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Full Length Article

Effects of Environmental Changes during Different Seasons on Hair Cortisol Concentration as a Biomarker of Chronic Stress in Korean Native Cattle

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Abstract

The effects of environmental changes during different seasons on hair cortisol concentration were evaluated as a stress biomarker in Hanwoo cattle. Nine cattle, including dams, heifers and calves (body weight (kg) = 545 ± 93.9, 380.7 ± 68.5 and 53.7 ± 1.4 and ave. age (months) = 55.9 ± 13.6, 17.2 ± 0.3 and 1.8 ± 0.6, respectively), were examined in this experiment. Hair samples were harvested on Aug 25, Oct11, Nov11 and Dec 23 of 2017 and on Feb 12 of 2018. Repeated-measures analyses were performed using the mixed procedure of SAS software. Lower hair cortisol concentration was observed in Oct compared with Feb and in Nov compared with Feb ($P < 0.05$). The hair cortisol concentration in Aug did not differ from those for the other dates. No differences were observed in the hair cortisol concentration of dams or heifers at different dates. In calves, higher hair cortisol concentration was observed in Aug *vs*. Oct and in Aug *vs*. Nov and lower hair cortisol concentration was observed in Oct *vs*. Feb and in Nov *vs*. Feb ($P < 0.05$). Relatively higher hair cortisol concentration was found in calves than dams in Aug and Feb and then heifers in Aug ($P < 0.05$). The effect of time (sampling date) and cattle type and the interactions between dam and calf, heifer and calf, and cattle and time were highly significant ($P < 0.01$). In conclusion, Hanwoo calves were relatively more susceptible to intense environmental conditions than were heifers and their dams.

Keywords: Chronic stress; Environmental conditions; Hair cortisol; Hanwoo cattle

Introduction

Korea's annual climate includes hot and humid summers, long cold winters, and short, temperate autumns and springs (Kang *et al*., 2016). Livestock are often subjected to temperature and humidity stressors that can impair their health and performance (Piao and Baik, 2015; Rojas-Downing *et al*., 2017). Animals have different thermal comfort tolerances (thermal comfort zone, or TCZ) for normal physiological functions (Rojas-Downing *et al*., 2017) and cattle are often exposed to heat stress (HS) and cold stress (CS). With the exception of neonatal calves (McDowell, 1972), the TCZ for Holstein cattle is 13–18°C, although this may change based on the type of cattle and their present physiological status. Hence, HS or CS may result when the environmental temperature surpasses or falls below the TCZ (Rojas-Downing *et al*., 2017).

Recently, studies have investigated the impact of global climate change and HS on the hair cortisol concentration of lactating cattle (Nejad *et al*., 2017a; Uetake *et al*., 2018) and sheep (Nejad *et al*., 2014, 2017b). Blood and saliva (Negrão *et al*., 2004), urine and feces (Salaberger *et al*., 2016) and milk (Gellrich *et al*., 2015) have all been used as biological matrices to measure cortisol, with each measure having its own limitations regarding sample collection and reliability as a metric for acute stress. Plasma cortisol is passively and consistently deposited into growing hair and can, therefore, be used to evaluate chronic stress that is a response to prolonged stress in cattle (Meyer and Novak, 2012; Comin *et al*., 2014; Yamanashi *et al*., 2016). Hair cortisol is a reliable, non-invasive measurement that is easy to collect and store (Uetake *et al*., 2018). Whereas numerous studies have demonstrated the influence of seasonal climate on cattle health (Thornton *et al*., 2009; Uetake *et al*., 2018), the use of hair as a retrospective biomarker of chronic stress in Korean native cattle has yet to be validated. We investigated the effects of environmental changes during different seasons on hair cortisol concentration as a retrospective biomarker of

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chronic stress in Hanwoo cattle. We hypothesized that seasonal changes act as a chronic stressor for Korean cattle, resulting in seasonal differences in cortisol accumulation in hair.

