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Authors' contributions Please specify the authors' role using this form.	Conceptualization and hypothesis: Yong-Ho *Jo,* Yves *Kamali,* Hong-Gu *Lee*. Data curation: Yves *Kamali,* Yong-Ho *Jo* Formal analysis: Jalil *Ghassemi Nejad*, Jae-Sung *Lee* Methodology*:* Yves *Kamali*, Yong-Ho *Jo*, Jae-Sung *Lee*, Won-Seob *Kim*. Writing first draft Yves *Kamali*, Yong-Ho *Jo*. Writing, reviewing, and editing: Jalil *Ghassemi Nejad*, Won-Seob *Kim*. Preparation, experiments, and discussion: Jalil *Ghassemi Nejad*, Jae-Sung *Lee*, Won-Seob *Kim*. Supervised the experiment, supporting the experiment financially: Hong-Gu *Lee*.
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Abstract

This study investigated the effects of L-glutamine (Gln) supplementation on growth performance, physiological traits, heat shock proteins (*HSP*s), and gene expression related to muscle and adipose tissue development in Hanwoo steers under heat stress (HS) conditions. Eight Hanwoo steers (initial body weight (BW) 570.7±43.6 kg, months of age 22.3±0.88) were randomly separated into two groups, control and treatment, and supplied with the concentration (1.5% of BW kg/day/head) and rice straw (1.5 kg/day/head). The treatment group were fed the Gln supplementation (0.5% of concentration, as-fed basis) once a day at 0800 h. Blood samples for the assessment of haematological and biochemical parameters and the separation of peripheral blood mononuclear cells (PBMCs) were collected four times, at 0, 3, 6, and 10 weeks of the experiment. Feed intake was measured daily. BW to analyze growth performance and hair follicle collection to analyze the expression of *HSPs* were executed four times at 0, 3, 6, and 10 weeks. To analyze gene expression, *longissimus dorsi* muscle samples were collected by biopsy at the end of the study. As a result, growing performance, including final BW, average daily gain, and gain-to-feed ratio, were not different between the two groups. Leukocytes including lymphocytes and granulocytes, tended to increase in the Gln supplementation group ($p = 0.058$). There were also no differences in biochemical parameters shown between the two groups, except total protein and albumin, both of which were lower in the Gln supplementation group ($p < 0.05$). Gene expressions related to muscle and adipose tissue development were not different between the two groups. As temperature–humidity index increased, *HSP70* and *HSP90* expression in the hair follicle showed a high correlation. *HSP90* in the hair follicle was decreased in the treatment group compared with the control group at 10 weeks

($p < 0.05$). Collectively, dietary Gln supplementation (0.5% of concentration, as-fed basis) may not be influential enough to affect growth performance and gene expression related to muscle and adipose tissue development in steers. However, Gln supplementation increased the number of immune cells and decreased *HSP90* in the hair follicle implying HS reduction in the corresponding group.

Keywords: L-glutamine; Hanwoo steer; heat shock protein; heat stress; immunity

Introduction

Korea is a peninsula surrounded by the sea on three sides with hot and humid summers, experiencing an average temperature of 24.4 °C (mean minimum temperature of 20.7 °C, and mean maximum temperature of 29.1 °C) and relative humidity of 77.3% from 2011 to 2021 (1). In general, beef cattle require more energy due to the increased maintenance energy requirement for thermo-emission, while the energy intake decreases with diminished feed intake during heat stress (HS) (2). Energy for beef cattle is important because limited energy causes reduced growth performances and productivity. Under HS conditions, insulin secretion increases, resulting in increased glucose absorption in cells and inhibited lipolysis in fat tissue compared with thermoneutral conditions. In addition, insulin inhibits beta-oxidation, which synthesizes energy using fat and increases the availability of monosaccharide glucose and amino acids (AAs) (3, 4). Additionally, protein metabolism is suppressed under HS. As the phenomena continue, first, blood flows into peripheral tissues to dissipate heat, the blood flow to digestive organs reduces, and the absorption of nutrition, including AAs, also reduces (5). Second, HS inhibits ammonia utilization of the ruminal microbes and reduces the proliferation of ruminal microbes, which eventually results in a decline in ruminal microbial protein synthesis (6, 7). Third, the production of heat shock protein (*HSP*) 70, which is a protein that prevents the denaturation of other proteins in HS, increases by 200 times in lymphocytes due to HS (8). Finally, the amount of glucogenic AAs in the blood reduces by approximately 17.1% followed by the production of glucose due to the negative energy balance caused by HS (9). However, supplementing heat stressed cows with a high dosage of dietary protein could reduce productivity due to an

63 excessive urea cycle (10), thus the optimum dosage, or its component (AA), should
64 be identified with regard to the different physiological effects.
65 Gln is a conditionally essential AA in the body of ruminants associated with normal
66 physiological functions, lipid and protein metabolism, and muscle growth (11-13).
67 Gln is also responsible for various functions in the body including: 1) synthesizing
68 other AAs using Gln; 2) increasing immunity with a proliferation of lymphocyte and
69 increasing-decreasing cytokines; and 3) using respiratory fuel in the mitochondria
70 of intestinal epithelial cells and myocytes (14, 15). Our hypothesis is that the
71 supplementation of glutamine will reduce heat stress and improve immunity in a lack
72 of energy and protein supply due to reduced intake during HS, and furthermore, it
73 has a positive effect on genes related muscle development.
74 A previous study in our laboratory revealed that *in vitro* supplementation with Gln
75 at 0.5% has positive effects on fermentation end products (VFAs) and on the
76 degradability of nutrients without adverse effects on ammonia-N or total gas
77 production compared with 2% and 3% supplementation (16). However, *in vivo*
78 studies investigating the effects of Gln supplementation on beef cattle under HS
79 conditions are scant. Moreover, the possible effects of Gln on the prevention of
80 excessive nitrogen sourced from dietary nitrogen in Hanwoo steers under HS has
81 yet to be investigated. Therefore, and following the previous *in vitro* study in our
82 laboratory (16), the objective of this study was to explore the effects of Gln
83 supplementation on growth performance, physiological traits, *HSPs*, and gene
84 expression related to muscle development in Hanwoo steers under HS conditions.
85
86 # Materials and Methods
87

88 **Animals, treatments, feeding, and housing management**

89 All experimental procedures were followed according to the Konkuk University
90 “Guidelines for the Care and Use of Experimental Animals” (Approval no: KU20001).
91 Originally, eight Korean native beef cattle were included in this study. The animals
92 (initial BW = 570.7 kg (SD = 43.6), months of age = 22.3 (SD = 0.88)) were randomly
93 separated into two groups (supplemented Gln group, *n* = 4; control group, *n* = 4).

94 The diets were supplied with a commercial concentration (Hanwoo-love-max,
95 Cargill, Seongnam, Korea) (1.5% of BW as-fed basis) and 1.5 kg of rice straw (as-
96 fed basis) (Table 1). The animals were fed quantitatively according to 1.5% of BW
97 at 0800 and 1600 h. The feed residual was measured daily at 0700 h. BW was
98 measured at 0, 3, 6, and 10 weeks. After evaluating BW, the amount of feeding was
99 reset based on the measured BW. Water was available *ad libitum*. In the treatment
100 group, an additional average of 51.45 g Gln/day/head (SD = 3.41, Daesang, Seoul,
101 Korea) was supplied via oral administration to each animal for 9 weeks, except for
102 an adaptation period of 1 week. The experiment period was from 27 June to 13
103 September, 2020, although the summer period in Korea starts from 31 May and
104 ends in September (approximately 4 months) (17). The amount of Gln fed to the
105 animals was 0.5% of the total diet on an as-fed basis. According to a previous study,
106 the addition of more than 2% Gln showed negative effects on rumen fermentation
107 and a degradability of nutrients *in-vitro*; however, Gln at an amount of 0.5% of the
108 total diet improved rumen fermentation and the degradability of nutrients (16). The
109 steers were housed in the same individual 5 m^2 pens (2 m (length) x 2.5 m (breadth)).
110

111 **Rectal temperature and behavior parameters under heat stress**

Two experimenters visually checked the behavior parameters from 1000 to 1800 h four times at 0, 3, 6, and 10 weeks. The frequency of lying and standing at 0, 3, 6, and 10 weeks was calculated from 1000 to 1800 h. The rectal temperature (RT) was checked for four times at 0, 3, 6, 10 weeks at 1400 h.

