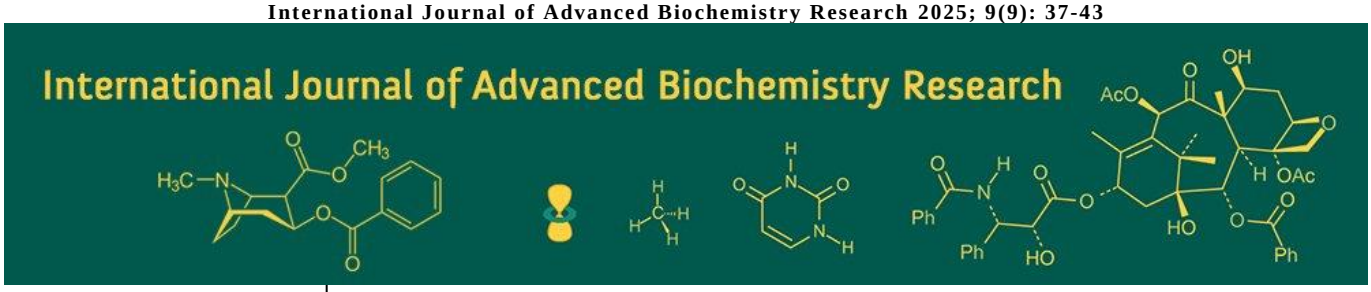

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Dr. Navin B
MVSc Scholar, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Dr. Karthiayini K
Professor and Head, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Dr. Beena V
Professor, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Dr. Zarina Aziz
Associate Professor and Head, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Dr. Harikumar S
Associate Professor and Head, Instructional Livestock Farm Complex, Pookode, Wayanad, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Dr. Sajana OK
Teaching Assistant, Department of Statistics, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Dr. Shalinee GV
MVSc Scholar, Department of Animal Nutrition, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, Indi

Dr. Haripriya K
MVSc Scholar, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Corresponding Author:
Dr. Navin B
MVSc Scholar, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (KVASU), Kerala, India

Comparative antioxidant and non-invasive physiological assessment of crossbred calves exposed to thermoneutral and acute heat stress conditions in a climate-controlled chamber

Navin B, Karthiayini K, Beena V, Zarina Aziz, Harikumar S, Sajana OK, Shalinee GV and Haripriya K

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Abstract
Heat stress due to climatic change in cattle affects their overall health, production and performance. Cattle are homeotherms, initiate thermoregulatory mechanisms to heat stress. They employ antioxidant responses to counteract the oxidative stress due to environmental challenges. Antioxidant response is their endogenous ability to neutralise reactive oxygen species produced during heat stress. Enzymatic antioxidants like glutathione peroxidase (GPx) can quench the free radicals and prevent from cellular damage during heat stress. Measuring this bio-marker played a significant role in assessing the impact of high temperature and humidity on oxidative stress levels of cows. Rectal temperature is the ideal physiological bio-marker of quantifying heat stress responses in cattle. Surface temperatures measured at different anatomical locations by infrared thermometry is a precise, alternative non-invasive method for physiological assessment in heat-stressed cattle and can be an indicator of animal welfare. Hence the present study was conducted to assess the antioxidant and non-invasive physiological responses of crossbred calves to acute heat stress (Treatment) and compared it with thermo-neutral zone (Control) in a climate-controlled chamber. Venous blood samples with anti-coagulant were collected for spectrophotometric estimation of GPx activity in both the groups. Rectal temperature and surface temperatures of eye, forehead and flank were recorded on alternate days of experiment in both the groups. The study evidenced that the biochemical antioxidant responses were significantly increasing (p<0.001) from day one to ten of exposure to heat stress and remained constant in TNZ. The rectal temperature and surface temperatures of eye, forehead and flank were significantly increased (p<0.001) in the heat-stressed calves when compared to TNZ. The correlation studies shown that eye temperature was strongly correlated with rectal temperature under heat stress. It was evident from the findings that crossbred calves maintain thermal homeostasis by adopting antioxidant and physiological adaptations when they were exposed to environmental challenges under controlled climatic conditions. Furthermore, the study thrown light on the normal physiological and cellular functioning in crossbred calves under thermo-neutral zone.