Materials and Methods

Experimental Site, Animals and Sampling Dates

The experimental protocol was approved by The Animal Welfare and Ethics Authority of Kangwon National University in Chuncheon, Republic of Korea. The cattle were obtained from the university farm between the summer of 2017 and the winter of 2018; they were housed in one barn and fed twice a day at 08:00 and 18:00. Fresh water was freely available. Calves were fed with their dam's milk at approximately 10% of their initial body weight until 4 month of age, when the experimental period ended. Nine healthy Korean (Hanwoo) cattle, including dams (n = 3), heifers (n = 3) and calves (n = 3), with initial average body weight (kg) of 545 ± 93.9, 380.7 ± 68.5 and 53.7 ± 1.4 and an average age (month) of 55.9 ± 13.6, 17.2 ± 0.3, 1.8 ± 0.6, respectively, were examined prior to the beginning of the experiment, on Aug 25. Hair samples were collected from the forehead of each individual on Aug 25, Oct 11, Nov 11, and Dec 23 of 2017 and on Feb 12 of 2018.

Hair Collection and Cortisol Analysis

Hair samples were collected from the forehead of each individual. The forehead was chosen as the collection site because of its accessibility and cleanliness (no feces contamination), which mean it is likely to represent the whole body of the animals. Hair samples from tails are discouraged, as fecal contamination can profoundly affect hair cortisol concentration (Meyer and Novak, 2012; Nejad *et al*., 2014; Yamanashi *et al*., 2016). Thus, use of the sampling location that is the cleanest and thus the least exposed to fecal contamination has been recommended.

To prepare hair samples for cortisol extraction (Nejad *et al*., 2014, 2017a), they (> 1 g) were carefully shaved from a 10-20 cm section of the forehead as close to the skin as possible using an electric hair shaver (Hair clippers, Model 7200, Rikei trading co., Ltd, Seoul, Korea). The hair samples were immediately folded into sheets of aluminum foil, numbered, placed into dry polypropylene tubes (50 mL conical tube, HM Hyundai Micro Co., Korea), stored in a plastic bag, transported to the laboratory and stored at room temperature. Samples were then washed. After drying, cortisol was extracted using methanol and analyzed using a hair cortisol Enzyme Immunoassay (EIA) kit (Salimetrics, State College, PA 16803. U.S.A.) according to the manufacturer's instructions (Fig. 1). The dried hair samples were thawed, 400 μL of phosphate buffer was added, vortexed and centrifuged at 1500 x *g* for 15 min. Each sample (25 μL) was run in duplicate to improve assay accuracy and reliability. Samples outside the standard curve were considered outliers and omitted. The optical density of the plate was read using a microplate reader (Synergy TM H1 BioTek, USA) set at 450 nm to μg dL^{-1}. A 4-parameter non-linear regression curve fit was then using MyAssay Analysis Online Software Solutions.

Concentration of the samples was obtained from the software converted into pg mg^{-1} using the following equation:

$$F = 10{,}000E\,(A/B)\,(C/D)$$

Where F = the final value of the hair cortisol concentration in (pg mg^{-1}), E = the vol. (mL) of the assay buffer used to reconstitute the dried extract, A = (μg dL^{-1}) from the assay output, B = the weight (mg) of the hair subjected to extraction, C = the vol. (mL) of methanol added to the powdered hair and D = the vol. (mL) of methanol recovered from the extract and subsequently dried down (Meyer *et al*., 2014).

Meteorological Measurements

The mean temperature (ºC) and relative humidity (% RH) of the area where cattle were housed were obtained from the Korean Meteorological Administration. Average Korean weather conditions between 1981 and 2010 are provided in (Table 1); the agency samples these data once per minute. Data for the time blocks used in this study were averaged from agency reports. The daylight hours during each season and the sampling times are presented in (Table 2).

The following equation was applied to calculate THI:

$$THI = (1.8 \times \text{Temperature} + 32) - (0.55 - 0.0055 \times \text{Humidity}) \times (1.8 \times \text{Temperature} - 26).$$

The THI values obtained were as follows: < 71, absence of heat stress; 72–79, mild heat stress; 80–89, moderate heat stress and > 90, severe heat stress (Armstrong, 1994).

