrients to the small intestine of lactating cows. *J. Dairy Sci.* 1990, 73:3526-3537.
35. Bunting LD, Fernandez JM, Fornea RJ, White TW, Froetschel MA, Stone JD, et al. Seasonal Effects of Supplemental Fat or Undegradable Protein on the Growth and Metabolism of Holstein Calves¹. *J. Dairy Sci.* 1996, 79:1611-1620.
36. Kim WS, Lee JS, Jeon SW, Peng DQ, Kim YS, Bae MH, et al. Correlation between blood, physiological and behavioral parameters in beef calves under heat stress. *Asian Australas J Anim Sci* 2018, 31:919-925.
37. Ghassemi Nejad J, Lohakare JD, Son JK, Kwon EG, West JW, Sung KI. Wool cortisol is a better indicator of stress than blood cortisol in ewes exposed to heat stress and water restriction. *Animal* 2014, 8:128-132.
38. Mitev S, Dinevska-Kovkarovska S, Miova B. Effect of the acclimation to high environmental temperature on the activity of hepatic glycogen phosphorylase (a+ b and a), liver glycogen content and blood glucose level in rats. *J. Therm. Biol* 2005, 30:563-568.
39. Rahimi G. Effect of heat shock at early growth phase on glucose and calcium regulating axis in broiler chickens. *Int J Poult Sci* 2005, 4:790-794.
40. Achmadi J, Yanagisawa T, Sano H, Terashima Y. Pancreatic insulin secretory response and insulin action in heat-exposed sheep given a concentrate or roughage diet. *Domest Anim Endocrinol* 1993, 10:279-287.

41. Itoh F, Obara Y, Rose MT, Fuse H, Hashimoto H. Insulin and glucagon secretion in lactating cows during heat exposure. *J. Anim. Sci* 1998, 76:2182-2189.
42. Mersmann H. Nutritional and endocrinological influences on the composition of animal growth. *Progress in food & nutrition science* 1987, 11:175-201.
43. Vernon RG. Effects of diet on lipolysis and its regulation. *Proceedings of the Nutrition Society* 1992, 51:397-408.
44. Sanders SR, Cole LC, Flann KL, Baumgard LH, Rhoads RP. Effects of acute heat stress on skeletal muscle gene expression associated with energy metabolism in rats. *Federation of American Societies for Experimental Biology*; 2009, 23:598.7.
45. Pearce SC, Upah NC, Harris A, Gabler NK, Ross JW, Rhoads RP, et al. Effects of heat stress on energetic metabolism in growing pigs. *Federation of American Societies for Experimental Biology*; 2011, 25:1052.5.
46. Sano H, Takahashi K, Ambo K, Tsuda T. Turnover and oxidation rates of blood glucose and heat production in sheep exposed to heat. *J. Dairy Sci.* 1983, 66:856-861.
47. Shwartz G, Rhoads M, VanBaale M, Rhoads R, Baumgard L. Effects of a supplemental yeast culture on heat-stressed lactating Holstein cows. *J. Dairy Sci.* 2009, 92:935-942.
48. Dale H, Stewart R, Brody S. Rumen temperature. 1. Temperature gradients during feeding and fasting. *Cornell veterinarian* 1954, 44:368-374.
49. Van Milgen J, Noblet J. Partitioning of energy intake to heat, protein, and fat in growing pigs. *J. Anim. Sci* 2003, 81:E86-E93.
50. 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.* 2015, 98:2356-2368.

51. 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* 1972, 23:717-725.
52. Kim YS, Lee JS, Lee Y, Kim WS, Peng DQ, Bae MH, et al. Effect of glutamine on heat-shock protein beta 1 (HSPB1) expression during myogenic differentiation in bovine embryonic fibroblast cells. *Food Sci* 2018, 27:829-835.
53. Parsell a, Lindquist S. The Function of Heat-Shock Proteins in Stress Tolerance: Degradation and Reactivation of Damaged Proteins. *Annu. Rev. Genet.* 1993, 27:437-496.
54. Kim WS, Ghassemi Nejad J, Roh SG, Lee HG. Heat-Shock Proteins Gene Expression in Peripheral Blood Mononuclear Cells as an Indicator of Heat Stress in Beef Calves. *Animals* 2020, 10:895.
55. Ananthan J, Goldberg AL, Voellmy R. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* 1986, 232:522.
56. Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R. Repression of Heat Shock Transcription Factor HSF1 Activation by HSP90 (HSP90 Complex) that Forms a Stress-Sensitive Complex with HSF1. *Cell* 1998, 94:471-480.
57. Hansen PJ. Physiological and cellular adaptations of zebu cattle to thermal stress. *Anim. Reprod. Sci.* 2004, 82-83:349-360.
58. Kristensen TN, 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 CHAPERON* 2004, 9:143-149.
59. Lacetera N, Bernabucci U, Scalia D, Basiricò L, Morera P, Nardone A. Heat Stress Elicits Different Responses in Peripheral Blood Mononuclear Cells from Brown Swiss and Holstein Cows¹. *J. Dairy Sci.* 2006, 89:4606-4612.

669 **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

670 * 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
671 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,
672 216 mg/kg, vitamin A, 300,000 IU/kg, vitamin D, 85,000 IU/kg and vitamin E, 2,300 IU/kg.
673 THI = Temperature humidity index, $THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]$.
674 Threshold = THI 70 to 73, Mild = THI 74 to 76, Moderate = THI 81 to 83, Severe = THI 89 to 91.
675 LP = Low protein (12.5%), MP = Medium protein (15%), HP = High protein (17.5%).

676 **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
Analysed										
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

677 ¹Values were estimated using NRC (2016).

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

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

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

681 **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		
Analysed												
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

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

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

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

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

686 **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

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

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

689 **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

690 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).

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

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

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

694 **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

695 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).

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

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

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

699 **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±1.1	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±1.7	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	221.1±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

700 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).

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

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

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

704 **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

705 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).

706 THI = Temperature humidity index.

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

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

709 **Figure 1. Agarose gel electrophoresis of HSP70 gene.** Lane 1 to 15 are standard for CC type except
710 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 claves 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.