Keywords: Oxidative stress, heat stress, thermo-neutral zone, crossbred calves, climatic chamber, non-invasive, body temperature

1. Introduction
Heat stress (HS) due to climatic change affects the overall production, health and productivity in livestock. Generally, livestock maintains thermal homeostasis by adopting various thermo-regulatory mechanisms, which can significantly promote their welfare and survival in stressed conditions (Indu and Pareek, 2015) [25]. Heat stress occurs when the endogenous heat production of cattle is greater than its capacity to lose heat (Bernabucci *et al*., 2010) [3]. The biochemical and physiological functions of cattle will remain normal in thermo-neutral zone (TNZ) or comfort zone where there won't be any energy expenditure for thermoregulation. When the ambient temperature exceeds the upper critical limit of their zone of homeothermy, cattle experience HS (Bagath *et al*., 2019) [2]. Heat stress often results in the production of various reactive oxygen species (ROS) such as peroxide, superoxide and hydroxyl radicals in cattle (Ganaie *et al*., 2013) [17]. The endogenous antioxidant mechanism is the first line of defense responses to overcome the oxidative stress due to HS.

The overproduction of ROS ultimately results in lipid peroxidation of plasma membrane and disruption of normal cellular metabolism (Gupta *et al*., 2013) [20]. Antioxidant enzymes synthesised in the body during HS protect the cells from ROS generated due to oxidative stress (Gupta *et al*., 2021) [21]. Their predominant role is to scavenge the intracellular and extracellular superoxides and inhibit the lipid peroxidation of plasma membrane (Zhang, *et al*., 2017) [51]. Measuring these enzymatic bio-markers warranted a significant role in assessing the impact of temperature-humidity challenge on oxidative stress levels of cows. Glutathione peroxidase (GPx) is a selenium dependent antioxidant enzyme converts hydrogen peroxide (H_2O_2) to water. Higher antioxidant enzyme activity often results from the upregulation of mRNA expressions of certain antioxidant genes such as GPx, SOD and CAT. This ultimately enhances the ability of cells to maintain redox homeostasis (Akbarian *et al*., 2014) [1]. Physiological adaptation in heat stressed animals is generally an early warning signal and ideal for quantifying HS response. Quantifying non-invasive physiological based indicators in heat stressed cattle such as internal body temperature (rectal temperature) and external or surface body temperatures is sensitive, more promising and reflected their heat load (Hoffmann *et al*., 2020) [22]. Rectal temperature is the gold standard physiological bio-marker for quantifying HS response in cattle (Falkenberg *et al*., 2014) [16]. The primary objective of non-invasive physiological assessment is ensuring without exposing cattle to discomfort and for quick and precise identification of HS response. Generally, the body surface temperature is an indicator of heat absorption during thermogenesis and heat dissipation during thermolysis, which may reflect the core temperature of cattle. According to Montanholi *et al*. (2008) [31], change in body surface temperature is one of the first identifiable physiological responses that occur in cattle to HS. These might be due to increased peripheral vasodilation that allow the blood flow to circulate in peripheral vessels of body for effective heat dissipation. Infrared thermometry and infrared thermography (IRT) are primarily used to record body surface temperatures (Wijffels *et al*., 2021) [47]. Surface temperature measured at different anatomical locations of body can be used as an indicator of animal welfare and can be effectively used for precision farming (Collier *et al*., 2006; Zotti *et al*., 2011; Poikalainen *et al*. 2012; Roberto and De Souza, 2014) [11, 52, 35, 38]. This also reduce the risk of transmission of infections, since there is no contact with the animal (Soerensen and Pedersen, 2015) [43]. Usually, surface body temperatures are measured in the sublingual, eye, axilla, groin, neck, ear, thorax, flank and forehead regions (Sellier *et al*., 2014) [40]. But the skin surface temperature is influenced by environmental temperature (de Lima *et al*., 2013) [15]. If the skin surface temperature is above 35 °C, heat exchange mechanisms become very active (Coppola *et al*., 2002) [13], rectal temperature rises and cutaneous heat dissipation mechanism occurs (Pollard *et al*., 2005) [36]. The different anatomical locations differ in their ability in heat dissipation as the temperature gradient differ at different sites (Singh and Singh, 2006; Poikalainen *et al*., 2012; Hoffmann *et al*., 2013) [42, 35, 23]. In view of this, the present study was conducted with the objective of assessing the antioxidant and non-invasive physiological responses such as rectal temperature, body surface temperatures of eye, forehead and flank using infrared thermometry in crossbred calves exposed to acute heat stress (Treatment) and thermo-neutral zone (Control) in a climate-controlled chamber.