Measurement of temperature–humidity index (THI)

The THI was recorded inside and outside the barn at 1 second intervals using a sensor (MHB-382SD, ZL 2008 2, Lutron, China), and the daily average (0000 to 2400) values of ambient temperature and relative humidity (RH) were calculated. The THI was calculated based on dry bulb temperature (T_{db}) and RH using the following formula: $THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$, in accordance with a previous study (18). The daily THI data are presented in Figure 1.

Preparation of blood samples

Blood samples were collected from the jugular vein of each beef using 18-gauge needles at 0800 h at 0, 3, 6, and 10 weeks. Serum tubes, K2 EDTA (K2E) 7.2 mg Plus tubes (BD Vacutainer, Franklin Lakes, NJ, USA), and sodium heparin (10 IU/mL) (BD Vacutainer, Franklin Lakes, NJ, USA) were prepared, and the collected blood samples were stored on ice before being transferred to the laboratory, except for heparin-treated blood samples, which were used to isolate peripheral blood mononuclear cells (PBMCs). Next, to separate the serum and plasma, the blood samples were centrifuged at 2,740 × g for 15 min at 4 °C. The serum and plasma were then transferred to a 1.5 ml tube and stored in a freezer (–

80 °C) before further analysis. The K2 EDTA blood samples were temporarily stored at 4 °C for subsequent analysis.

Analyzing blood metabolites and amino acids

Analytical reagents for measuring blood urea nitrogen, glucose, total protein, albumin, globulin, and creatine phosphokinase levels were purchased from Fujifilm healthcare (Tokyo, Japan). All these parameters were analyzed using a biochemistry analyzer (Fuji Dri Chem 7000i, Tokyo, Japan).

Blood AAs were performed as described in the AA analyzer manual (Sykam 433, Gewerbering, Germany). Briefly, 200 µL of plasma was deproteinized with an equal volume of 10% (w/v) sulfosalicylic acid and incubated for 30 min at 4 °C, then centrifuged at 20,800 × g for 3 min at room temperature. The supernatant fluid was filtered using a 0.2 µm nylon filter. The AAs were measured photometrically at 570 nm. The run time for each sample was 130 min.

Isolation of PBMCs for RNA extraction

The isolation of PBMCs was performed with a minor modification to the previously described method (19). Blood samples were stored at room temperature and transferred to the laboratory for blood separation and further analyses. For the isolation of PBMCs, the blood samples were processed within 8 h 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 as per the manufacturer's instructions. The isolated PBMCs were

161 washed twice with 1 × PBS. TRIzol Reagent (Life Technologies, Seoul, Korea) was
162 then added to the pellet of PBMCs, and it was stored at –80°C until RNA extraction.
163
164 **Collection of hair follicles for RNA extraction**
165 The collection of hair follicles was performed according to the previously
166 described method (20). Tail hair (25 to 30 hairs) was pulled from each steer at 0800
167 h. Individual hairs were grasped as close to the skin as possible and then rapidly
168 pulled out. The hair follicles were washed using diethylpyrocarbonate (DEPC)-
169 treated water. After the follicles were washed, the bottom centimeter of each hair,
170 containing the hair follicle, was cut, and placed in a 5 ml specimen jar filled with
171 RNAlater™ (Ambion, Austin, TX, USA). The samples were stored at room
172 temperature for 1 to 14 days until RNA extraction. For RNA extraction, only the hair
173 follicle was cut and placed in 1 mL of TRIzol, then finely ground with a homogenizer.
174
175 ***Longissimus dorsi* muscle sampling and analysis**
176 *Longissimus dorsi* muscle sampling and total RNA extraction were performed by
177 surgical biopsy according to previously described methods minor modifications (21).
178 Tissue samples (approximately 2 g) were collected from the *longissimus dorsi*
179 muscles of the steers (n = 8) via biopsy at week 10 of the experiment. The biopsy
180 procedure followed the Konkuk University “Guidelines for the Care and Use of
181 Experimental Animals”. Briefly, local anesthetic agents were administered at 6
182 equidistant points around the resection area, namely, the *longissimus dorsi* muscle
183 at positions 12 to 13 of the ribs. The veterinarian resected 5 cm of skin in the
184 direction of the muscle and then dissected the *longissimus dorsi* muscle tissue using
185 surgical equipment. The collected tissue was immediately washed with DEPC-

treated distilled water (diethylpyrocarbonate, Sigma-Aldrich, Seoul, Korea), placed in liquid nitrogen until completely frozen, and placed on dry ice until transferal to the laboratory. The tissue samples were then ground into powder in liquid nitrogen. Total RNA was extracted using TRIzol Reagent. An amount of 0.1 g of tissue sample was mixed with 1 mL of TRIzol. Next, the sample was homogenized using a homogenizer (IKA T10 basic, Seoul, Korea). The muscle tissue was stored at –80°C until RNA extraction.

Total RNA extraction

The method used for extracting total RNA from the PBMCs, hair follicles, and muscle tissue were the same. The samples were centrifuged at 4 °C and 12,000 × g for 10 min; then, the upper aqueous phase was transferred into a new 1.5 mL tube. Next, 200 μL of chloroform was added, and the mixture was incubated for 2 min at room temperature after vortexing, followed by centrifugation at 12,000 × g for 15 min at 4 °C. The upper transparent aqueous phase was transferred into a new 1.5 mL tube. Isopropanol (500 μL) was added, and the sample was incubated for 10 min at room temperature after vortexing, followed by centrifugation at 12,000 × g for 10 min at 4°C to precipitate the RNA. The supernatant was then discarded, and the RNA was washed by adding 1 mL of 75% ethanol to the tube. After vortexing, the mixture was centrifuged at 4 °C and 12,000 × g for 10 min. To collect the RNA, the wash step was repeated with 1 mL of 100% ethanol following the same procedure. After washing, the extracted RNA was dried under a vacuum for approximately 15 min, redissolved in 30 μL of DEPC-treated distilled water, and then incubated at 60 °C for 10 min. The RNA concentration was determined using spectrophotometric analysis (NanoDrop 1000, Thermo Scientific, Seoul, Korea).

Design of primers

In the muscle tissue, fatty acid–binding protein 4 (*FABP4*), glycerophosphate dehydrogenase (*GDP*), heat shock protein beta 1 (*HSPB1*), lipoprotein lipase (*LPL*), myoblast determination (*MyoD*), myogenic factor 5 (*MYF5*), myogenic factor 6 (*MYF6*), myogenin (*MyoG*), peroxisome proliferator-activated receptor-gamma (*PPARγ*), stearoyl-CoA desaturase (*SCD*), 18S ribosomal RNA (*18S*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein lateral stalk subunit P0 (*RPLP0*) primers were designed using the National Center for Biotechnology Information (NCBI) Primer-BLAST (Table 3). In the PBMCs, heat shock protein 70 (*HSP70*), heat shock protein 90 (*HSP90*), beta-2-microglobulin (B2M), and ribosomal protein S15a (*RPS15A*) primers were also designed using the NCBI Primer-BLAST (Table 3). In the hair follicle, we used *HSP70*, *HSP90*, and *GAPDH* designed above.

Synthesis of cDNA and gene expression analysis

To synthesize cDNA, 1 μg of RNA was reverse transcribed in a 100 μL reaction volume with an iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed on duplicate samples by using a CFX Connect™ Real-Time System (Bio-Rad, Seoul, Korea) with IQTM SYBR Green Supermix reagents (Bio-Rad, Seoul, Korea). The following PCR conditions were used: 95 °C for 3 min and 40 cycles at 95 °C for 10 s, 51–65 °C for 30 s and 72 °C for 30 s. The threshold cycles for each sample were normalized to housekeeping genes (muscle tissue: *RPLP0*, *GAPDH*, *18S*; PBMCs: *B2M*, *RPS15A*; hair follicle: *GAPDH*). The relative expression of the target gene was

quantified as the fold change of the expression of the target gene relative to the expression of the thermoneutral control according to the 2-ΔΔrCT method (22).

