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Physiology

The relationships between environmental parameters in livestock pen and physiological parameters of Holstein dairy cows

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ABSTRACT. There has been an increase in temperature and the incidence of extreme weather events, such as heat wave, due to global warming, which has promoted the incidence of livestock diseases. Therefore, it is important to examine the effect of changes in environmental parameters on livestock performance. The aim of this study was to examine the relationship between ambient environmental conditions in livestock pen and the physiological parameters of Holstein dairy cows. The results showed that there was a decrease in the red blood cell counts, hemoglobin concentrations, and mean corpuscular hemoglobin concentration of the cows with increasing pen temperature, wet bulb globe temperature (WBGT), and temperature humidity index (THI). Additionally, high daily variation in temperature caused a decrease in the serum albumin levels of the cows. Moreover, the lowest serum calcium, inorganic phosphorus, and magnesium concentrations were observed in November, and were negatively correlated with the 24-hr temperature, WBGT, and THI range of the pen prior to sampling. Multiple regression analysis showed a positive correlation between serum cortisol concentration and 24-hr WBGT range of the pen prior to samplings and packed cell volume. However, serum cortisol and total protein concentrations were negatively correlated. Overall, the findings of the study suggest that large variation in temperature induced stress in the cows, which could be overcome by increased water consumption and improved protein digestion and absorption by the animals, and the addition of minerals, such as calcium to the diet.

KEYWORDS: blood examination value, cortisol, Holstein dairy cow, temperature humidity index, wet bulb globe temperature

Recently, there has been an increase in the incidence of livestock diseases due to climate change, indicating the need for further research [33]. Particularly, climate change affects the productivity of dairy and beef cattle considerably by influencing environment factors, such as temperature and humidity, in pens. Several studies have examined the combined effect of ambient temperature and humidity on the performance of Holstein dairy cows using the temperature humidity index (THI) [15, 18, 26, 38, 41].

The wet bulb globe temperature (WBGT) is used to assess the risk of heat stroke in humans [8]. Individuals in the median-based heat exposure category with the highest WBGT have a higher prevalence of heat-related symptoms than those in the lowest category, providing an indicator of the health effect of global warming [7]. WBGT has been examined in thermal comfort studies in cattle as well [17, 40].

Additionally, serum cortisol levels have been used as a stress marker in animals [4, 21]. A significant increase in serum cortisol concentration has been reported in dairy cows used for practical training, indicating stress caused by the training [9]. Although several studies [6, 13, 37] have examined the effects of ambient environmental conditions on the performance of cattle, studies are yet to examine the relationship between environmental variables, such as temperature, humidity, WBGT, and THI and the milk yield, somatic

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cell count (SCC) in milk, hematological and serum parameters, and serum cortisol concentration of Holstein dairy cows.

Therefore, the aim of this study was to examine the relationship between ambient environmental conditions, such as temperature, humidity, WBGT, and THI and the rectal temperature (RT), respiratory rate (RR), body condition score (BCS), rumen fill score (RFS), fecal score (FS), milk yield, SCC in milk, hematological and serum parameters, and serum cortisol levels of Holstein dairy cows.

MATERIALS AND METHODS

Farm

The experiment was performed in a farm located in Imabari City, Ehime Prefecture, Japan (34.0447495°N, 132.988554°E, 11.9 m above sea level). A total of 87 cows were used in this farm, and the type of housing was free pen with one 833.3 m^2 pen for milking cows and one 166.6 m^2 pen for dry cows. The cowshed was a single L-shaped structure facing the southwest direction, with steel framed posts and beams and slate roof. Fans were installed in 12 locations in the pen and operated all year round. The milking system consisted of a waiting area in the northern part of the pen and a walk-through milking parlor in the eastern part, where a total of 12 cows are milked in the morning and evening daily.

Feeds

Total mixed rations (TMR) for milking cows were prepared twice a day using TMR mixer and fed at 9:00 am and 7:00 pm. In the dry period, each cow was fed Sudan Hay and dry milk formula. Feeds used in the study were purchased from the manufacturer (Zenno, Tokyo, Japan). The ingredients and chemical composition of the TMR and dry-lactation diets are shown in Table 1. The composition and feed quantity administered did not change during the study period.

Table 1. Ingredients and chemical composition of total mixed ration (TMR) and dry period feed

Item	Value	
	TMR	Dry period feed
Ingredients (% of DM[1])		
Formula feed[2]	49.6	14.3
Corn flakes	3.5	
Brewers grains	6.4	
Sudan hay	21.0	85.7
Timothy hay	11.6	
Flowering alfalfa hay	7.2	
Chemical composition (% of DM[1])		
TDN[3]	70.8	61.7
CP[4]	14.3	8.4
UIP[5]	5.3	3.1
SIP[6]	3.2	1.9
Ca[7]	0.6	0.5
P[8]	0.4	0.3
Fat	3.3	1.8
ADF[9]	21.0	29.9
NDF[10]	35.9	48.5
Starch	25.7	14.0
NFC[11]	38.2	11.0
Ash	5.0	5.0

[1]: Dry matter, [2]: different types of lactation and dry period, [3]: total digestible nutrients, [4]: crude protein, [5]: undegraded intake protein, [6]: soluble intake protein, [7]: calcium, [8]: phosphorous, [9]: acid detergent fiber, [10]: neutral detergent fiber, [11]: non fibers carbohydrate.

Measurement of temperature and humidity

The temperature and humidity in the cowsheds were automatically measured 16 times a day at 90-min intervals by a temperature and humidity memory meter (SK-L200TH IIα, SATO KEIRYOKI MFG Co., Ltd., Tokyo, Japan). The temperature and humidity memory meter was placed approximately 1.8 m from the floor, between the waiting area and the free pen fence, to ensure uniform and representative temperature and humidity values. Data from the temperature and humidity storage meters were periodically recorded on a laptop computer. WBGT was derived from the conversion table (relationship between WBGT value, temperature, and relative humidity) in the "Guidelines for Prevention of Heat Stroke in Daily Life" of the Japanese Society of Biometeorology [14]. THI was calculated using the following formula: $(1.8 \times \text{dry-bulb temperature} + 32) - [(0.55\text{–}0.0055 \times \text{relative humidity}) \times (1.8 \times \text{dry-bulb temperature} - 26.8)]$ [19].

Cows

A total of 10 milking Holstein dairy cows of healthy, with average age of 53 months (37–120 months) were randomly selected for the study. Among the 10 cows, three cows had one obstetric history, six had two, and one had six. The mean number of days in milk (DIM) at the beginning of the study was 228 days (± standard deviation 98 days). The cows were observed for health status once in the middle of the month throughout the year from September 2020 to August 2021. The cows were observed promptly, restrained in interlocking stanchions on an empty stomach immediately after morning milking (8:00 to 9:00 am), and the following parameters were recorded: RT, RR, heart rate (HR), BCS, RFS, locomotion score (LS), FS, and presence of mastitis. Determination of BCS, RFS, LS and FS was done using the determination method reported in previous paper. [3]. The same parameters above were also observed for dry cows. This study was conducted with the approval of the Committee for Experimental Animals, Okayama University of Science (Approval number: Jitsu 2021-030).

Milk yield and SCC

Milk yield was recorded by the milking system in the morning during observation and milking time and during milking in the previous night, and the sum of these records was used as the daily milk yield. Milk collected in the previous night and the following day were from a single cow were pooled for SCC analysis. The SCC in milk samples (10–15 ml per sample) was determined using a simplified somatic cell counting device (ADAM-scc, Nano En Tek Co., Ltd., Hwaseong, Korea). Cows in dry period were excluded from the milk yield and SCC measurements. During the study period, a cow was dry in December, January, May, July, and August, and three cows were dry in February.

Blood collection

Approximately 12 ml of blood was collected from the median caudal vein of each cow using vacuum collection tube, of which 10 ml was transferred in tube for serum analysis (Beneject II vacuum collection tube VP-P100K, TERMO Co., Ltd., Tokyo, Japan) and 2 ml was transferred into EDTA bottle for hematological analysis (Beneject II vacuum collection tube VJ-DK052E004, TERMO Co., Ltd.). Blood serum was separated by centrifugation at 3,500 rpm for 10 min, and the serum was stored at −80°C for further analysis.