Statistics

Repeated-measures analyses were performed to compare the hair cortisol concentration among the cattle and the environmental conditions using the MIXED procedure of SAS software (Version 9.3, SAS Institute Inc., N.C., U.S.A.). The difference between means was tested using Tukey's test and then calculated with adjusted p-values. The mean hair cortisol concentration for each month was compared with the mean hair cortisol concentration of the other months. Variance and covariance assumption structures (AR (1), UN, CS, etc.) were tested; then, AR (1), as the best covariance structure for final analysis, was selected. Comparisons were also made within time (sampling dates) among the cattle type where multiple comparison procedures were used to analyze these data. The

Table 1: Average temperature, rainfall and daylight hours at the experimental location from 1981 to 2010 (Data from Korea Meteorological Administration; http://www.weather.go.kr)

Element	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Minimum temperature (ºC)	-9.9	-6.9	-1.3	4.4	10.8	16.5	20.8	20.8	14.8	7.1	0.1	-6.4
Maximum temperature (ºC)	1.3	5.1	11.1	19.1	23.9	27.6	29.1	29.8	25.5	19.6	11.0	3.8
Rainfall (mm)	20.3	23.8	41.7	62.3	104.0	123.1	383.8	317.5	160.9	44.3	44.7	20.9
Monthly daylight hours (hr)	165.7	172.4	197.6	215.5	221.3	200.6	144.2	169.1	173.8	174.1	141.3	148.7

Table 2: Meteorological measurements of the experimental site from Jul 2017 to Feb 2018. [1] Mean±SD, [2] temperature-humidity index (THI)

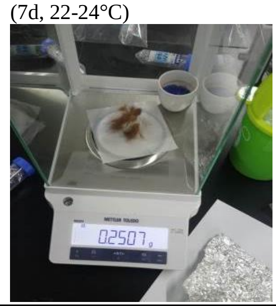
Items	Jul 1st - Aug 25th	Aug 25th - Oct 11th	Oct 11th - Nov 11th	Nov 11th - Dec 23rd	Dec 23rd - Feb 12th
2017					2018
Temperature (ºC)					
Mean	26.1 ± [1]3.0	19.0 ± 4.0	10.8 ± 5.1	-2.1 ± 5.4	-5.5 ± 6.0
Max.	31.4	26.8	18.4	8.1	5.2
Min.	18.9	10.1	-1.9	-5.4	-12.3
Relative humidity (%)					
Mean	85.4 ± 15.6	80.8 ± 19.9	74.6 ± 22.4	67.2 ± 24.5	60.5 ± 21.9
Max.	99.9	99.9	99.9	99.9	81.7
Min.	27.5	41.1	17.4	48.9	17.7
THI[2]					
Mean	77.4	65.3	52.3	33.6	29.9
Max.	88.5	80.3	65.1	46.6	43.0
Min.	62.8	52.6	41.9	32.3	31.6
Average daylight hours (hour day^{-1})					
	13.8	12.1	10.5	9.7	10.2

(1). Method of hair cortisol concentration in Hanwoo cattle[1]

(2). Hair collection: Shaving (> 1g) from the forehead of Hanwoo cattle

(3). Placed hair in aluminum foil & numbering - storage

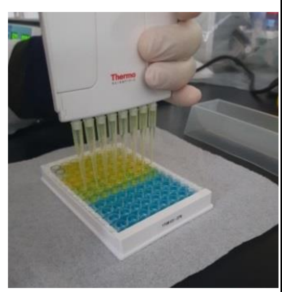
(4). Hair sample preparation: Weighing (250 mg) & washing hair by isopropanol (5mL), 3 min on rotator, & repeat twice). Sample drying (7d, 22-24°C)



(5). Hair sample grinding: Weighing (50 mg) & grinding by bead beater (8min-50Hz)

(6). Hair cortisol extraction: Methanol (1 mL), rotation (30 rpm & 24h)

(7). Hair cortisol analysis: By Salimetrics EIA kit



(8). Reading optical density: Using microplate reader (450 nm)

Fig. 1: Info-graphic of hair cortisol analysis.1 (Ghassemi Nejad *et al.*, 2014, 2017a)

estimates and standard errors provided in this work were calculated from the least square means of the fixed effects in the model. *P*-value <0.05 was considered significant. Three EIA kits were used to measure hair cortisol; the inter- and intra-assay coefficient of variations was 8.75 and 9.79%, respectively, from the duplicated samples.

Analytical Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus two standard deviations (SDs) of 10 sets of duplicates at the 0 μg dL^{-1} level. The minimal concentration of cortisol that can be distinguished from 0 was 0.007 μg dL^{-1} (Salimetrics kit).

Results

Our results suggest that reliable and detectable data on cortisol concentration can be obtained from hair samples of Hanwoo cattle. The mean hair cortisol concentration ranged from 3.7-7.3 ± 0.44 (pg mg^{-1}).