Statistical analysis

Growth performance, complete blood cell count, blood metabolite, and blood AA were analyzed using SAS 9.4 Proc Mixed and repeated-measures analysis (SAS Institute Inc., Cary, NC, USA). The model used was as follows:

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

where Y_{ijk} is the observation of Hanwoo steers *k* at sampling time *j* for given treatment *i*; μ represents the overall mean, α*i* denotes the fixed effect of treatment *i* (control and Gln supplementation group); γ(α)*ijk* is the random effect of Hanwoo steers *k* nested in treatment *i*, and sampling time *j* (3, 6, 10 weeks, or every day); and ε_{ijk} represents the residual effect. Covariance structures (autoregressive order 1 and unstructured) for the repeated measures model were tested. 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 the adaptation period 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 with standard errors.

Data of behavior, RT, and mRNA expression from each sample including muscle, PBMCs, and hair follicle, were assessed using independent (unpaired) sample t-tests for unequal variances. The model used was as follows:

$$Y_{ik} = \mu + \beta_i + + \varepsilon_{ik}$$

where Y_{ik} represents the observation of Hanwoo steers *k* for given treatment *i*; μ is the overall mean; b*i* denotes the fixed effect of treatment *i* (control and Gln supplementation group); and ε_{ik} is the residual effect.

Data of behavior, RT, and mRNA expression from the PBMCs and hair follicles were determined using the one-way ANOVA procedure with Tukey's test. The model used was as follows:

$$Y_{ik} = \mu + \delta_i + + \varepsilon_{ik}$$

where Y_{ik} represents the observation of Hanwoo steers *k* for given sampling times *i*; μ is the overall mean; δ_i was the fixed effect of sampling times *i* (3, 6, and 10 weeks); and ε_{ik} stands for the residual effect. Differences observed between means were considered significant at $p < 0.05$, and the tendency was declared at $0.05 \leq p < 0.1$.

Results and discussion

Effect of Gln supplementation on rectal temperature and behavior characteristics.

For this study, which was conducted at the hottest time of the year in Korea, 17 July to 31 August, the animals were under HS conditions, as revealed by an average temperature of 26.2 °C (range 24.3–28.9 °C), a relative humidity of 84.0% (range 69.6–93.7%), and an average THI of 76.9 (range 73.1–80.2) (Figure 1). The average THI for the entire experiment was 74.6, the average maximum THI was 80, and the average minimum THI was 69.9 (Figure 1). In the ambient temperature 25 °C and relative humidity 45–50% (THI 72.8–73.3) which is upper critical temperature because *Bos taurus* increased slightly the respirations but remained

RT and heat production in the THI (23). Furthermore, fattening cows have difficultly dissipating heat because their surface area relative to weight becomes smaller, increasing the insulation effect by increasing the body condition score (2).

Based on the studies mentioned above, in this experiment, the Hanwoo steers experienced sufficient HS, and the RT measurements are shown in Figure 2B. In Figure 2A, the mean THI, maximum THI, and minimum THI were identified at the measuring time for RT and behavior. The average THI was maintained at approximately 75.1 until 67 days after the start of the experiment, and the average THI decreased over time (Figure 1). Thus, the average RT of 38.6 °C at 10 weeks was 0.26 °C lower than the average RT at 0, 3, and 6 weeks. The difference in RT resulting from Gln supplementation was not shown ($p > 0.05$).

Through looking at standing behavior time and lying behavior time, we could confirm whether there was a difference in behavior according to Gln supplementation. The average standing time in the control group was 426.3 min/480 min, and in the treatment group, it was 420.8 min/480 min (Figure 2C). Lying time in the control group was 53.8 min/480 min, and in the treatment group, it was 59.2 min/480min (Figure 2C). Although Gln supplementation resulted in no difference in behavior ($p > 0.05$), the standing time was approximately 7.5 times longer than the lying time (from 1000 to 1800 h). This long standing time results in an increase in surface area contact with outside air to reflect heat in HS (24, 25). Consistently, Pinto and Hoffmann (26) reported that a longer standing time increases the efficiency of respiration in cows.

Effect of L-glutamine supplementation on blood amino acids in Hanwoo steers.

The tendency of L-citrulline and L-glutamic acid to increase compared with the control groups was confirmed by Gln supplementation under HS ($p < 0.10$). Blood glutamic acid (Glu) increased 2.27 times compared with the control group to show a concentration of 28.2 μmol/L ($p = 0.083$). Gln is transferred from the arterial blood to the inner cell. The Gln that enters the cell is converted into Glu by glutaminase, and Glu acts as an energy producer or intermediate, or is discharged into venous blood (27). As a result, the amount of blood Glu increased in the 0.5% Gln supplemented group compared with the control group.

Blood L-citrulline increased 1.3 times compared with the control group to show a concentration of 30.2 μmol/L ($p = 0.061$). According to a previous study, approximately 6% of the carbon is used to form L-citrulline through Gln metabolism, and 34% of the nitrogen from Gln is used to produce L-citrulline (28). Moreover, cyclical L-citrulline increases as the clearance of L-citrulline is reduced because Gln and L-citrulline share transporters (29).

Effect of L-glutamine supplementation on blood metabolite profile in Hanwoo steers.

HS reduced the feed intake but increased the maintenance energy requirement for sweating and panting to reduce body temperature (3). Gln could be a respiratory fuel in immune cells, muscles, the liver, and digestive organs (13). As Gln was used as energy, it was able to increase blood glucose by reducing the amount of use of blood glucose; however, there was no difference between the two groups (Table 5, $p = 0.104$). When multiparous Holstein cow duodenums were infused with Gln 300g/day, which amount of Gln was a higher amount of Gln than in this study, there

were no differences in energy metabolisms such as glucose, non-esterified fatty acids, and beta-hydroxybutyrate (30).

There was no difference in the amount of blood urea nitrogen (p = 0.837) and creatine phosphokinase (p = 0.684) according to Gln supplementation. Previous studies have confirmed the increase in blood urea nitrogen due to Gln treatment. This study supplied Gln at approximately 51.45 g per day, which is less than Gln 300 g per day in previous studies (30, 31).

Gln supplementation tended to reduce total protein in the blood (p = 0.097) and albumin (p = 0.059). The total protein decreased due to the decrease in albumin. Albumin has various functions, such as regulating the plasma oncotic pressure and transporting nutrients. Contrary to this study, a previous study found that albumin was increased as the formalin-treated Gln was increased by 150, 250, and 350 g per day (32). Moreover, the intake of Gln increases albumin in situations where albumin reduced due to surgery (33). Albumin concentrations decrease as albumin escapes into the urine as a result of renal difficulties, or or as albumin synthesis decreases due to liver disorders (34). However, given the albumin concentrations in this study are within the normal range of 2.73 to 3.65 g/dL, they were not altered by renal or liver disorders (35). The results of this experiment need further research to identify the effect of different ratios of Gln infusion or supplementation on blood metabolites and to warrant the obtained results.

Effect of L-glutamine supplementation on growth performances in Hanwoo steers

To the best of our knowledge, there have been no experiments to confirm growth performances as a result of the addition of Gln to cows. Until now, there have been

studies that provided Gln to cows to boost the immune system and milk production (30, 32). Even though there have been no previous studies on an improvement in growth performance with the addition of Gln in cows, it was confirmed that growth performance, with the addition of Gln, was improved under HS conditions in broilers (36, 37). We supplied Gln at 0.5% of the total diet (as-fed basis) for 9 weeks under HS conditions, but we observed no difference in feed intake, average daily gain, final body weight, or gain-to-feed ratio ($p > 0.05$). The reason why there were no differences in growth performances could be explained by the following two speculations. First, the addition of 0.5% Gln in the presence of HS may be considered insufficient to indicate a change in growth performance. Second, because the purpose of this study was to focus on physiological phenomena during HS conditions, the experiment period was relatively short (10 weeks). A longer experiment period may result in the observation of significant changes in growth performance parameters, and this could be a hypothesis for further research. It is suggested that various amounts of Gln be supplied to cattle during HS to observe the possible positive effect on performance; however, the amount of supplementation should be limited to an amount of Gln lower than 2% of the total diet, due to possible toxic effects. This is because, if Gln supplementation exceeds 2% of the total diet, the ammonia concentration in rumen fluid exceeds the normal range (16).

Effect of L-glutamine supplementation on complete blood cell counts in Hanwoo steers.