Blood Examination

EDTA blood was immediately collected and analyzed for white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet counts (PLT), using an automated blood cell analyzer (Celltac Alpha MEK-6558, Nihon Kohden Co., Ltd., Tokyo, Japan). Packed cell volume (PCV) was measured by placing EDTA blood in a hematocrit capillary tube (EM Meister Hematocrit Capillary Plain Asone Co., Ltd., Osaka, Japan) and centrifuging at 10,000 rpm for 5 min. Serum parameters were determined using an autoanalyzer (Hitachi autoanalyzer, Hitachi High-Technologies Co., Ltd., Tokyo, Japan). Serum parameters include aspartate transferase (AST), gamma-glutamyl transpeptidase (γ-GTP), blood urea body nitrogen (BUN), total protein (TP), albumin (Alb), blood glucose (Glu), total cholesterol (T-cho), calcium (Ca), inorganic phosphorus (iP), magnesium (Mg), and non-esterified free fatty acid (NEFA) concentrations. A/G was calculated as Alb/TP-Alb.

Measurement of serum cortisol level

Serum cortisol concentration was determined using a commercially available cortisol ELISA kit (Cortisol ELISA Kit Item No. 500360, Cayman Chemical Co., Ltd., Ann Arbor, MI, USA).

Statistical analysis

Statistical analysis was performed using EZR (EasyR version 4.0.3, Jichi Medical University Saitama Medical Center, Saitama, Japan). The mean, maximum, minimum, and range values for temperature, humidity, WBGT, and THI of the cows, 24 hr prior to observation were analyzed using Kruskal-Wallis test. Similarly, monthly data, including RT, RR, HR, BCS, RFS, LS, FS, presence of mastitis, milk yield, and SCC were analyzed using Kruskal-Wallis test. Additionally, hematological and serum parameters and cortisol concentrations were analyzed using Kruskal-Wallis test.

Furthermore, simple regression analysis was performed to determine the relationships between the environmental parameters highlighted above and physiological (RT, RR, HR, BCS, RFS, LS, FS, milk yield, and SCC), hematological, and serum parameters. The relationships among serum albumin, calcium, inorganic phosphorus, and magnesium concentrations were determined using simple regression analysis.

Multiple regression was performed among the parameter to determine the dependent and independent/causal parameters. Statistical significance was set at $P<0.05$.

RESULTS

The mean, maximum, minimum, and range data of the environmental parameters 24 hr prior to sampling are shown in Fig. 1. Throughout the year, temperature, humidity, WBGT, and THI were highest in August 2021 in the summer and lowest in January 2021 in the winter. The largest variations in WBGT and THI values were observed in November 2020. Additionally, there was no incident of mastitis or lameness among the experimental animals. The RT of the cows was highest ($P<0.01$) in August 2021, whereas RR was highest in September 2020, July and August 2021 (Fig. 2). The mean RT and RR of the cows were significantly positively correlated ($P<0.01$) with the temperature, heat index, and THI (mean, maximum, and minimum, respectively) of the pen 24 hr prior to sampling (Table 2).

There were no significant differences in the daily DIM, milk yield, and SCC of the cows between the months (Table 3). Additionally, there was no significant correlation with the parameters of the barn environment (results not shown).

Compared with the other months, there was a significant decrease ($P<0.001$) in the MCHC of the cows in June, July, and August 2021 (Fig. 3). The mean RBC of the cows was significantly negatively correlated ($P<0.05$) with pen temperature, humidity, WBGT, and THI (mean, maximum and minimum, respectively) 24 hr prior to sampling (Table 4). Similarly, the mean HGB levels of the cows were negatively correlated with the temperature, WBGT, and THI (mean, maximum, and minimum, respectively) of the pen 24 hr prior to sampling. Additionally, the MCHC of the cows was negatively correlated with the temperature (mean, maximum and minimum), humidity (mean and minimum), WBGT (mean, maximum and minimum), and THI (mean, maximum and minimum) of the pen 24 hr prior to sampling, and positively correlated with the 24-hr temperature, humidity, and THI range (Table 4).

Furthermore, there was a significant decrease ($P<0.05$) in the serum BUN, TP, Alb, Glu, T-cho, Ca, iP, and Mg concentrations of the cows in November compared with the other months (Figs. 4 and 5). Although there was no significant difference in the serum cortisol levels of the cows, the highest value was obtained in November 2020 (Fig. 5). The correlations between temperature, humidity, heat index, and THI of the pen 24 hr prior to sampling and the serum biochemistry parameters and cortisol levels of the cows are shown in Table 5. The mean serum AST levels of the cows were negatively correlated with the 24 hr temperature and WBGT range of the pen. Moreover, mean serum γ-GTP and TP concentrations were negatively correlated with the 24-hr temperature, WBGT, and THI range of the pen prior to sampling. Furthermore, the mean serum Alb concentrations of the cows