Our results indicate a high concentration of hair cortisol in Aug (HS), lower concentration in Nov and Dec, and higher concentration during Feb (CS); (Fig. 2). There were no significant differences in the hair cortisol concentration between Aug and the other sampling dates, particularly between Aug and Feb (P=0.323*)*, when HS and CS were observed (Fig. 2). However, the hair cortisol concentration was significantly lower in Oct and Nov than in Feb ($P < 0.05$); (Fig. 2).

There were no significant differences in the hair cortisol concentration of dams or heifers at different sampling dates (Fig. 3A and B). In calves, the hair cortisol concentration was significantly higher in Aug than Oct ($P < 0.05$) or Nov ($P < 0.05$); however, there were no differences between Aug and Dec or Feb (Fig. 3C). There was a significant difference in calf hair cortisol concentration between Oct and Feb ($P < 0.05$), but we found no difference between Oct and Nov or Dec (Fig. 3C). There was a significant difference in calf hair cortisol concentration between Nov and Feb ($P < 0.05$), but no difference was found between Nov and Dec (Fig. 3C).

Hair cortisol concentration was significantly higher in calves than in dams and heifers in Aug ($P < 0.05$). There was no significant difference between dams and heifers among the sampling dates (Fig. 4). There were no significant differences in the hair cortisol concentration of Hanwoo cattle in Oct, Nov and Dec (Fig. 4). The hair cortisol concentration of calves and dams significantly differed in Feb ($P < 0.05$); however, there were no differences between dams and heifers or between heifers and calves in Feb (Fig. 4).

The effects of time (sampling month) and cattle type were highly significant ($P < 0.01$). However, the interactions between sampling month were not significant ($P > 0.05$). Within cattle type, the differences between dams and calves and between heifers and calves were highly significant ($P < 0.01$), whereas there was no difference ($P > 0.05$) between dams and heifers. The interaction between cattle type and time was highly significant ($P < 0.01$).

Discussion

Our study used hair from Hanwoo cattle to measure cortisol concentration as an indicator of chronic stress related to various environmental conditions. Hair cortisol is a unique biomarker of long-term hypothalamic–pituitary–adrenal (HPA) axis activity (Meyer and Novak, 2012). Analysis of cortisol concentration using hair yields a cumulative

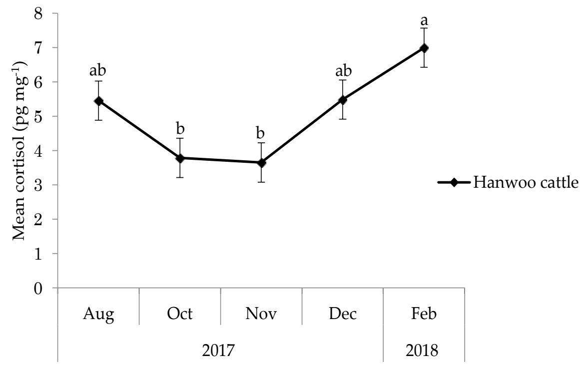


Fig. 2: Comparison of mean hair cortisol concentration (pg mg^{-1}) in different months in Hanwoo cattle. Values are expressed as means ± SEM. Different letters indicate significant differences ($P < 0.05$)

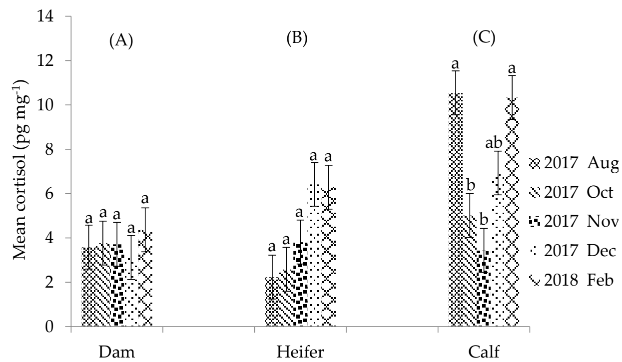


Fig. 3: Comparison of mean hair cortisol concentration (pg mg^{-1}) in dams (**A**), heifers (**B**) and calves (**C**) from Aug, 2017 to Feb, 2018. Values are expressed as means ± SEM. Different letters indicate significant differences ($P < 0.05$)