2. Materials and Methods

2.1 Ethical approval

The experiment was conducted at the climatic animal chamber in Climate Controlled Research Complex (CCRC) of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS), KVASU. It was proceeded following the approval by Committee for the Control and Supervision of Experimentation on Animals (CCSEA), New Delhi (No: V-11011(13)/7/2024-CPCSEA/DADF, dated 08/08/2024).

2.2 Layout of the experiment

Six healthy crossbred female calves of 10 months of age with identical body characteristics were selected randomly from University Livestock Farm (ULF), Mannuthy. The same selected calves were distributed in two groups: thermo-neutral zone (TNZ) or control group; acute heat stress or treatment group (Fig.1). The calves were given an acclimatisation period of 10 days each in the animal holding facility and in the climatic chamber. It was immediately followed by experimental period of 10 days each housed under TNZ and acute HS. In TNZ, the chamber was maintained at 27 °C temperature. For acute HS study, maximum temperature of 40 °C was simulated for three hours a day for 10 days. The relative humidity of 55-65 percent was maintained for both TNZ and HS. After the experiment, the animals were re-shifted to the respective cattle sheds of the farm for rehabilitation and reuse, as recommended by CCSEA.

Fig 1: Animals selected for experiment kept in climate-controlled chamber.

2.3 Collection of Venous Blood samples

Blood samples were collected by jugular venipuncture in lithium heparinised vacutainers in animals on both the experimental groups on days zero, one, five and ten of the experiment and was used for the preparation of haemolysate for the estimation of enzymatic antioxidant GPx activity.

2.4 Assessment of Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase activity was assessed by UV-VIS spectrophotometer using GPx activity assay kit (Origin Diagnostics and Laboratory, Kerala).

2.4.1 Principle of GPx estimation

Glutathione peroxidase converts free radicals like hydrogen peroxide (H_2O_2) to reduced glutathione to produce H_2O and oxidised glutathione (GSSG). The glutathione activity can be calculated by measuring the consumption of reduced glutathione. Hydrogen peroxide (H_2O_2) and reduced glutathione can react without catalysis of GPx, and hence the fraction of GSH reduction by non-enzymatic reaction should be subtracted. GSH can react with di-nitrobenzoic acid to form 5-thio-dirutrobenzoic acid anion, which show a stable yellow colour.

2.4.2 Procedure of Haemolysate preparation for GPx assay

Haemolysate samples were prepared from the collected venous blood samples and used for the GPx assay. RBC was spin down by centrifugation at 3000 rpm for 10 min at 4 ℃. Plasma was removed from the cells and buffy coat was removed and discarded. RBC pellet was washed with saline at 4 ℃ and centrifuged at 3000 rpm for 10 min at 4 ℃. The clear saline from top was discarded and repeated once. An equal volume of deionised water was added to an aliquot of RBC, and vortexed well for complete lysis of the cells. Using Drabkin's solution (Agappe), hemoglobin concentration estimation of the 1:2 diluted RBC hemolysate was performed. The unit was converted from g/dl to g/L. Hemolysate sample aliquots were stored at <-65 ℃ freezer until spectrophotometric analysis. Frozen samples were thawn out and vortexed well before analysis. Thawed out red blood cell lysate was diluted in deionised water to 6-7 g/L of hemoglobin. Before analysis, mixed well and left the diluted samples on ice.