Gln supplementation increased the number of WBC by 1.26 times ($p = 0.058$), lymphocytes by 1.30 times ($p = 0.068$), and granulocytes by 1.28 times ($p = 0.031$),

but the proportion decreased as it did not affect the number of monocytes (p = 0.058) (Table 4). HS reduced bovine lymphocyte proliferation because HS may impair the T helper cell 1 and T helper cell 2 balance (38). However, Gln supplementation increased the lymphocyte and granulocyte under HS conditions. The first reason for this could be that Gln is utilized as energy sources for immune cells, especially lymphocytes (15). The second reason could be that Gln can convert a substrate for nucleotide synthesis (14). Finally, Gln stimulates cytokines including interferon γ, Interleukin 2, interleukin 4, and interleukin 10, for direct or indirect lymphocyte proliferation (14).

In addition, there were no differences in erythrocyte instruments and platelet instruments between the two groups (Table 4, $p > 0.05$).

Effect of L-glutamine supplementation on *HSP* gene expression in the hair follicle and PBMCs of Hanwoo steers

At the cellular level, all animals respond to HS by synthesizing *HSPs*, which protect cells from heat-induced injury. *HSP70* and *HSP90* are molecular chaperones that protect cells from HS by refolding denatured proteins back into their correct conformations (39). There were no differences in *HSP70* and *HSP90* expressions in PBMCs between the groups ($p > 0.05$, Figure 2A and 2B). Only *HSP70* tended to change in the control group according to the sampling weeks (p = 0.093, Figure 2A).

HSP70 and *HSP90* gene expression were changed according to sampling weeks except *HSP70* in the treatment group ($p < 0.05$, Figure 2C and 2D). *HSP90* gene expression in the hair follicle was decreased in the treatment group at 10 weeks ($p < 0.01$). The expression of *HSP70* and *HSP90* in the hair follicle is among the indicators of HS responses (20). In the treatment group (0.5% Gln), the

409 expression of these two *HSPs* was less than in the control group, meaning that Gln
410 had a positive effect on the reduction in HS in 10 weeks. Thus, supplementation with
411 Gln at 0.5% may attenuate HS at 10 weeks.
412
413 **Effect of L-glutamine supplementation on gene expression related to**
414 **myogenesis and adipogenesis, lipogenesis, and lipolysis in *longissimus***
415 ***dorsi* muscle tissue of Hanwoo steers**
416 There were no differences between the groups in gene expression related to
417 muscle tissue, including *HSPB1*, *MyoD*, *MYF5*, *MYF6*, and *MyoG*, and gene
418 expression related to fat tissue, including *FABP4*, *GDP*, *LPL*, *PPARγ*, and *SCD*
419 (Figure 3, $p > 0.05$). Gln is known to be related to the development of muscle cells,
420 such as the role of respiratory fuel in muscle cells, the synthesis of proteins of
421 myosin heavy chain, and precursors for nucleotide (13). Moreover, Gln reduced the
422 HS in cells by increasing the gene expression of *HSPB1* in bovine embryonic
423 fibroblast cell (11). As Gln was added by 0, 1, 2, and 4 mM, the expression of *HSPB1*
424 was linearly increased (11). *HSPB1*-increased gene expression related to the
425 differentiation of myocytes including *MyoG* and *Desmin*, and the increased cellular
426 protein of myocytes (11). Therefore, it could be expected that the expression of
427 muscle development-related genes would sufficiently increase by the above
428 functions of Gln under HS; however, there was no difference between the two
429 groups in the current study ($p > 0.05$). The reasons could be speculated as follows.
430 First, the supplementation of Gln was used in immune cells rather than muscle cells.
431 The increase in immune cells, which is an essential factor for survival, is more
432 important than muscle development. Second, as the amount of Gln stored varies
433 depending on the muscle type, the reaction may differ depending on the Gln

supplementation. Type I muscle fibers have approximately three times more Gln storage that type II fibers (12). Type I muscle fibers have a higher activity of Gln synthetase and higher availability of ATP for Gln synthesis (12). In the case of *longissimus dorsi* muscle sampled in this study, the number of type I muscle fiber was approximately 33.1%, and the area of type I muscle fiber was approximately 24.3% of muscle fiber in Hanwoo steers (40). The amount of Gln in the *longissimus dorsi* muscle was higher than for round and chuck (41). Thus, additional Gln treatment may be less effective because the *longissimus dorsi* muscle already stores a lot of Gln. Furthermore, we need to confirm the effect of Gln supplementation on muscle development according to the parts of muscle or the type of muscle fiber in cows.

It was important to analyze the gene expression related to adipose developments in the *longissimus dorsi* muscle because this part of the muscle has interaction between muscle tissue, intra-, and intermuscular fat tissue. Carbone sourced from Gln was synthesized to fatty acid in adipocyte. Gln stimulated the gene expression of *fatty acid synthase* and *GPD* (13). However, there was no difference between the control and supplementation groups for *FABP4*, *GDP*, *LPL*, *PPARγ*, *SCD*, *HSPB1*, *MyoD*, *MYF5*, *MyF6*, and *MyoG* in this study.

Conclusion

Short-term supplementation with Gln at 0.5% of the total diet (as-fed basis) stimulated lymphocyte and granulocyte proliferation despite the HS that caused immune system decline. Despite no differences in physiological and behavioral characteristics between the control and Gln supplementation group, our results indicated that *HSP90* was more resistant to HS in the Gln supplementation group.

Gln may prioritize immune improvement under HS condition as shown in this study by boosting immune parameters, including GRA, LYM, MON, and WBC. Thus, supplementation with 0.5% Gln could be beneficial in improving the immune system in beef cattle under HS conditions. Further study is needed to examine whether muscle development improves when higher concentrations of Gln are added for longer periods.

Acknowledgments

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Tables and Figures

Table 1. Composition and nutrient content of experimental diets for steer (DM basis).

Items	Concentrate[1]	Rice straw
Chemical composition (DM), %		
Moisture	4.70	12.29
Crude protein	15.35	4.45
Ether extraction	3.37	1.74
Neutral detergent fiber	25.43	66.70
Acid detergent fiber	7.66	45.13
Non-fiber carbohydrate [2]	47.58	12.43
Crude ash	8.27	14.68
Ca	0.78	0.33
P	0.44	0.13
Total digestible nutrition[3]	75.0	43.66
Digestible energy[4]	3.31	1.93

[1] Commercial feed (Cargill Agri Purina, Seoul, Korea)

[2] Calculated value using NRC, 2001 equation is 100 - (CP + EE + NDF - NDIP + ASH)

[3] Calculated value using NRC, 2001 equation is truly digestible (td)NFC + tdCp + (tdFA × 2.25) + tdNDF -7

[4] Calculated value using NRC, 1989 equation is 0.04409 × TDN

589 **Table 2.** Primer sequences used in qPCR assays.

Gene symbol	Gene name	Annealing temperature	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
FABP4	Fatty acid binding protein 4	60.8°C	TGTCACTGCCA CCAGAGTTT	TGGACAACGTAT CCAGCAGAA
GDP	glycerophosphate dehydrogenase	65.0°C	CACGAAGTCCA TCTCCCGAA	GTTGTCCACTTT CCACCTGCT
LPL	Lipoprotein lipase	60.0°C	TACCCTGCCTG AAGTTTCCAC	CCCAGTTTCAG CCAGACTTTC
PPARγ	Peroxisome proliferator-activated receptor gamma	61.0°C	ACTTTGGGATCA GCTCCGTG	TCCTCATAGTGC GGAGTGGA
SCD	Stearoyl-CoA desaturase	60.0°C	TCCGACCTAAG AGCCGAGAA	GCAGGATGAAG CACAACAACAG
HSPB1	Heat shock protein beta 1	60.0°C	CCTGGACGTCA ACCACTTC	GCTTGCCAGTG ATCTCCAC
MYOD	Myoblast determination protein	59.6°C	AGAGTTGCTTTG CCAGAG	CTGCCTGCCGT ATAAACA
MYF5	Myogenic factor 5	62.5°C	TCCTGATGTACC AAATGTATATGC C	ATCCAGGTTGCT CTGAGTTGG
MYF6	Myogenic factor 6	60.7°C	GAAGGAGGGAC AAGCATTGA	GAGGAAATGCT GTCCACGAT
MYOG	Myogenin	65.0°C	TACAGACGCCC ACAATCTGC	GGTTTCATCTGG GAAGGCCG
HSP70	Heat shock protein 70	60.0°C	TACGTGGCCTT CACCGATAC	GTCGTTGATGA CGCGGAAAG
HSP90	Heat shock protein 90	60.0°C	GGAGGATCACT TGGCTGTCA	GGGATTAGCTC CTCGCAGTT
18S	18S ribosomal RNA	51.0°C	ACCCATTCGAAC GTCTGCCCTATT	TCCTTGGATTGT GGTAGCCGTTT CT
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	60.0°C	CGTGGAGGGAC TTATGACCAC	CGCCAGTAGAA GCAGGGATG
RPLP0	Ribosomal protein lateral stalk subunit P0	62.5°C	CAACCCGGCTC TGGAGAAACTG	ACTTCACACGG CGCTATGG
B2M	beta-2-microglobulin	60.0°C	GACACCCACCA GAAGATGGA	CAGGTCTGACT GCTCCGATT
RPS15A	ribosomal protein S15a	60.0°C	CCGTGCTCCAA AGTCATCGT	GGGAGCAGGTT ATTCTGCCA

590 **Table 3.** Effect of 0.5% L-glutamine supplementation on blood amino acid
591 concentration in Hanwoo steers.