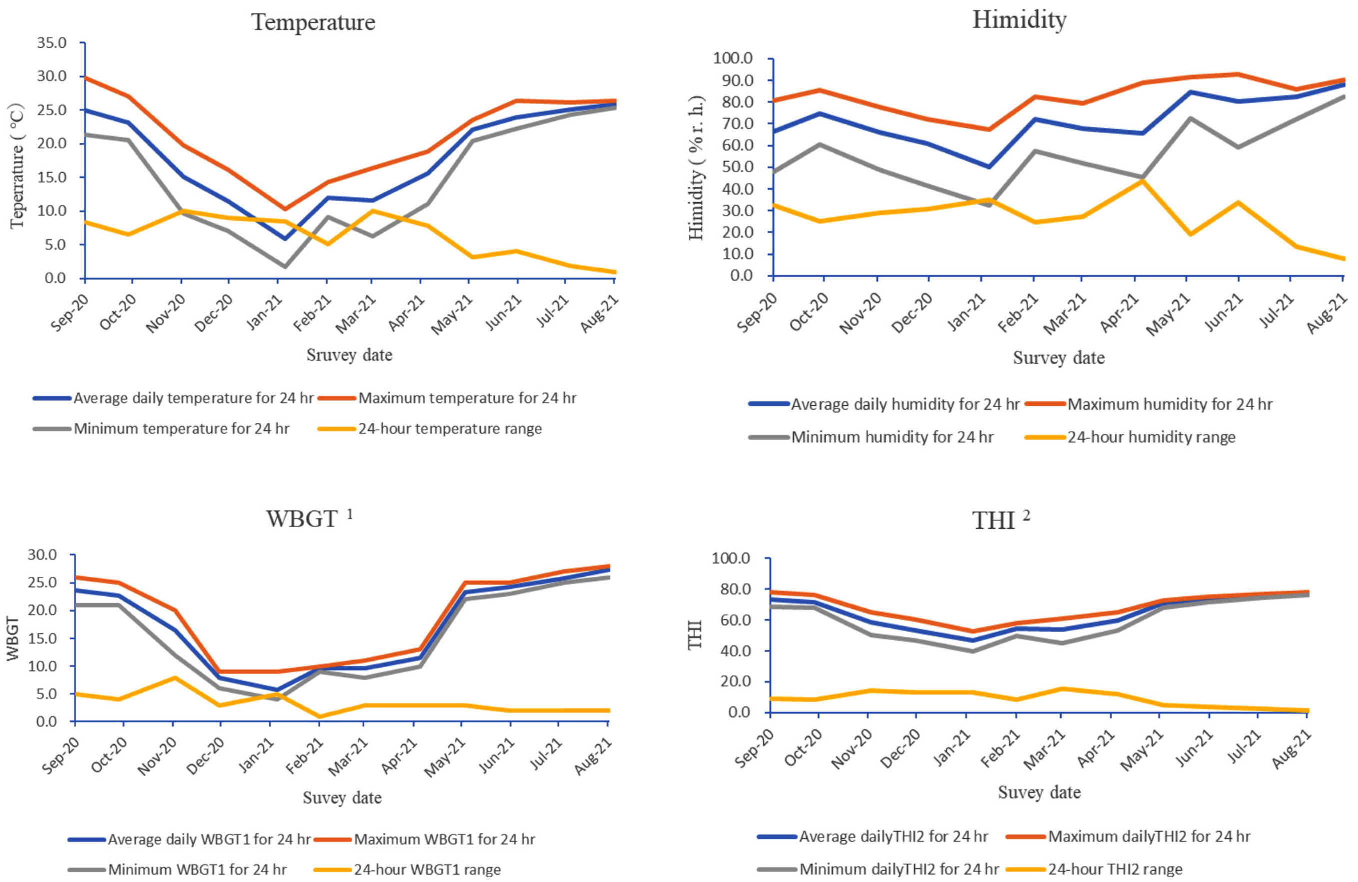

Fig. 1. Temperature, humidity, Wet Bulb Globe Temperature (WBGT) and temperaturehumidity index (THI) of 24 hr prior to the time of observation of the cows during the year. Temperature and humidity data were recorded every 90 min from 9 am the day before to 9 am on the day of blood sampling. The heat index shows the temperature and humidity recorded every 90 min from 9:00 am on the previous day to 9:00 am on the day of blood sampling by the conversion table of the Japan Weather Association. The temperature and humidity index were calculated by a formula for the temperature and humidity recorded every 90 min from 9:00 am on the previous day to 9:00 am on the day of blood sampling.

were significantly negatively correlated with the 24-hr temperature and WBGT range of the pen prior to sampling. The monthly serum Ca, iP and Mg concentrations of the cows were negatively correlated with the 24-hr temperature, WBGT of the pen prior to sampling. Additionally, there was a significant negative correlation between the monthly serum cortisol levels of the cows and the 24-hr WBGT range of the pen prior to sampling.

Furthermore, there were significant positive correlations between the serum ALB levels of the cows and serum Ca, iP, and Mg concentrations (r=0.9945, r=0.9377, and r=0.9806, $P<0.001$, respectively) (Fig. 6). Positive correlations ($P<0.001$) were observed between serum Ca and iP concentrations (r=0.9398), Ca and Mg concentrations (r=0.9678), and Mg and iP concentrations (r=0.9286) (Fig. 7).

Multiple regression analysis showed a significant causal relationship ($P=0.0078$) between the mean serum cortisol concentrations of the cows (dependent variable) and the 24-hr WBGT range of the pen prior to sampling (r=0.7221), mean serum TP concentration (r= −0.6092), and PCV values (r=0.5116) (explanatory variables) (Table 6).

DISCUSSION

The production limit temperature for Holstein dairy cows has been reported to be 24°C [2].WBGT represents the effect of heat on physical work capacity, and is used to assess heatstroke risk in humans [8]. A WBGT of 25 or more is considered high-risk for heatstroke, 28 or more is considered alarming, and 31 or more is considered dangerous [8]. WBGT has also been applied in thermal comfort studies of cattle [17]. Studies has shown that THI of 72 and above can negatively affect Holstein dairy cows [26, 38]. In this study, the average THI of the pen 24 hr prior to sampling was above 72 in September, June, July, and August, and the minimum was above 72 in July and August. Moreover, the highest temperature, WBGT, and THI range of the pen 24 hr prior to sampling were observed in November.

Heat stress has been shown to reduce the appetite of dairy cows, resulting in lower milk output and reproductive performance [27]. In the present study, the temperature, WBGT, and THI of the pen 24 hr prior to sampling were positively correlated with

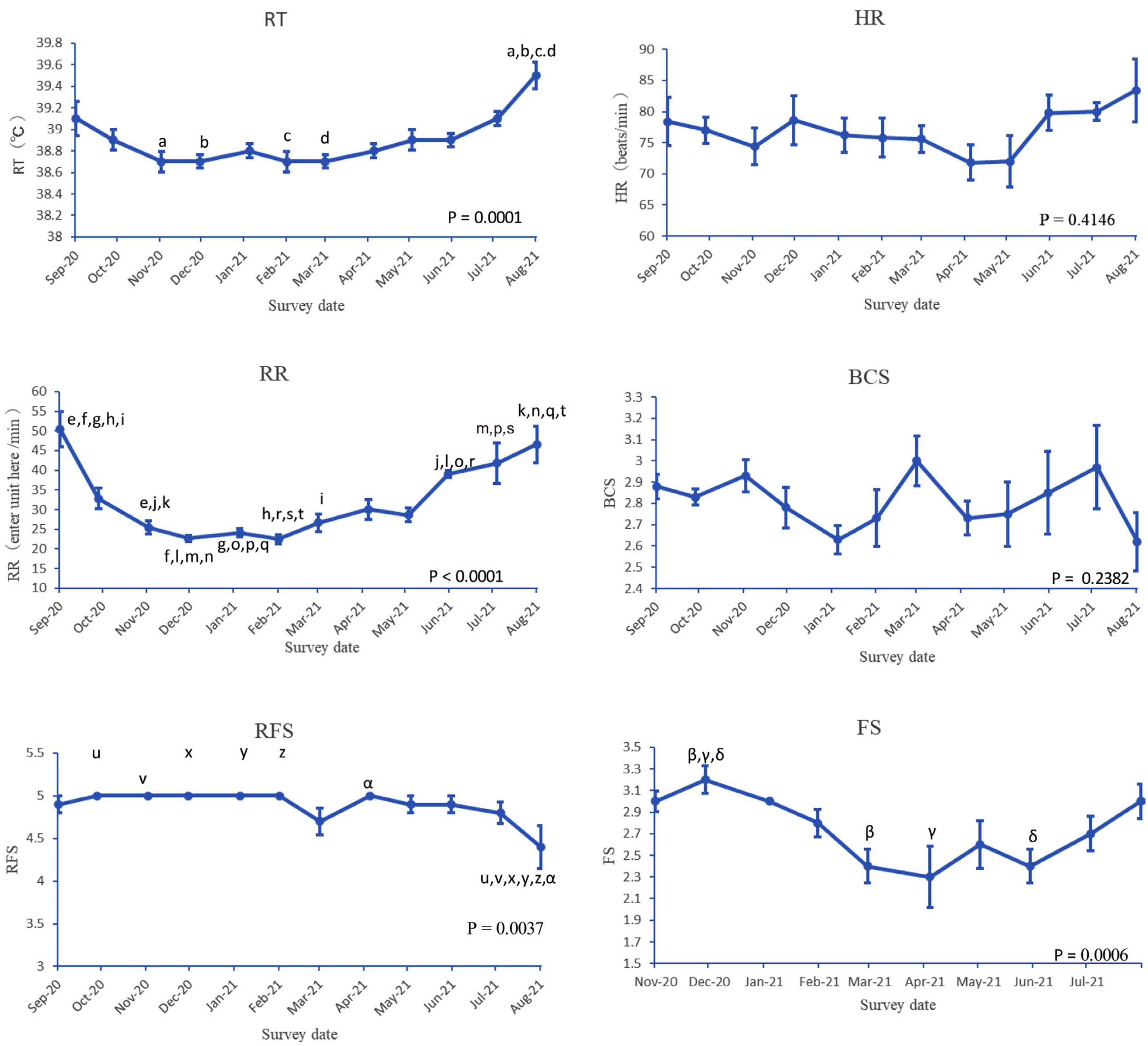

Fig. 2. The eucrasia of cows at time of observation. RT: rectal temperature, HR: heart rate, RR: respiratory rate, BCS: body condition score, RFS: rumen fill score, FS: fecal score. The same 10 cows were used for each month's examination. The same 10 cows were used for each month's examination. Each value represents the mean ± standard error of the mean (SEM) of 10 cows. Values with the same letters are significantly different (a, b, d–h, l–n, p–r, t, u, v, x–z, α, β, γ and δ: *P*<0.01. c, i, j, k, o and s: *P*<0.05).

the monthly RT and RR of the cows, which could be attributed to increase in RT and RR of the cows with increase ambient temperature of the pen. The monthly RFS of the cows were negatively correlated with the mean humidity of the pen 24 hr prior to sampling, with August being the most humid month. This suggests that high temperature and humidity may contribute to reduced appetite in cows.