2.4.3 Spectrophotometric assessment of GPx activity

Primarily, preheated the UV-VIS spectrophotometer (Perkin Elmer Lambda) for 30 minutes and adjusted the wavelength to 412 nm and set zero with distilled water. Diluted the standard solution (80 µL mol/mL) to 0.08 µmol/mL with diluent. Subsequent assay was performed in a 1.5 mL microcentrifuge tube. Then the reagents were added in the order as mentioned in the Table 1. Then, centrifuged at 4000 rpm at room temperature for 5 min and transferred the supernatant into a new 1.5 mL microcentrifuge tube and the final procedure of assay was performed as summarised in Table 2. Mixed well and then placed at room temperature for 15 min and the absorbance at 412 nm was measured. The recorded absorbance were A_T. A_C, A_S and A_B respectively.

$\Delta A_T = A_C - A_T$

$\Delta A_S = A_S - A_B$

Table 1: Initial Procedure of Spectrophotometric GPx assay

Reagent	Test (T)	Control (C)
Haemolysate sample	20 µL	-
I Reagent	20 µL	20 µL
Incubation for 5 min at 37 ℃		
II Reagent	10 µL	10 µL
Incubation for 5 min at 37 ℃		
III Reagent	200 µL	200 µL
Haemolysate sample	-	20 µL

Table 2: Final Procedure of Spectrophotometric GPx assay

Reagent	Test (T)	Control (C)	Standard (S)	Blank (B)
Diluent	-	-	-	100 µL
Supernatant	100 µL	100 µL	-	-
Working standard	-	-	100 µL	-
IV Reagent	100 µL	100 µL	100 µL	100 µL
V Reagent	25 µL	25 µL	25 µL	25 µL

Calculation

GPx (U/mL) = ΔAT ÷ (ΔAS ÷ CS) × 1000 × VEV ÷ VS ÷ T = 200 × ΔAT ÷ ΔAS

Hence, GPx (U/mL) = 200 x ΔAT ÷ ΔAs

Since 1:10 dilution was done, dilution factor was 10.

Actual value = GPx (U/mL) × 10

GPx (U/L of Haemolysate) = (Actual value)/100

2.5 Non-invasive physiological assessment in cattle

2.5.1 Recording of Rectal temperature

The rectal temperature was recorded by inserting 6-7 cm inside the rectum inclined towards the wall of the rectum, at three-hour interval from 8.00 a.m. to 6 p.m. on alternate days of the experiment using a clinical thermometer in both experimental groups

2.5.2 Recording of body surface temperature

Body surface temperatures of different anatomical sites of animal body such as eye, forehead and flank were recorded on alternate days of the experiment using EASYCARE (German-tech) LCD display non-contact infrared thermometer by keeping it 5 to 7 cm away from the desired surface site.

2.7 Statistical analysis

The results were expressed as Mean ± standard Error. The statistical significance of the data was assessed by linear mixed model analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) version 24.0 software. Correlation studies between body temperatures were done using Spearman's rho correlation coefficient method.

3. Results and Discussion

3.1 Antioxidant response

The overall enzymatic GPx activity in crossbred calves was significantly higher (p<0.001) in the acute heat stressed group when compared to the TNZ (Table. 3). The enzymatic antioxidant activity remained constant without significant difference in various days of the TNZ. In contrast to this, it was gradually increasing from day one to day ten of exposure to HS (Fig. 2). The highest GPx value was recorded on day 10 of HS and the lowest on day zero or before HS exposure.

Table 3: Glutathione peroxidase activity of crossbred calves exposed to thermo-neutral zone and acute heat stress under climate-controlled conditions (Mean ± SE, n=6).