Items, μmol/L	CON	TRT	SEM	*P*-value
Ammonium chloride	50.9	46.8	3.44	0.278
Glycine	62.1	65.5	3.88	0.410
L-Alanine	48.8	52.6	4.05	0.378
L-Arginine	22.7	26.0	1.84	0.123
L-Citrulline	23.3	30.2	3.01	0.061
L-Glutamic acid	12.4	28.2	7.58	0.083
L-Histidine	11.7	11.5	1.21	0.896
L-Isoleucine	24.0	26.4	2.86	0.421
L-Leucine	36.6	43.1	5.00	0.244
L-Lysine	37.1	37.7	8.72	0.944
L-Methionine	6.3	5.5	0.66	0.302
L-Ornithine-monohydrochloride	21.4	24.6	3.45	0.391
L-Phenylalanine	23.8	21.1	1.44	0.104
L-Tryptophan	6.3	7.7	0.76	0.132
L-Tyrosine	19.6	19.2	2.07	0.840
L-Valine	56.8	65.5	8.30	0.338
Taurine	12.0	14.4	1.93	0.256

592

593 **Table 4.** Effect of 0.5% L-glutamine supplementation on biochemical parameters in

594 Hanwoo steers.

Items	CON	TRT	SEM	*p*-value
Blood urea nitrogen, mg/dL	18.21	17.84	1.68	0.837
Glucose, mg/dL	71.88	66.68	2.71	0.104
Total protein, g/dL	7.50	7.12	0.19	0.097
Albumin, g/dL	3.43	3.25	0.08	0.059
Globulin, g/dL	4.06	3.84	0.14	0.146
A/G ratio	0.84	0.86	0.04	0.616
Creatine phosphokinase, μ/L	111.50	121.50	23.41	0.684

595

596

Table 5. Effect of 0.5% L-glutamine supplementation on haematological parameters in Hanwoo steers.

Items	CON	TRT	SEM	*p*-value
Leukocyte indices				
WBC, 10^3/μL	10.20	12.88	1.15	0.058
LYM, 10^3/μL	5.78	7.51	0.78	0.068
GRA, 10^3/μL	3.96	5.07	0.40	0.031
MON, 10^3/μL	0.45	0.31	0.21	0.520
LYM, %/WBC	57.19	58.06	2.50	0.740
GRA, %/WBC	38.59	39.71	2.31	0.644
MON, %/WBC	4.53	2.33	0.94	0.058
Erythrocyte indices				
RBC, 10^6/μL	9.93	8.84	0.73	0.185
HGB, g/dL	14.24	13.56	0.64	0.328
HCT, %	40.01	38.06	1.86	0.334
MCH, pg	14.41	15.42	0.68	0.188
MCHC, g/dL	35.64	35.63	0.56	0.988
MCV, fL	40.26	43.15	2.01	0.200
RDW, %	21.75	20.48	0.63	0.090
Platelet indices				
PLT, 10^3/μL	352.35	310.92	60.84	0.521
PCT, %	0.25	0.22	0.05	0.600
MPV, fL	6.85	7.15	0.24	0.259
PDW, %	33.25	33.07	1.19	0.888

WBC: white blood cell; LYM: lymphocyte; GRA: granulocyte; MON: monocyte; RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; MCH: mean corpuscular

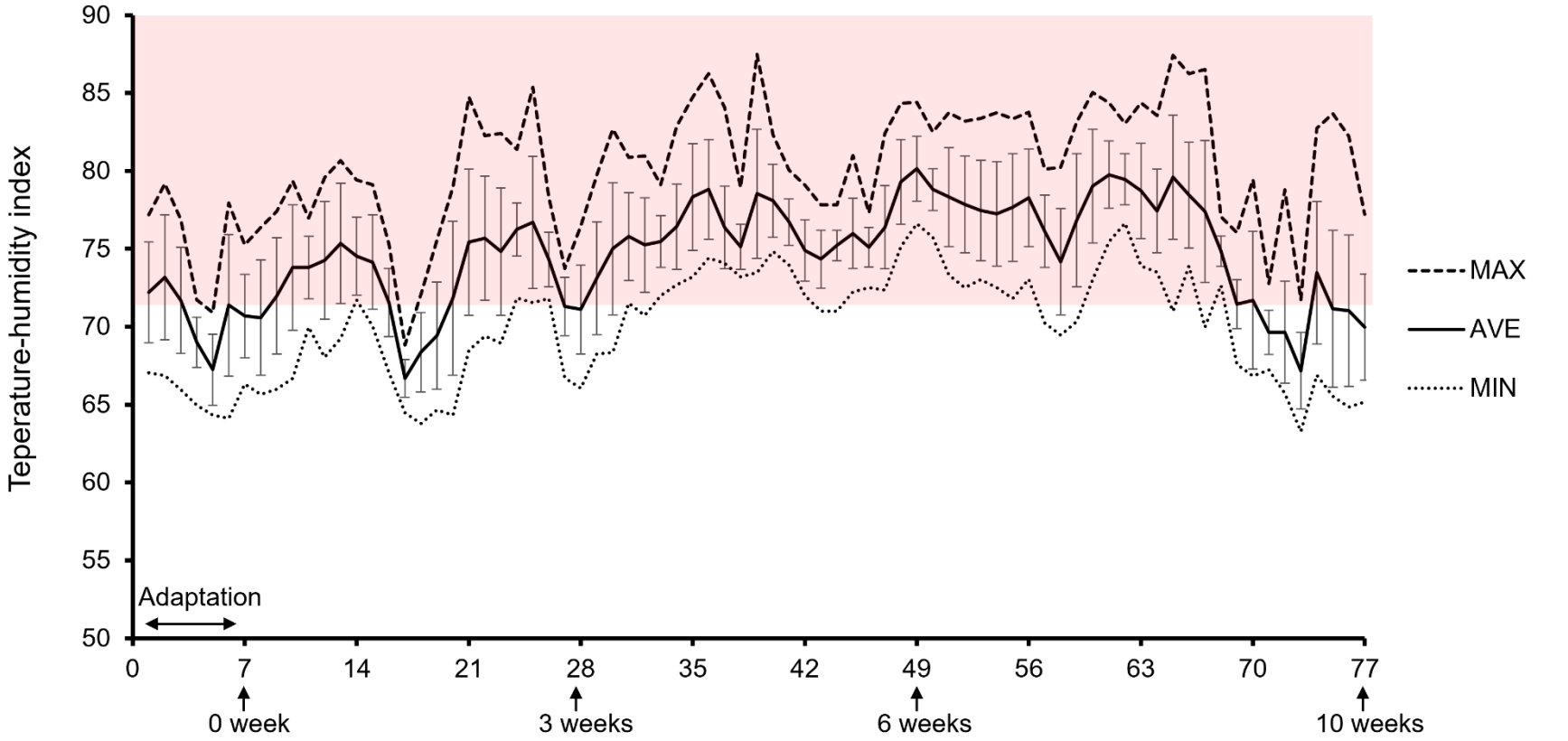
602 hemoglobin; RDW: red blood cell width; PLT: platelet; MPV: mean platelet volume;

603 PCT: plateletcrit; PDW: platelet distribution width.

604 **Table 6.** Effect of 0.5% L-glutamine supplementation on growth performance in
605 Hanwoo steers.