A previous study found that the RBC of humans was significantly lower in summer and fall than in winter and spring, and MCHC was lower in summer [20]. Chaudhary *et al*. reported that high THI significantly reduced the MCHC of Indian buffaloes [5]. A significant decrease in blood oxygen saturation was observed in Holstein dairy cows in Oman at a THI of 93 compared to a THI of 72 [31]. In this study, the RBC, HGB value and MCHC of the cows were negatively correlated with the temperature, humidity, WBGT, and THI of the pen 24 hr prior to sampling. Based on these results, it can be inferred that high ambient temperature and humidity decreased the oxygen carrying capacity of cattle, as evidenced by the decrease in the RBC, HGB, and MCHC of the cows with increasing pen temperature, humidity, WBGT, and THI.

In humans, it has been reported that passive whole-body heating significantly increases the PLT count in circulating blood [24]. In this study, PLT was positively correlated with the temperature, humidity, WBGT, and THI of the pen 24 hr before sampling. This

Table 2. Relationship between temperature, humidity, Wet Bulb Globe Temperature (WBGT), temperature-humidity index (THI) data for 24-hr at the time of observations and the eucrasia of cattle

Parameter	Eucrasia of cows					
	RT	HR	RR	BCS	RFS	FS
Average daily temperature for 24 hr	0.7741	ns	0.8471	ns	ns	ns
Maximum temperature for 24 hr	0.7173	ns	0.8516	ns	ns	ns
Minimum temperature for 24 hr	0.8020	ns	0.8306	ns	ns	ns
24-hr temperature range	−0.6797	ns	ns	ns	ns	ns
Average daily humidity for 24 hr	0.5860	ns	ns	ns	−0.5791	ns
Maximum humidity for 24 hr	ns	ns	ns	ns	ns	−0.5820
Minimum humidity for 24 hr	0.6302	ns	ns	ns	−0.6665	ns
24-hr humidity range	ns	ns	ns	ns	0.6933	ns
Average daily WBGT for 24 hr	0.7835	ns	0.8165	ns	ns	ns
Maximum WBGT for 24 hr	0.7623	ns	0.8042	ns	ns	ns
Minimum WBGT for 24 hr	0.7949	ns	0.8041	ns	ns	
24-hr WBGT range	ns	ns	ns	ns	ns	ns
Average daily THI for 24 hr	0.8006	ns	0.8469	ns	ns	ns
Maximum daily THI for 24 hr	0.7672	ns	0.8533	ns	ns	ns
Minimum daily THI for 24 hr	0.8142	ns	0.8311	ns	ns	ns
24-hr THI range	−0.7566	ns	−0.6442	ns	ns	ns

RT: rectal temperature, HR: heart rate, RR: respiration rate, BCS: body condition score, RFS: rumen fill score, FS: feces score. The numbers indicate Pearson's product moment correlation coefficient (r value). The correlation between the temperature and humidity 24 hr before the monthly sampling in one year and the physical condition of 10 cows was shown. ns: No significant correlation. Underline is P<0.01. No line is P<0.05.

Table 3. Days in milk, milk yield and somatic cell count in cows on sampling days

Parameter	Survey date												P-value*
	Sep-20	Oct-20	Nov-20	Dec-20	Jan-21	Feb-21	Mar-21	Apr-21	May-21	Jun-21	Jul-21	Aug-21	
Days in milk (days)	228.3 ± 30.9	255.3 ± 30.9	290.3 ± 30.9	318.3 ± 30.9	284.1 ± 53.9	275.2 ± 61.5	159.1 ± 52.0	159 ± 49.4	187 ± 49.4	215.0 ± 49.4	249.0 ± 49.4	171.7 ± 33.4	0.0937
Milk yield[1] (kg)	27.7 ± 1.6	30.08 ± 2.3	27.31 ± 2.4	25.2 ± 3.3[3]	24.9 ± 4.3[3]	27.6 ± 4.3[4]	31.5 ± 3.2	34.5 ± 3.6	36.4 ± 2.8[3]	34.6 ± 3.7	32.8 ± 2.5[3]	31.5 ± 1.8[3]	0.0850
Somatic cell counts[2] (×10^3 cell/ml)	137.4 ± 30.9	196.4 ± 71.8	210.4 ± 76.4	267.5 ± 223.2[3]	173.1 ± 61.2[3]	140.9 ± 44.3[4]	207.9 ± 70.6	326.9 ± 228.8	302.3 ± 241.9[3]	679.3 ± 440.3	371.5 ± 124.1[3]	476.6 ± 339.5[3]	0.7774

* Significant difference was shown on each sampling days. Each value represents the mean ± SEM of 10 cows. [1] Milk yield was expressed as the sum of the morning of the day the sample was taken and the evening of the previous day. [2] Somatic cell counts were presented as an average of the morning of the day the sample was taken and the evening of the previous day. [3] The number of samples of was 9 because there were one dry cows. [4] The number of samples was 7 because they were 3 dry cows.

finding indicates that high temperature and humidity induced increase in circulating PLT in the peripheral blood of the dairy cows during the hot season.

Seasonal variations in blood AST levels have been reported in humans, with higher values in the winter due to cold stress and hormonal changes [22]. The serum AST levels of goats in Ethiopia have been reported to be higher during the dry season after a prolonged rainy season [35]. Similarly, the findings of this study showed that highest serum AST concentrations were observed in the hot and humid month of August. Moreover, the monthly serum AST and γ-GPT concentrations of the cows were positively correlated with the humidity of the pen 24 hr before sampling, and negatively correlated with the 24-hr temperature and WBGT range of the pen prior to sampling. Generally, feed quality is negatively affected under high humidity and temperature storage conditions. Based on these findings, it could be speculated that the high serum AST of the cows during hot and humid months could be attributed to the poor quality of administered feed during this period.

Grünwaldt *et al*. reported that the serum globulin and albumin concentrations of Argentine beef cattle varied considerably in February and May with seasonal changes in environmental temperature [11]. Timaran *et al*. reported that serum albumin concentration and albumin/globulin varied with calving season in Colombian dairy cows [35]. Moreover, prolonged exercise has been shown to decrease the plasma protein levels of horses, including albumin levels [29]. In the present study, the monthly serum TP and Alb concentrations of the cows were significantly lower in November, and TP was negatively correlated with the 24-hr temperature, WBGT, and THI range of the pen prior to sampling, while Alb was only correlated with the 24-hr temperature and WBGT range of the pen prior to sampling. Additionally, the monthly serum A/G ratios of the cows were negatively correlated with the temperature, WBGT, and THI (on mean, maximum, and minimum, respectively) of the pen 24 hr prior to sampling. These findings suggest that the cows were more active during the periods of high temperature variation, resulting in a decrease in serum