Antioxidant response	Days	Thermo-neutral zone	Acute heat stress	p-value
GPx activity (U/L of Haemolysate)	0	9.13^{aA}±0.33	9.17^{aA}±0.263	0.917^{ns}
	1	9.37^{aA}±0.63	13.83^{bB}±0.95	$< 0.01^{**}$
	5	9.25^{aA}±0.44	16.80^{bB}±1.01	$< 0.001^{***}$
	10	9.158^{aA}±0.32	21.33^{cB}±2.13	$< 0.01^{**}$
	Overall	9.23^{A}±0.23	15.28^{B}±0.641	$< 0.001^{***}$

Means bearing different superscripts within a row (A-B) and columns (a-c) differ significantly
***Significant at 0.001 level, **Significant at 0.001 level, ns-non-significant at 0.05 level

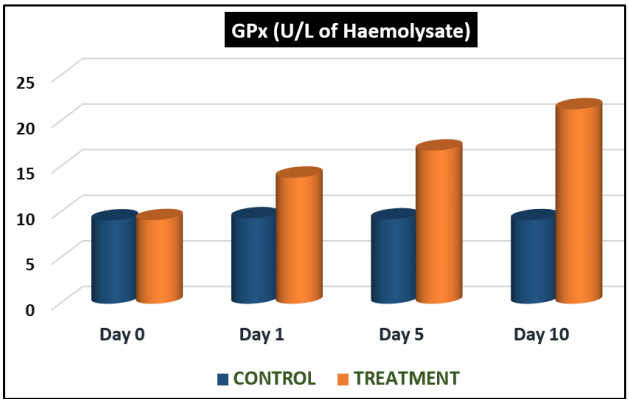

Fig 2: Enzymatic antioxidant activity of crossbred calves exposed to thermoneutral zone and acute heat stress in a climate chamber.

The significantly highest GPx value (p<0.001) on different days of acute heat stressed group when compared to TNZ agreed with the findings of Sakatani *et al.* (2012) [19], Lallawmkimi *et al.* (2013) [28], Chaudhary *et al.* (2015) [8] and Rathwa *et al.* (2017) [37]. Zeng *et al.* (2013) [50] found that enzymatic GPx activity was significantly higher HS group when compared to no-HS group which is consistent with the present findings. Chetia *et al.* (2017) [9] explained that increased antioxidant enzyme (GPx) activity during HS converts hydrogen peroxide (H_2O_2) to water using glutathione as a co-substrate. The increased production of H_2O_2 in HS might be due to increased activity of superoxide dismutase (SOD) which might result in a coordinated increase in GPx activity. This acted as antioxidant defense response in preventing the accumulation of harmful reactive oxygen species (ROS) and might counteract the oxidative stress during environmental challenge period. Hence the increase in enzymatic antioxidant activity during HS might have helped the calves to maintain and restore the redox homeostasis for maintaining normal cellular function and preventing oxidative damage.

3.2 Non-invasive physiological responses

The physiological parameters recorded such as rectal temperature and body surface temperatures of eye, forehead and flank were summarised in Table 4.

Table 4: Overall mean values of physiological recordings of crossbred calves exposed to thermo-neutral zone and acute heat stress conditions (Mean ± SE, n=6)

Physiological variables	Thermo-neutral zone	Acute heat stress	p-value
Rectal temperature	37.87^{A}±0.04	38.95^{B}±0.04	$< 0.001^{***}$
Eye temperature	37.36^{A}±0.04	38.81^{B}±0.04	$< 0.001^{***}$
Forehead temperature	37.27^{A}±0.05	38.73^{B}±0.05	$< 0.001^{***}$
Flank temperature	37.13^{A}±0.06	38.82^{B}±0.06	$< 0.001^{***}$

Means bearing different superscripts within a row (A-B) differ significantly,***Significant at 0.001 level

Table 5: Spearman's rho correlation coefficient of rectal temperature with body surface temperatures recorded in crossbred calves exposed to acute heat stress

Spearman's rho correlation coefficient		Eye temperature	Forehead temperature	Flank temperature
Rectal temperature	Correlation Coefficient	0.425^{**}	0.14	0.19
	Sig. (2-tailed)	0.01	0.41	0.26

**Correlation is significant at 0.01 level (2-tailed)

3.2.1 Rectal temperature

Compared to calves kept under TNZ, the mean rectal temperature was significantly (p<0.001) increased in heat stressed condition (Table 4) which was similar to the findings of Garner *et al.* (2017) [18] They evidenced that mean rectal temperature was significantly increased in cows exposed to 40 °C than at 25 °C. The significantly increased rectal temperature with increase in temperature and humidity simulated inside the chamber was an indication that the thermoregulatory responses of the heat-stressed calves were insufficient to fully counteract the heat load. Similar finding was also reported by Woo *et al.* (2024) [48] who suggested that rectal temperature increased with increase in climatic variables suggesting that rectal temperature was an early warning signal in heat stressed animals, where the balance between heat gain and heat loss in the animal might have disturbed.