Items	Control	Treatment	SEM	*p*-value
0 weeks				
Average BW	571.8	569.6	23.5	0.950
3 weeks				
Average BW	578.0	579.5	22.8	0.964
Increment	6.25	9.94	3.82	0.537
Average daily gain	0.30	0.47	0.18	0.533
Feed intake	9.39	9.89	0.55	0.539
Gain-to-feed ratio	0.03	0.05	0.03	0.354
6 weeks				
Average BW	584.5	591.1	25.2	0.859
Increment	6.50	11.6	3.90	0.388
Average daily gain	0.31	0.55	0.19	0.388
Feed intake	9.21	9.48	0.16	0.182
Gain-to-feed ratio	0.03	0.06	0.02	0.360
10 weeks				
Average BW	610.5	618.4	22.1	0.809
Increment	26.0	27.3	4.92	0.863
Average daily gain	0.90	0.94	0.17	0.863
Feed intake	9.40	9.47	0.19	0.738
Gain-to-feed ratio	0.10	0.10	0.02	1.000
Total				
Increment BW	12.9	16.3	3.71	0.395
Average daily gain	0.50	0.66	0.17	0.383
Feed intake	9.35	9.61	0.41	0.671
Gain-to-feed ratio	0.06	0.07	0.02	0.476

606



607

608 **Fig. 1.** Temperature–humidity index during the experimental period from 27 June 2020 to 13 September 2020.

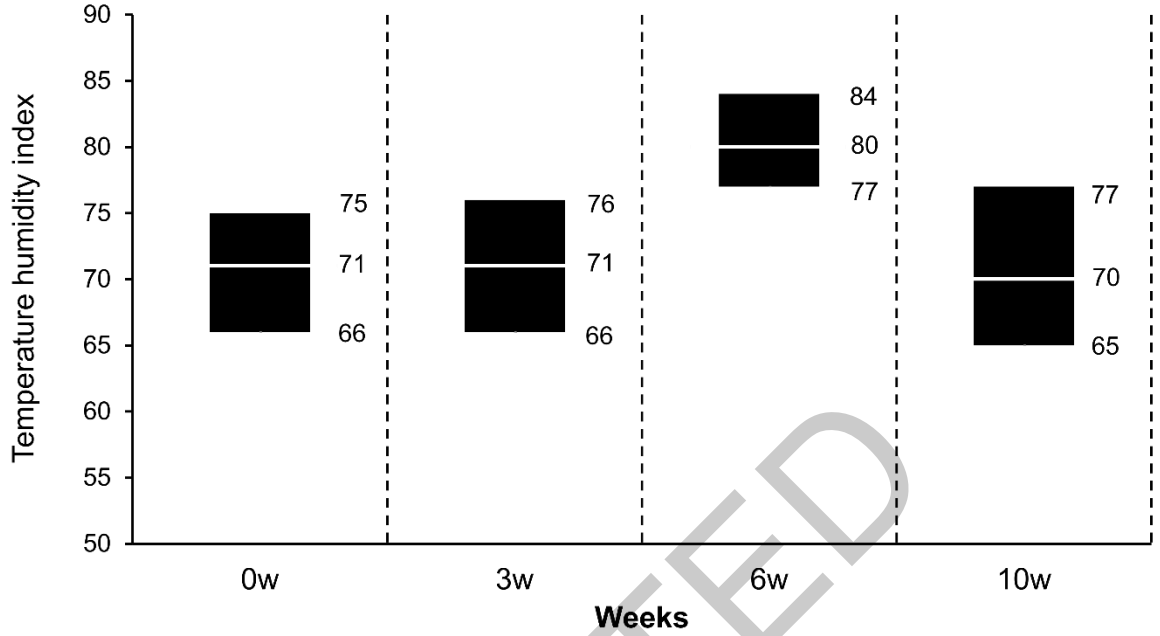
609 Temperature–humidity index calculated as follows: THI= $(1.8 \times T_{db}+32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$ (Council,

610 1971).

611 MAX: maximum THI; AVE: average THI; MIN: minimum THI. The error bar means standard deviation. The red part means above

612 THI 72, HS detected in cows.

613 **(A)**



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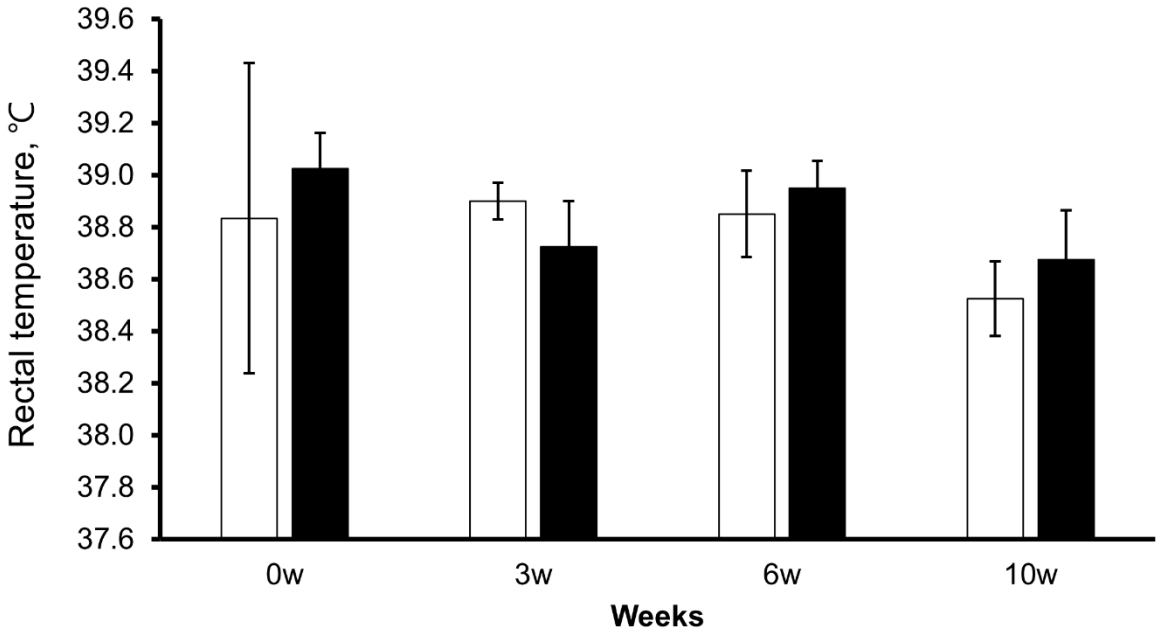
615 **(B)**



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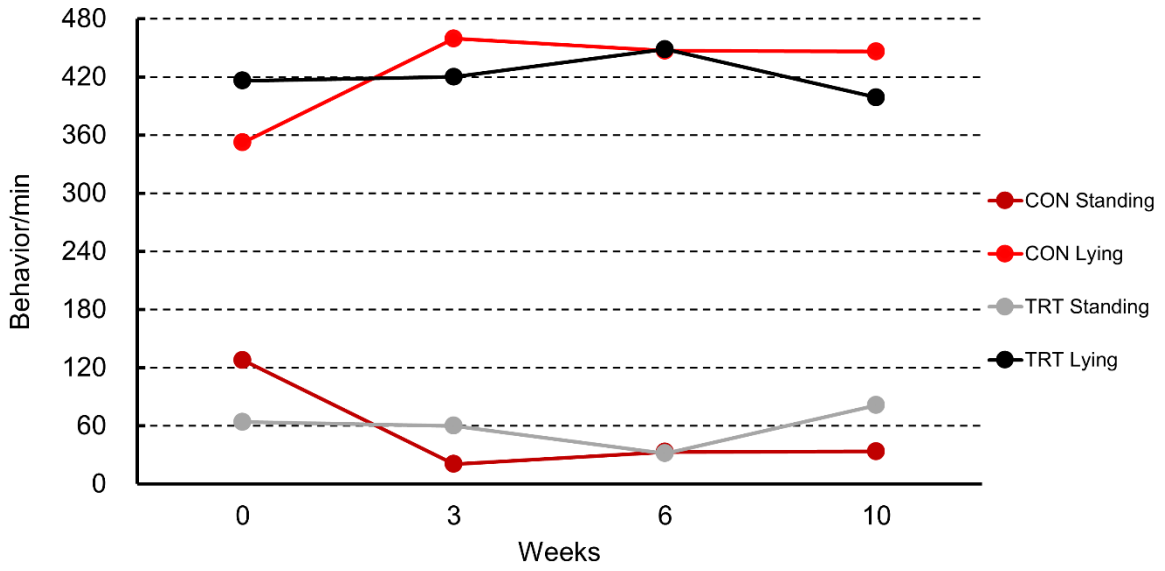
617 **(C)**



618

619 **Figure 2.** Rectal temperature and behavior including standing and lying (1000 to 1800

620 h) during the experimental period at 0, 3, 6, 10 weeks.