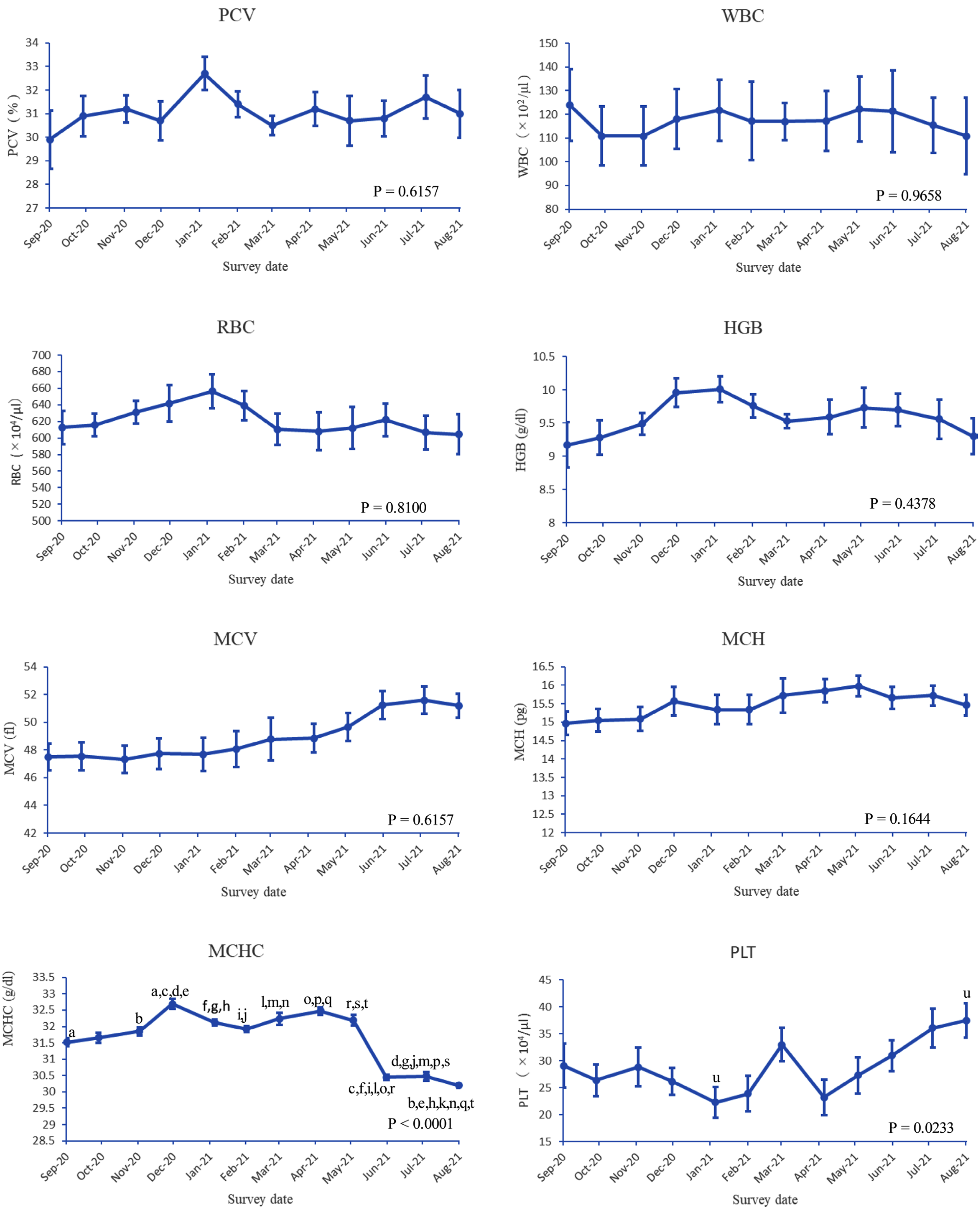

Fig. 3. Hematological values in cows on sampling days. The same 10 cows were used for each month's examination. PCV: packed cell volume, WBC: white blood cell counts, RBC: red blood cell counts, HGB: hemoglobin. MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, PLT: platelet counts. *P*-value: significant difference was shown on each sampling days. Each value represents the mean ± standard error of the mean of 10 cows. Values with the same letters are significantly different (c–h, k–s and t are siginificant as $P<0.01$ and a, b, i, j, u are at $P<0.05$).

Table 4. Relationship between temperature, humidity, Wet Bulb Globe Temperature (WBGT), temperature-humidity index (THI) data in the barn for 24-hr at the time of sampling and blood biochemical examination values of cattle

Parameter	Hematological values of 10 cows							
	PCV	WBC	RBC	HGB	MCV	MCH	MCHC	PLT
Average daily temperature for 24 hr	ns	ns	−<u>0.7548</u>	−0.7338	0.5783	ns	−<u>0.7279</u>	0.6161
Maximum temperature for 24 hr	ns	ns	−<u>0.7332</u>	−<u>0.7610</u>	ns	ns	−0.6494	0.5685
Minimum temperature for 24 hr	ns	ns	−<u>0.7155</u>	−0.6207	0.6440	ns	−<u>0.7706</u>	0.6217
24-hr temperature range	ns	ns	ns	ns	−<u>0.8203</u>	ns	<u>0.7302</u>	−0.5028
Average daily humidity for 24 hr	ns	ns	−0.7030	ns	<u>0.7668</u>	ns	−0.6839	0.6689
Maximum humidity for 24 hr	ns	ns	−<u>0.7689</u>	ns	0.6782	ns	ns	ns
Minimum humidity for 24 hr	ns	ns	−0.6735	ns	<u>0.7324</u>	ns	−0.6792	0.7032
24-hr humidity range	ns	ns	ns	ns	ns	ns	0.5809	−<u>0.7122</u>
Average daily WBGT for 24 hr	ns	ns	−0.6891	−0.6569	0.5985	ns	−<u>0.7810</u>	0.6564
Maximum WBGT for 24 hr	ns	ns	−0.7007	-0.6733	ns	ns	−<u>0.7487</u>	0.6205
Minimum WBGT for 24 hr	ns	ns	−0.6584	−0.6144	0.6572	ns	−<u>0.7952</u>	0.6554
24-hr WBGT range	ns	ns	ns	ns	-0.6105	ns	ns	ns
Average daily THI for 24 hr	ns	ns	−<u>0.7442</u>	−0.6625	0.6184	ns	−<u>0.7540</u>	0.6303
Maximum daily THI for 24 hr	ns	ns	−<u>0.7606</u>	−<u>0.7297</u>	ns	ns	-0.6942	0.6141
Minimum daily THI for 24 hr	ns	ns	−0.7048	−0.5979	0.6619	ns	−<u>0.7790</u>	0.6217
24-hr THI range	ns	ns	ns	ns	−<u>0.7982</u>	ns	<u>0.7966</u>	ns

Twenty four-hr meteorological data at the time of sampling and hematological values of cattle. PCV: packed cell volume, WBC: white blood cell counts, RBC: red blood cell counts, HGB: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, PLT: platelet counts. The numbers indicate Pearson's product moment correlation coefficient (r value). The correlation between the temperature and humidity 24 hr before monthly sampling in one year and the hematological test values of 10 cows was showed. ns: No significant correlation. Underline is $P<0.01$. No line is $P<0.05$.

Alb concentration.

Serum glucose concentrations in dairy cows have been reported to decrease under high temperature and humidity conditions, and is negatively correlated with THI [13]. In the present study, the lowest monthly serum Glu concentrations was observed in November, and was negatively correlated with the 24-hr WBGT range of the pen prior to sampling. This result was attributed to short resting time of cows due to high variations in the WBGT range of the pen.

A previous study indicated that serum cholesterol is involved in steroid hormone and lipid metabolism and that milk output is not affected by high temperature [28]. In the present study, the highest serum T-ch concentrations was observed in May. The significantly high serum T-ch concentrations in May can be attributed to the conducive temperature range of this period, which supported optimal energy metabolism in the body; moreover, milk yield was highest in May.

Staric *et al*. reported that there were no significant differences in the serum Ca, iP and Mg concentrations of Slovenian dairy cows fed TMR and grass-fed herds during summer and winter [32]. On the contrary, Abdelrahman *et al*. reported significant seasonal changes in serum Ca, iP and Mg concentrations in grazing dairy cattle in Sudan during the dry and rainy seasons, with lower serum concentrations observed in the late dry season [1]. The results of the study showed that the lowest serum Ca, iP, and Mg concentrations of the cows were observed in November, and were negatively correlated with the 24-hr temperature, WBGT, and THI range of the pen prior to samplings. However, serum Ca, iP, and Mg concentrations were positively correlated with mean humidity. The difference in results could be attributed to differences in study location. The present study was performed in a temperate monsoon zone, which could have influenced the serum Ca, iP, and Mg concentrations of the cows.