3.2.2 Body surface temperatures

In the current study, recorded body surface temperatures by infrared thermometry such as eye temperature, forehead temperature and flank temperature of the calves were significantly increased (p<0.001) in HS exposure when compared to exposure at comfort zone. This finding completely agreed with the reports of Blond *et al.* (2024) [5], Silva and Maia (2011) [41], Bhan *et al.* (2013) [4], Katiyatiya *et al.* (2017) [26], Yadav *et al.* (2017) [49] and Park *et al.* (2019) [32]. They suggested that body surface temperature

generally increase in HS when compared to TNZ. This increase in surface temperature might be a result of increased core body temperature which might have caused redistribution of blood flow and shifted the temperature from core to periphery for ensuring effective heat dissipation.

The present finding of increased eye temperature in HS is consistent with Church *et al*. (2014) [10]. Some studies shown that eye temperature, particularly in the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle, considered as an indicator of HS (Pavlidis *et al*., 2002; Cook *et al*., 2005 and Cardoso *et al*., 2015) [33, 12, 7]. According to Dai *et al*. (2015) [14] and Sutherland *et al*. (2020) [45], eye temperature is usually observed for infrared thermography because of lack of hair around eye region. Gloster *et al*. (2011) [19] suggested that increase in eye temperature in HS might be due to location of eye nearer to the hypothalamic thermosensitive area which might reduce lag time in stress response. Anatomically in the caruncula lacrimalis and posterior border of the eyelids, eyes have superficial capillaries beds, which are highly innervated by the sympathetic nervous system which might contribute to high eye temperature in heat-stressed cattle (Stewart *et al*., 2007) [44]. The Spearman's rho correlation (Table 5) shown that rectal temperature had a strong positive correlation with eye temperature in heat-stressed calves. This finding is precisely consistent with the findings of Brcko *et al*. (2020) [6] and Idris *et al*. (2021) [24].

The significantly higher forehead temperature of heat-stressed calves in the present study was consistent with the reports of Singh and Singh, 2006 [42] and Peng *et al*. (2019) [34]. Martello *et al*. (2016) [29] suggested that the forehead temperature was sensitive and suitable indicator for early monitoring of HS. In view of the current results and earlier reports of Weschenfelder *et al*. (2013) [46], recording temperature in forehead region which is in close proximity to higher brain centres regulating body temperature, might be a suitable indicator of core body temperature (Mccafferty, 2007 and Kessel *et al*., 2010) [30, 27]

Albeit flank temperature was significantly increased during HS, it was not positively correlated with rectal temperature. Due to its close distance to rumen, the flank temperature might be a good indicator to reflect rumen temperature of cattle rather than rectal temperature (Montanholi *et al*., 2008) [31].

4. Conclusion

It was evident from the study that cellular and physiological functions of crossbred calves were unaffected under TNZ. The study thrown light on the antioxidant and physiological adaptations of crossbred calves when they were exposed to temperature-humidity challenges under climate-controlled conditions. This study highlighted that non-invasive physiological assessment of HS using infrared thermometry is promising to quantify the body temperature of calves as they were significantly increased in HS than TNZ. Recording body surface temperature at different anatomical sites gave a clear picture about correlation of rectal temperature with eye temperature when exposed to HS. Hence eye temperature might be considered as reflective of rectal temperature in heat-stressed calves. Overall, the study suggested to maintain crossbred cattle in TNZ for their normal functions, performance, welfare and productivity.

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6. Conflict of Interest

The authors declared no conflict of interest.

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