621 (A) Maximum THI and average THI, minimum THI of the day, measured RT and

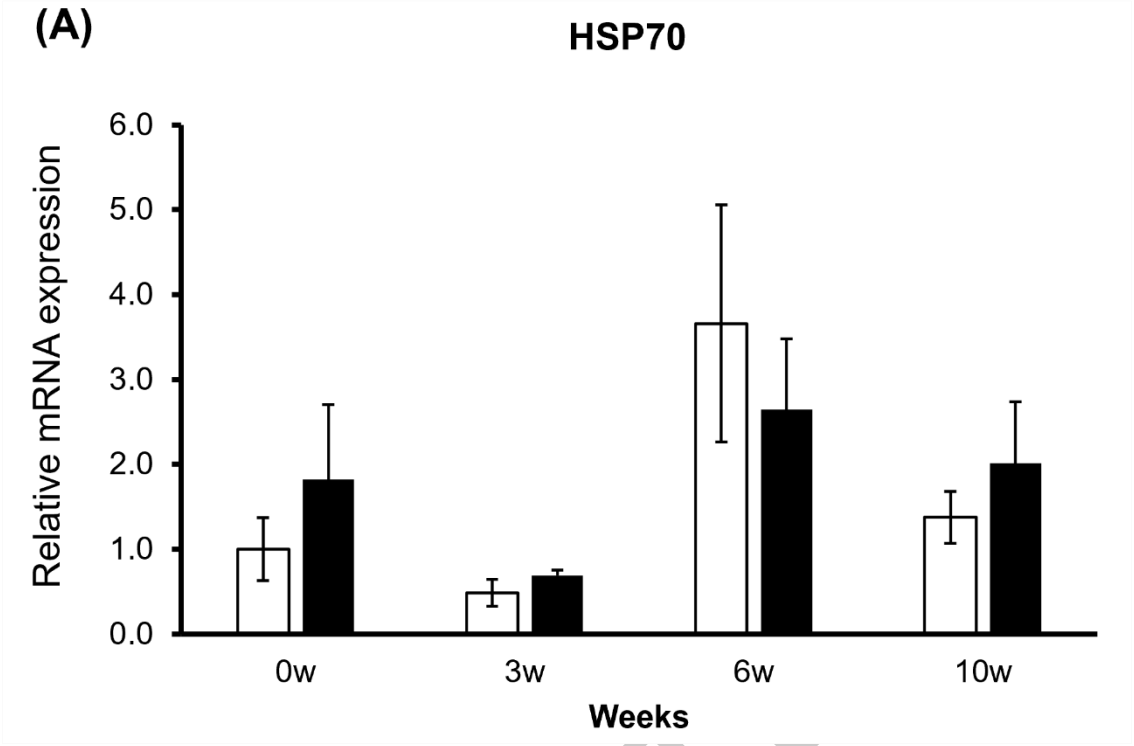
622 behavior. Temperature–humidity index calculated as follows: THI = (1.8 × T_{db}+32) –

623 [(0.55 – 0.0055 × RH) × (1.8 × T_{db} – 26.8)] (18). (B) The RT at 0, 3, 6, 10 weeks. Data

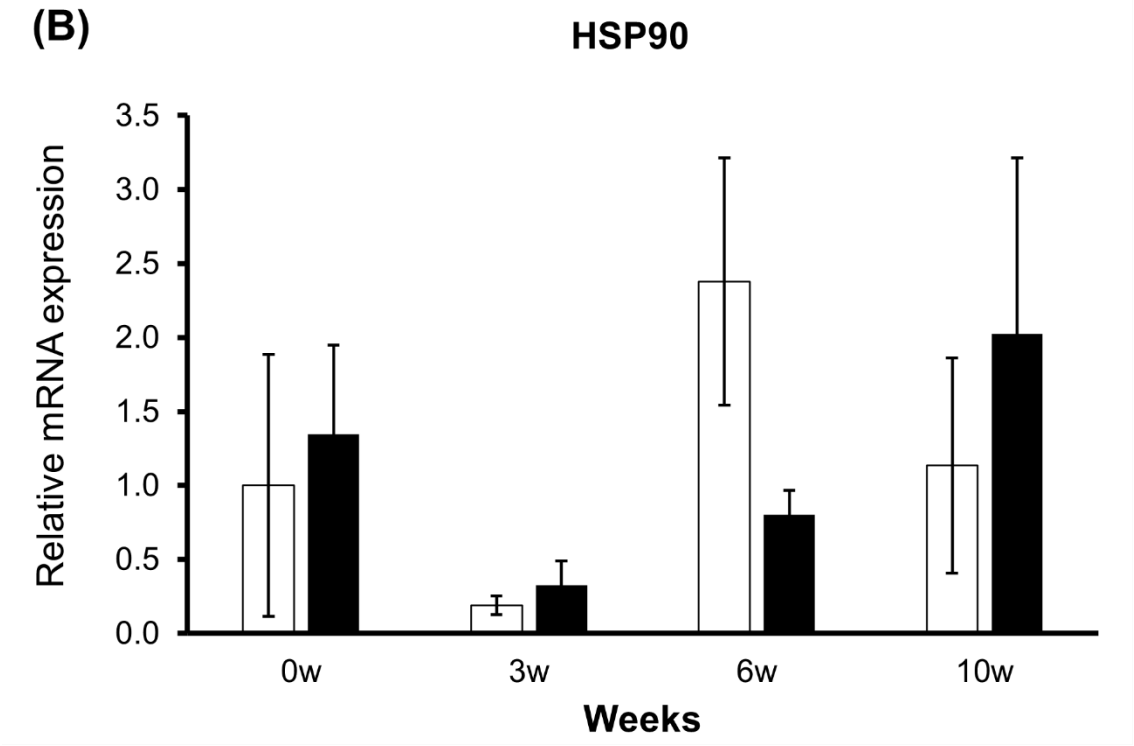
624 are presented as the means ± standard error. □ control ■ treatment, error bar means

625 standard error (n = 4) (p > 0.05). (C) Behavior including standing and lying in 0, 3, 6,

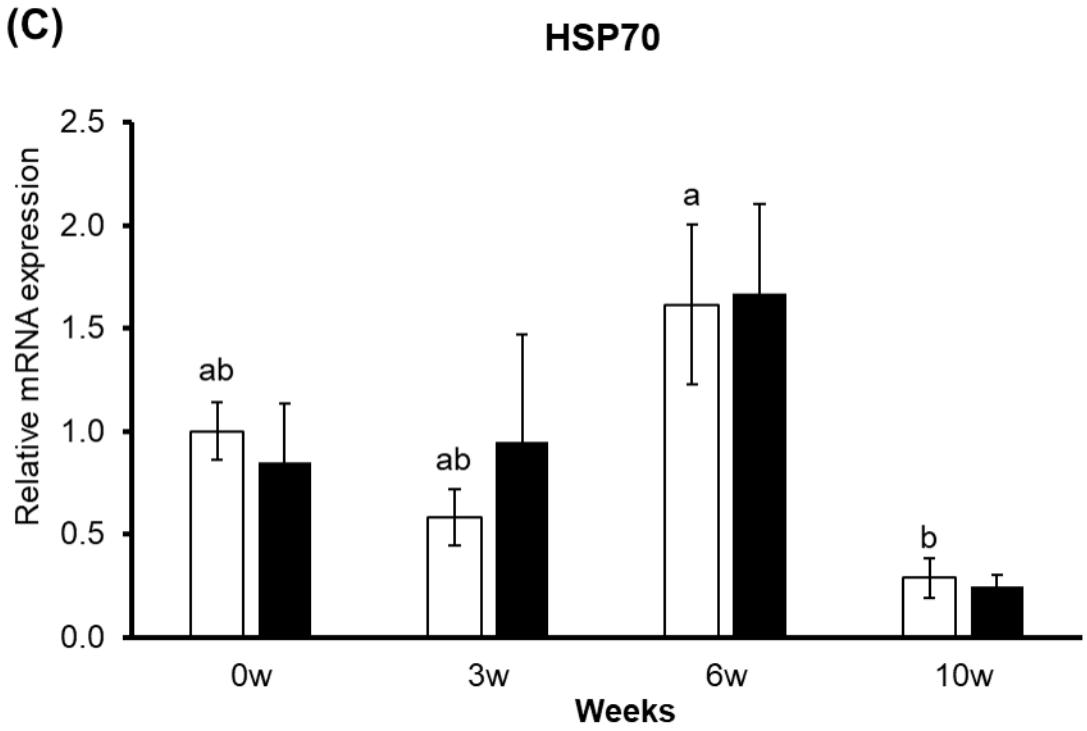
626 10 weeks (p > 0.05).