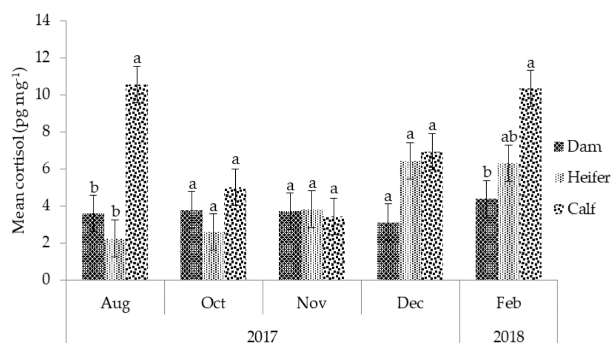


Fig. 4: Mean hair cortisol concentration (pg mg^{-1}) in dams, heifers, and calves from Aug, 2017 to Feb, 2018. Values are expressed as means ± SEM. Different letters indicate significant differences ($P < 0.05$)

cortisol profile of the hair growth period that can be used to evaluate chronic stress (Nejad *et al*., 2017a; Uetake *et al*., 2018). Furthermore, hair cortisol has been linked to stress-induced health conditions (Meyer and Novak, 2012; Nejad *et al*., 2017b). According to Uetake *et al*. (2018), hair cortisol concentration can be used as a retrospective indicator of stress during periods of approximately weeks to months; our study supported this. Burnett *et al.* (2014) reported a similar range of hair cortisol concentration in black and white Holstein dairy cows (3.8-7.8 ± 1.1 pg mg^{-1}); however, this study was the first to report hair cortisol concentration in native Hanwoo dams, heifers and calves with brown hair.

Despite other studies that reported higher hair cortisol concentration in Holstein cattle (Nedić *et al*., 2017) and ewes (Nejad *et al*., 2017b) during HS, we did not find any differences in the hair cortisol concentration between HS and TCZ. This trend in hair cortisol concentration in Hanwoo cattle supported our hypotheses of high concentration in Aug that decrease in Oct and Nov and increase from Dec to Feb. This may be attributed to the temperatures and relative humidity THI throughout the experiment, which were moderately high in summer (Aug), with a gradual decrease in autumn (Oct-Nov) and a severe decrease during winter (Dec-Feb). The absence of differences in the hair cortisol concentration between Aug and other sampling dates indicated that the THI during summer was not influential enough to significantly increase hair cortisol concentration. However, lower hair cortisol concentration in Oct and Nov than in Feb illustrate the impacts of CS. No differences between the hair cortisol concentration during the HS in Aug *vs*. during the CS in Feb were found, differs from the study by Pierre *et al.* (2018) that reported substantial effects of daylight hours on plasma cortisol concentration in humans. Although plasma cortisol concentration peaked during short winter days and decreased during long summer days (Pierre *et al*. 2018), we did not observe this effect in Hanwoo cattle. Although plasma cortisol is the main source of hair cortisol during a prolonged period, there are other sources that can deposit cortisol into hair, including sweat, deep skin and the environment (Meyer and Novak, 2012; Nejad *et al*., 2014; Salaberger *et al*., 2016). This result in Hanwoo cattle supported our hypothesis and was consistent with the results of Piao and Baik (2015) that suggested a higher susceptibility of Hanwoo cattle to CS compared to HS. It has been suggested that animal susceptibility to environmental stressors may also depend on other factors, such as breed, life history, and physiological status (Mader *et al*., 2010). In the current study, some fluctuations in hair cortisol concentration could be attributed to the aforementioned factors under HS and CS, particularly in calves.