In a previous study, we observed a positive correlation between serum ionized Ca and Alb concentrations at the time of initial diagnosis in cows with acute mastitis [12]. The findings of the present study showed that there were positive correlations between mean serum Alb, Ca, iP, and Mg concentrations. In humans, pregnant women at risk of preterm labor have significantly lower blood Ca, iP, Mg, and Alb concentrations compared with normal pregnant women [39]. Spaans *et al*. reported that postpartum blood Ca, iP and Mg concentrations were low in grass-fed dry cows [30]. The monthly serum Alb, Ca, iP and Mg concentrations of the cows were within normal range, and there was no significant monthly differences in DIM. Moreover, there were no effects of milking stage or feed ingredients in serum Alb, Ca, iP and Mg concentrations since the cows were fed the same purchased feed. The positive correlation between seasonal changes in mean serum Alb, Ca, iP and Mg concentrations in this study suggests that the cationic Ca and Mg were bound to the anionic Alb and iP in the blood and maintained equilibrium with each other.

Serum NEFA in dairy cows shows diurnal variation, with higher levels during fasting and significantly lower levels after TMR feeding [23]. Turk *et al*. investigated seasonal changes in lipid metabolism and reported that NEFA increased significantly after parturition in summer compared to winter, indicating negative energy balance [36]. In the present study, significantly lower serum NEFA concentrations were observed in November and December, whereas significantly higher values were observed in February, July, and August. In this farm, there were no changes in the type of feed or feeding pattern throughout the year. Overall, these findings suggests that changes in serum NEFA concentrations were most likely influenced by the quality of purchased hay and

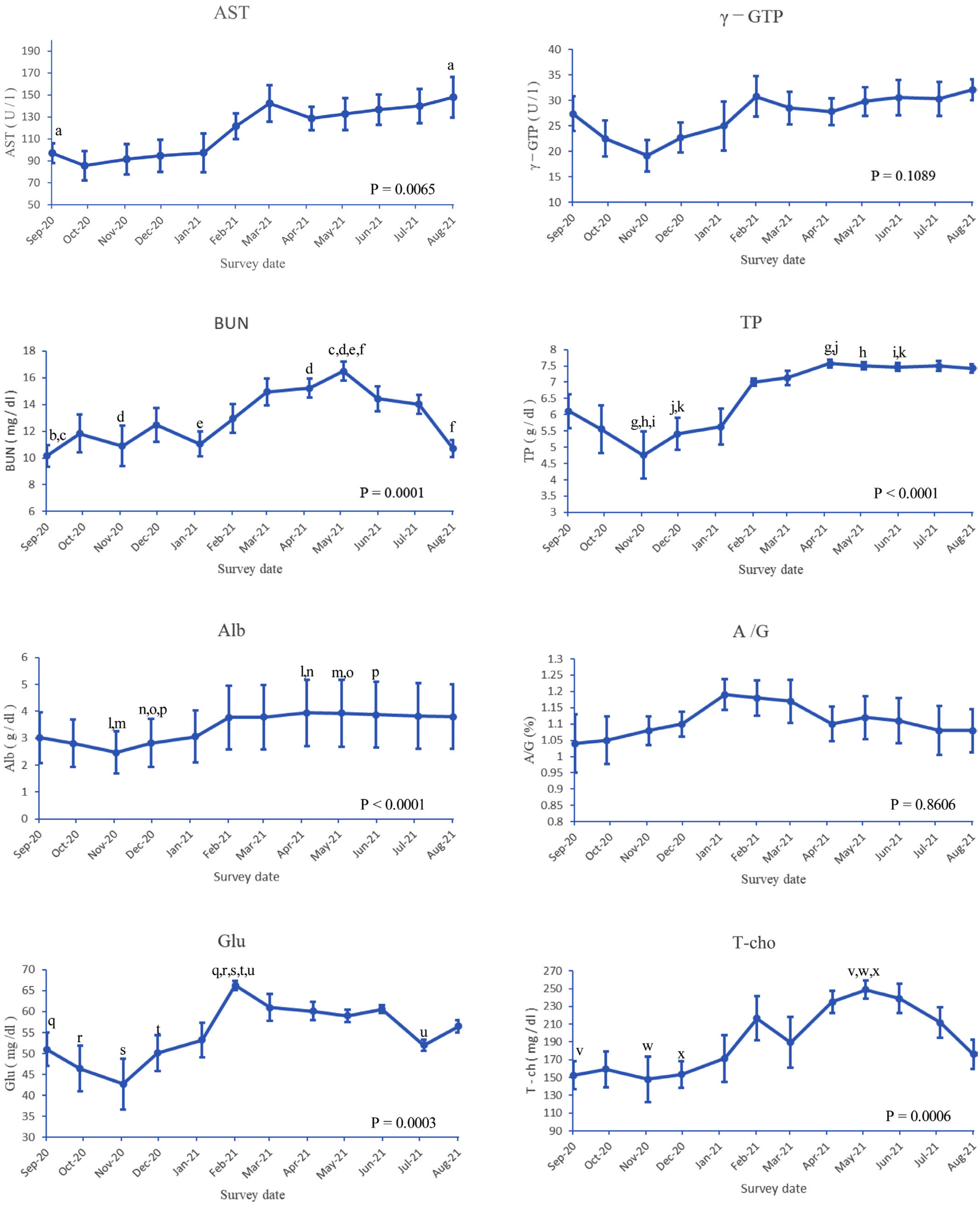

Fig. 4. Blood biochemical examination values (1) in cows on sampling days. AST: aspartic acid transferase, γ-GTP: γ-glutamyl transpeptidase, BUN: blood urea nitrogen, TP: total protein, Alb: albumin. A/G: albumin / globulin, Glu: glucose, T-cho: total cholesterol. The same 10 cows were used for each month's examination. *P*-value: significant difference was shown on each sampling days. Each value represents the mean ± standard error of the mean (SEM) of 10 cows. Values with the same letters are significantly different (a, c, f, s and u are siginificant as $P<0.01$ and b, d, e, g–j, k–r, t, v, w and x are at $P<0.05$).