(A)
HSP70
Relative mRNA expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0w
3w
6w
10w
Weeks

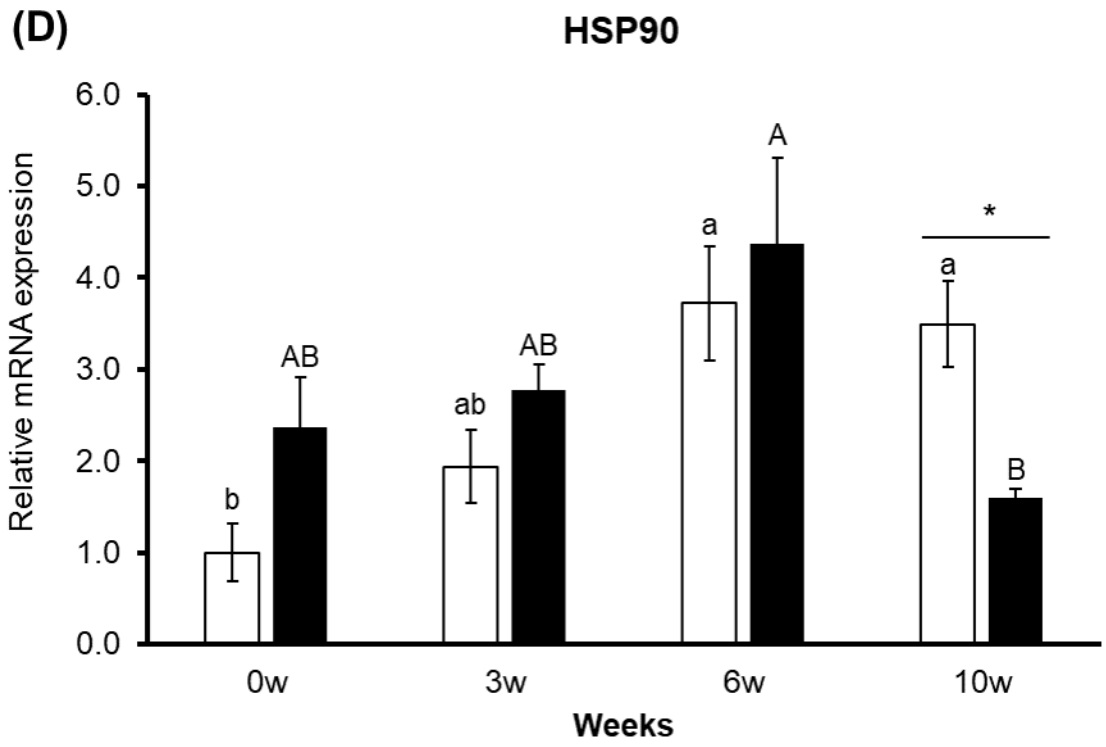
627


(B)
HSP90
Relative mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0w
3w
6w
10w
Weeks

628


(C)
HSP70
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
ab
ab
a
b
0w
3w
6w
10w
Weeks

629


(D)
HSP90
Relative mRNA expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
b
AB
ab
AB
a
A
a
*
B
0w
3w
6w
10w
Weeks

630

Figure 3. Heat shock protein 70 and 90 gene expression on peripheral blood mononuclear cells (PBMCs) and hair follicles in Hanwoo steers from the control group and L-glutamine supplementation group.

(A) Validation by qPCR of *HSP70* gene in PBMCs. (B) Validation by qPCR of *HSP90* gene in PBMCs. (A–B) qPCR values are shown as the fold change in expression after normalization to the control genes at week 0 *RPS15A* and *B2M*. (C) Validation by qPCR of *HSP70* gene in the hair follicle. (D) Validation by qPCR of *HSP90* gene in the hair follicle. (C–D) qPCR values are shown as the fold change in expression after normalization to the control genes at week 0 *GAPDH*. □ Control group (n = 4), ■ Treatment group (n = 4). Data are presented as the means ± standard error. * Means with different superscripts differ significantly between groups ($p < 0.01$). $^{a, b}$ Means with different superscripts differ significantly in the control group ($p < 0.05$) among the sampling weeks. $^{A, B}$ Means with different superscripts differ significantly in the treatment group ($p < 0.05$)

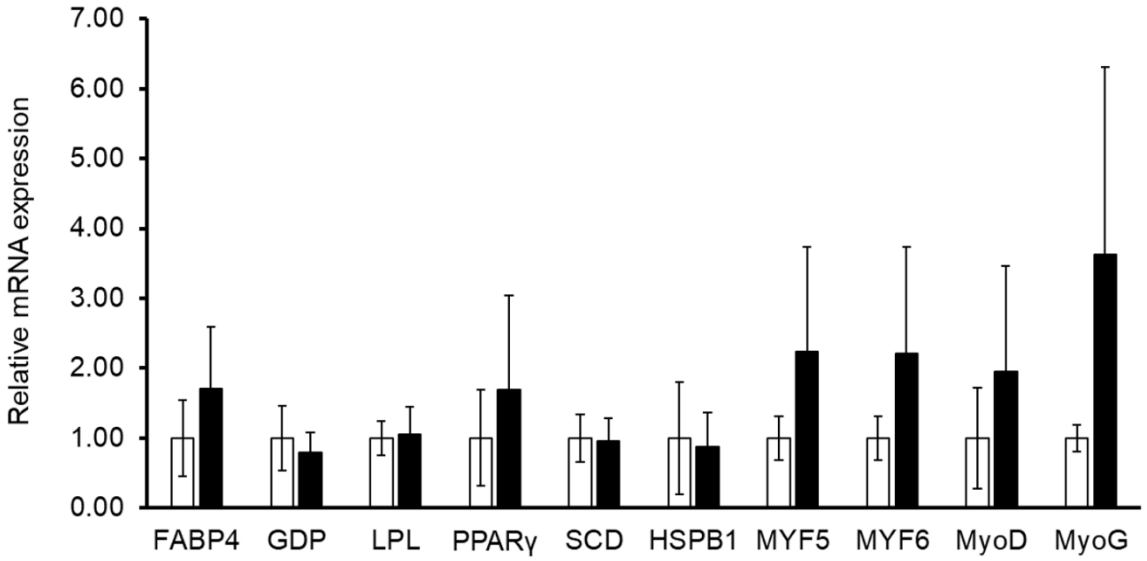


Figure 4. Gene expression study in Hanwoo steer *longissimus dorsi* muscles from the control group and L-glutamine supplementation group.

Validation by qPCR of 13 genes in *longissimus dorsi* muscles. The qPCR values are shown as the fold change in expression after normalization to the control genes 18S, GAPDH, and RPLP0. □ Control group (n = 4), ■ Treatment group (n = 4). Data are presented as the means ± standard error. The full names of the genes are fatty acid-binding protein 4 (*FABP4*), glycerophosphate dehydrogenase (*GDP*), lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor-gamma (*PPARγ*), stearoyl-CoA desaturase (*SCD*), heat shock protein beta 1 (*HSPB1*), myoblast determination (*MyoD*), myogenic factor 5 (*MYF5*), myogenic factor 6 (*MYF6*), and myogenin (*MyoG*). $p > 0.05$.