Environmental conditions during HS could not affect hair cortisol concentration in both heifer and dams in different sampling dates. This may be attributable to the higher tolerance of Hanwoo dams and heifers to hot and humid environmental conditions. This result is similar to the results of Piao and Baik (2015) and Kang *et al*. (2017), who reported a higher tolerance to HS in mature Hanwoo cattle. However, the optimal temperature ranges and relative humidity (THI) in Hanwoo cattle have not yet been established (Kang *et al*., 2017). Additionally, the range of the optimal temperature zone of dairy Holstein cows was estimated to be -0.5°C to 20°C (Johnson, 1987), whereas Berman *et al*. (1985) indicated that the upper critical air temperature for dairy cows ranged from approximately 25°C to 26°C. In the present study, the THI was not high enough in the region where the experiment was conducted to cause sufficient HS to increase hair cortisol concentration. Furthermore, the cattle in our experiment were provided with shelter and were thereby protected from direct solar radiation; thus, the heat load may not have been enough to increase hair cortisol concentration. Hence, shelter may improve the comfort of Hanwoo cattle during summer. The higher hair cortisol in calves could be attributed to the fact that they were newborn, and their acclimation to the new environmental conditions may have increased their cortisol concentration. The stress levels in calves (as indicated by hair cortisol concentration in this study) were significantly higher than those in their dams and heifers under HS and CS, suggesting that calves are more vulnerable to intense environmental conditions during seasonal changes. The decline in the hair cortisol concentration in calves from Oct to Nov TCZ could be explained by the decline in the THI during that period. The substantial decrease in the THI in Dec and Feb was, as expected, associated with an increase in hair cortisol concentration. This result indicates that the effect of CS on calves is remarkably strong, possibly because calves have thinner skin than older animals. Additionally, the calves in this experiment suffered from skin disease (*Dermatophytosis* caused by *Trichophyton verrucosum*) (Dalis *et al*., 2014) during Dec and Feb, which might have generated additional stress. The absence of significant differences in the hair cortisol concentration between Aug and Feb indicated that the calves responded similarly to both HS and CS; this is inconsistent with results reflecting the effects of daylight hours on plasma cortisol concentration reported by another study (Pierre *et al.* 2018).

We found that calves had higher hair cortisol concentration than mature non-pregnant heifers and dams. Given that the neonatal calves in this study were newborn, we would expect them to have higher stress levels due to their need to habituate to a new environment after birth and to their physiological status. Calves experience more variation in their physiological status compared with heifers and dams. The instability of the physiological status of calves may be influenced by different factors, such as the transition from being mono-gastric to poly-gastric animals, the transition from milk to dry matter, the acclimatization to a new environment (Piao *et al.* 2015; Rojas-Downing *et al*., 2017) and the higher potential of disease exposure due to their undeveloped immune system (Kang *et al*., 2016). These results were consistent with other studies that reported higher levels of stress in neonatal calves and young heifers (González-de-la-Vara *et al*., 2011). Moreover, higher hair cortisol concentration in young calves confirmed this result. The reason that heifers showed lower hair cortisol concentration than calves in Aug is unclear. The variations in hair cortisol concentration in dams were less pronounced than those in heifers. This could be explained by the higher hair cortisol concentration in calves than dams, but not heifers. Korean native cattle (Hanwoo) have been reported to have considerably lower milk production than Holstein cows (Lee *et al*., 2014) and have been bred primarily for beef production (Lee *et al*., 2014). Thus, the impact of production stress that was noted in a previous study (Nedić *et al*., 2017) was not applicable to the current study. This could be why dams showed lower stress levels than their offspring. In the present study, we duplicated each sample to validate the data and the power of the test. However, future studies would likely benefit from also increasing the number of subjects. The reason why we exploited three animals in each group (3 animals × 2 hair sample duplication = 6 data replications each), was that due to the herd size in Korea. In Korea, small farms known as family farm are common including the University farm. Therefore, it wasn't likely to have more than three calves with similar physiological conditions such as weight and age and plus their mothers in the experiment.

We expected the effects of sampling time or season and type of cattle to be significant. As the cattle in this experiment differed in their physiological status, such as their growth stage, these discrepancies are logical. The differences among dams, calves, and heifers could be due to the fact that younger individuals experience stress during acclimatization to changes in the environment, such as climate conditions (temperature, humidity, daylight hours, etc.) and barn management (changes in feed, barn operation, etc.). The interaction between the differences in cattle, particularly regarding the physiological status of calves, dams, and heifers and the substantial (THI) differences in different environments during different seasons may have caused this phenomenon. Pierre and Rao (2018) reported that seasonal changes in cortisol secretion, with the highest concentration in winter and the lowest concentration in summer, result in seasonal modifications of the alignment of biological activities with the environment. They concluded that the primary interaction between the (HPA) axis and the circadian clock may have led to circadian misalignment of the synchronization of peripheral regulatory processes. Future research should focus on the effects of daylight hours in different seasons on both plasma and hair cortisol concentration.

Conclusion

This study validated the use of hair cortisol concentration of Hanwoo cattle as a retrospective biomarkers of chronic stress. These data add to the extant information on TCZ in Hanwoo cattle. Consistent with previous research, the present study found that calves were more susceptible to environmental changes during different seasons, than were adults. Increasing the number of cattle and adding blood or saliva cortisol measurements to the hair cortisol data could further validate the obtained results.

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