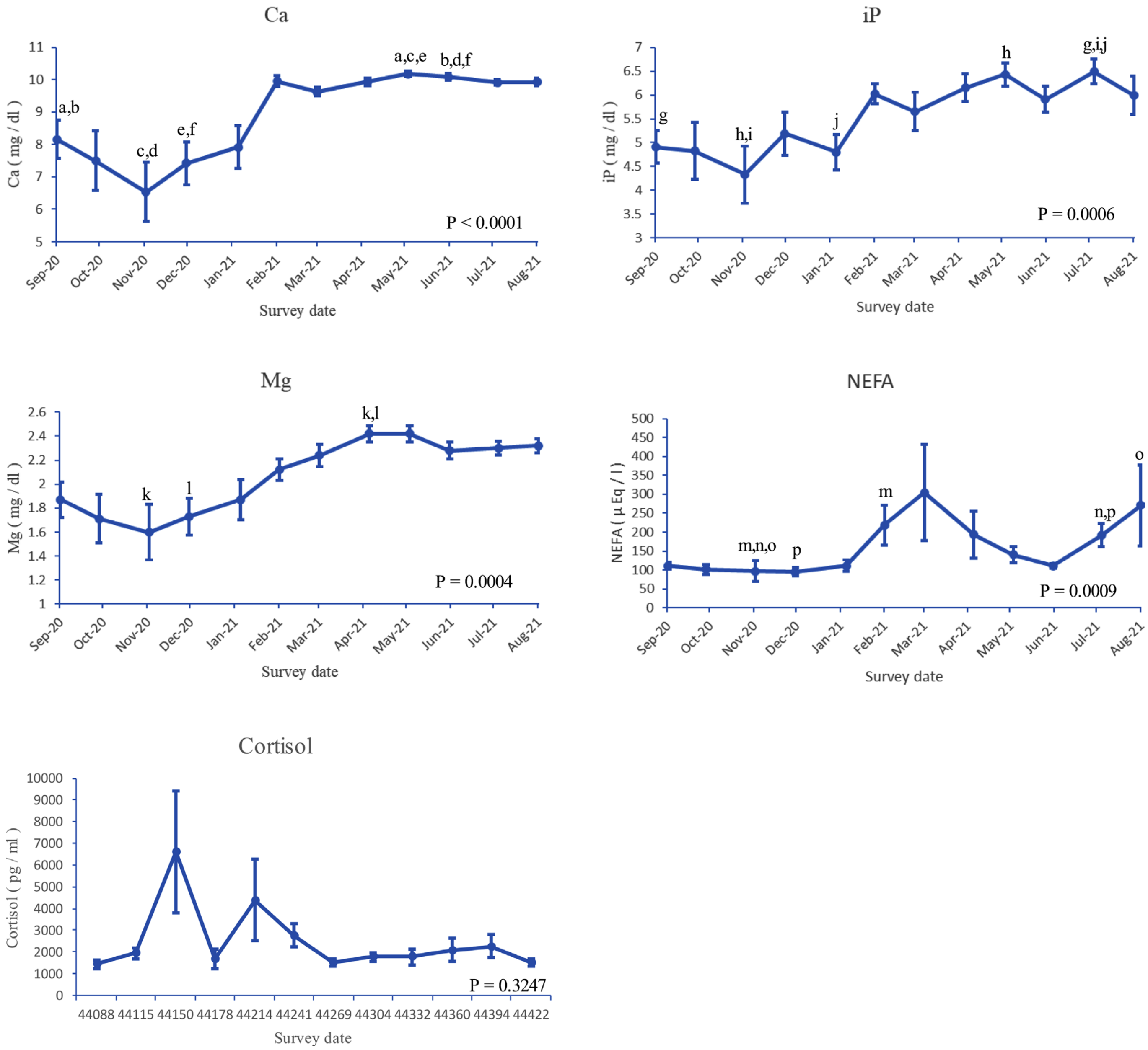

Fig. 5. Blood biochemical examination values (2) and blood cortisol level in cows on sampling days. Ca: calcium, iP: inorganic phosphorus, Mg: magnesium, NEFA: nonesterified fatty acid. The same 10 cows were used for each month's examination. *P*-value: significant difference was shown on each sampling days. Each value represents the mean ± standard error of the mean (SEM) of 10 cows. Values with the same letters are significantly different (c, e and n are siginificant as $P<0.01$ and a, b, d, f–m, o are p at $P<0.05$).

other products.

Kasimannickam *et al*. reported that when cattle were subjected to heat stress, serum cortisol levels increased significantly compared to normal cattle [16]. In contrast, the serum cortisol concentrations of the cows were not significantly affected by seasonal variations in pen temperature, and were significantly positively correlated with the 24-hr WBGT range of the pen prior to sampling. Olsson *et al*. reported an increase in the serum cortisol and hematocrit levels of breeding season goats, with increasing water intake under dehydration conditions [25]. Similarly, Stull *et al*. reported an increase in both serum cortisol levels and hematocrit levels in horses during 24-hr transport in summer [34]. Goncharova reported that the A/G ratio of serum total protein changed with serum cortisol concentration in calves from birth to 3 months, with the inhibition of globulin absorption under high blood cortisol concentration and increase in globulin concentration under low blood cortisol concentration [10]. Moreover, Yang *et al*. reported thar changes in temperature altered intracellular protein transport and impaired intracellular proteolytic enzyme activity in a human colon cell line, Caco-2 cells [42]. Furthermore, multiple regression analysis showed a positive correlation between the 24-hr WBGT range of the pen prior to sampling and the monthly PCV levels of the cows, and a negative correlation between mean

Table 5. Relationship between temperature, humidity, Wet Bulb Globe Temperature (WBGT), temperature-humidity index (THI) data in the barn for 24-hr at the time of sampling and blood biochemical examination values of cattle

Parameter	Blood biochemical examination values of 10 cows												
	AST	γ-GTP	BUN	TP	Alb	A/G	Glu	T-cho	Ca	iP	Mg	NEFA	Cortisol
Average daily temperature for 24 hr	ns	ns	ns	ns	ns	−0.7657	ns	ns	ns	ns	ns	ns	ns
Maximum temperature for 24 hr	ns	ns	ns	ns	ns	−0.8434	ns	ns	ns	ns	ns	ns	ns
Minimum temperature for 24 hr	ns	ns	ns	ns	ns	−0.6975	ns	ns	ns	ns	ns	ns	ns
24-hr temperature range	−0.6186	−0.7219	ns	−0.6604	−0.6129	ns	ns	ns	−0.6605	−0.7175	−0.6138	ns	ns
Average daily humidity for 24 hr	0.6456	0.6137	ns	0.6369	ns	ns	ns	ns	0.6093	0.6499	0.5802	ns	ns
Maximum humidity for 24 hr	0.6241	0.5968	ns	0.7278	0.6538	ns	ns	0.6592	0.6791	0.6679	0.6867	ns	ns
Minimum humidity for 24 hr	0.6270	0.5955	ns	0.5856	ns	ns	ns	ns	ns	0.6163	ns	ns	ns
24-hr humidity range	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Average daily WBGT for 24 hr	ns	ns	ns	ns	ns	−0.7140	ns	ns	ns	ns	ns	ns	ns
Maximum WBGT for 24 hr	ns	ns	ns	ns	ns	−0.7388	ns	ns	ns	ns	ns	ns	ns
Minimum WBGT for 24 hr	ns	ns	ns	ns	ns	−0.6581	ns	ns	ns	ns	ns	ns	ns
24-hr WBGT range	−0.6840	−0.8097	ns	−0.7801	−0.7905	ns	−0.7620	−0.6174	−0.8122	−0.8288	−0.6983	ns	0.7221
Average daily THI for 24 hr	ns	ns	ns	ns	ns	−0.7410	ns	ns	ns	ns	ns	ns	ns
Maximum daily THI for 24 hr	ns	ns	ns	ns	ns	−0.8119	ns	ns	ns	ns	ns	ns	ns
Minimum daily THI for 24 hr	ns	ns	ns	ns	ns	−0.6853	ns	ns	ns	ns	ns	ns	ns
24-hr THI range	ns	−0.6835	ns	−0.6091	ns	ns	ns	ns	−0.5929	−0.6314	ns	ns	ns

AST: aspartic acid transferase, γ-GTP: γ-glutamyl transpeptidase, BUN: blood urea nitrogen, TP: total protein, Alb: albumin, A/G: albumin / globulin, Glu: glucose, T-ch: total cholesterol. Ca: calcium, iP: inorganic phosphorus, Mg: magnesium, NEFA: nonesterified fatty acid. The numbers indicate Pearson's product moment correlation coefficient (r value). The correlation between the temperature and humidity 24 hr before monthly sampling in one year and the mean blood chemistry test values and serum cortisol levels of 10 cows was showed. ns: No significant correlation. Underline is $P<0.01$. No line is $P<0.05$.

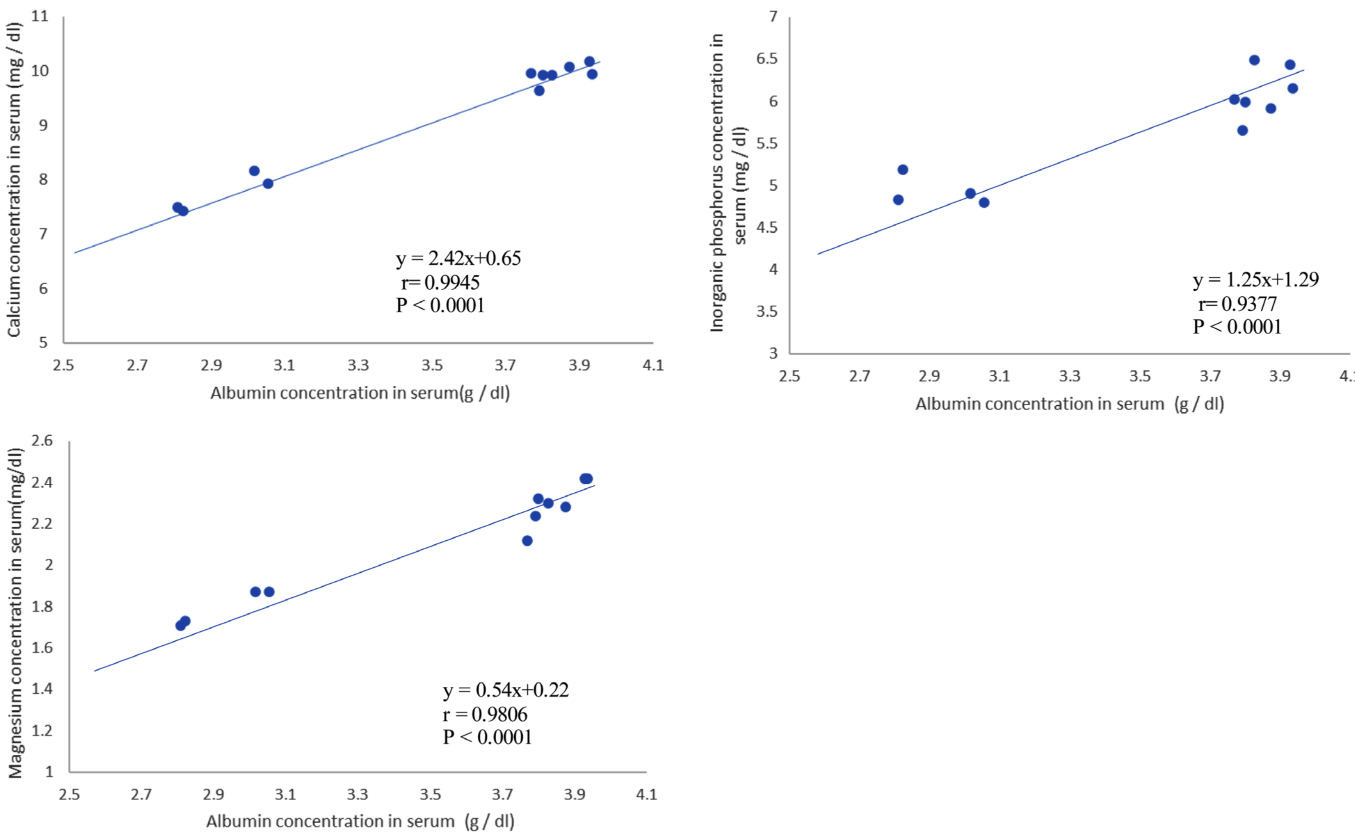

Fig. 6. Correlations between mean serum albumin concentration and mean serum calcium concentration, mean serum inorganic phosphorus concentration and mean serum magnesium concentration at each 12-month period.

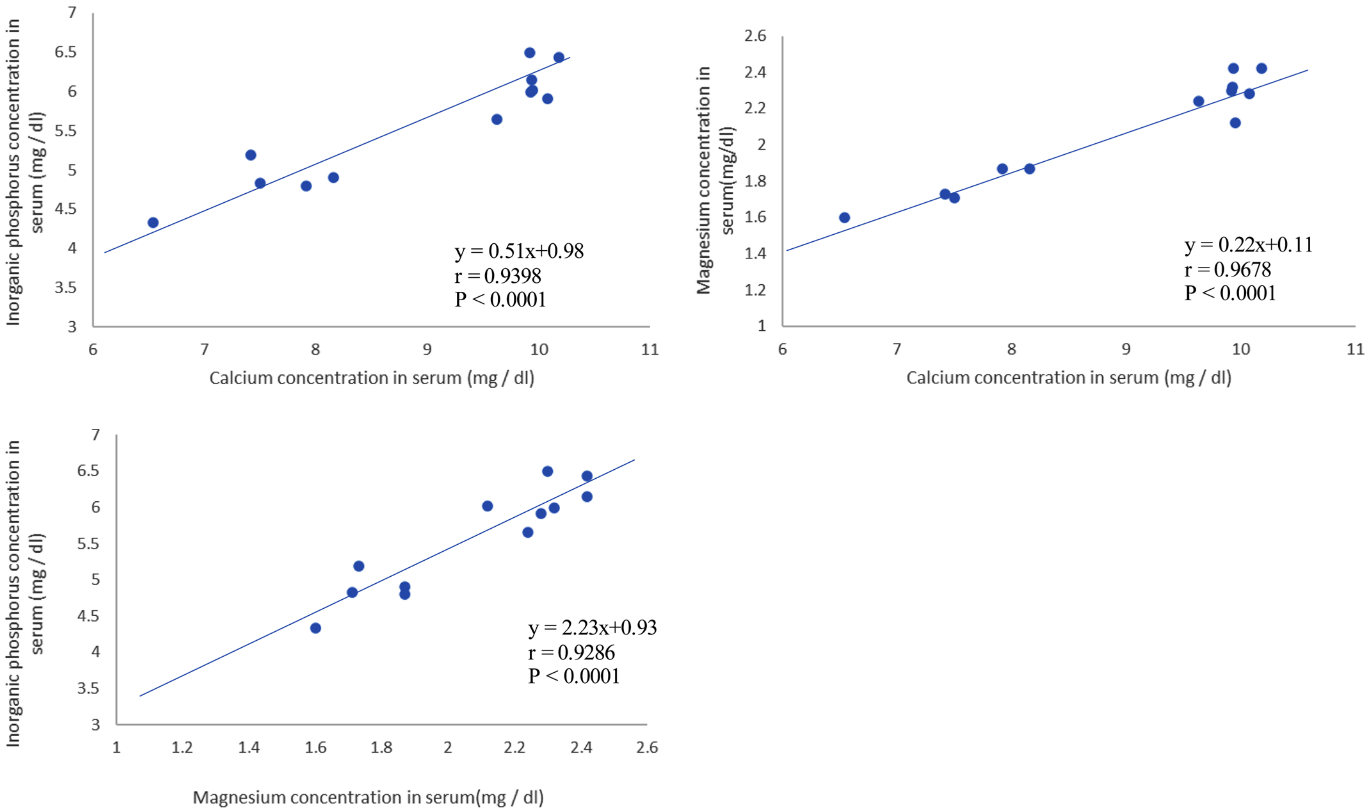

Fig. 7. Correlation between mean serum calcium and mean serum inorganic phosphorus concentrations and mean serum magnesium concentrations at each 12-month period.

Table 6. Multiple regression analysis of cortisol concentration in serum and daily difference in Wet Bulb Globe Temperature (WBGT), total protien in serum and packed cell volume

Summary of multiple regression analysis					
Model	R	R^2	Flexibility decision finished multiple correlation coefficient	Adjusted R-square	
1	0.8697	0.7563	0.8154	0.6649	
Analysis of variance[a)]					
Model	Sum of squares	Degree of freedom	Unbiased variance	Variance ratio	Level of significance
Total variation	25,918,194.79	11			
Variation by regression	19,602,434.99	3	VR=6,534,144.99	F=8.277**	*P*=0.0078
Residual variation	6,315,759.804	8	VE=785,469.976		
Result of analysis					
Explanatory variable	Coefficient[a)]				
	B[b)]	SE	β[c)]	r	
(Constant)	−32,320.2261	443.1152			
Daily difference in Wet Bulb Globe Temperature	560.5207	0.5429	0.6868	0.7221	
Total protein	−32.4674	0.2826	−0.0216	−0.6092	
Packed cell volume	1,066.5539	0.2007	0.4831	0.5116	

Dependent variable is cortisol concentration in serum. Explanatory variable is daily difference in WBGT, total protien in serum, packed cell volume. [a)] Dependent variable cortisol concentration in serum, [b)] partial regression coefficient, [c)] estimated regression coefficient. ***P*<0.01.

serum TP and cortisol concentrations. Based on these results, it can be inferred that the increase in serum cortisol concentrations could be attributed to an increase in PCV levels, which was caused by an in increase in the 24-hr WBGT range of the pen prior to sampling. Additionally, the increase in serum cortisol concentration was associated with a decrease in serum TP concentration, suggesting that it may have affected the digestion and absorption of protein from the gastrointestinal tract.

The findings of the study showed that large variations in temperature induced stress in the cows, which could be overcome by increased water consumption and improved protein digestion and absorption by the animals, and the addition of minerals, such as calcium to the diet. In the future, extreme weather events may cause severe changes in temperature and humidity in cowsheds; therefore, it is necessary to continually monitor cowshed environment and ensure efficient nutritional management and optimal housing conditions for improved dairy cow performance.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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