

IMPACT OF ACUTE HEAT STRESS ON CROSSBRED CALVES UNDER CONTROLLED CLIMATIC CONDITIONS

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**NAVIN B.
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THESIS

Submitted in fulfilment of the requirement for the degree of

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Kerala Veterinary and Animal Sciences University**



**DEPARTMENT OF VETERINARY PHYSIOLOGY
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KERALA, INDIA**

2024

DECLARATION

I hereby declare that this thesis entitled **“Impact of acute heat stress on crossbred calves under controlled climatic conditions”** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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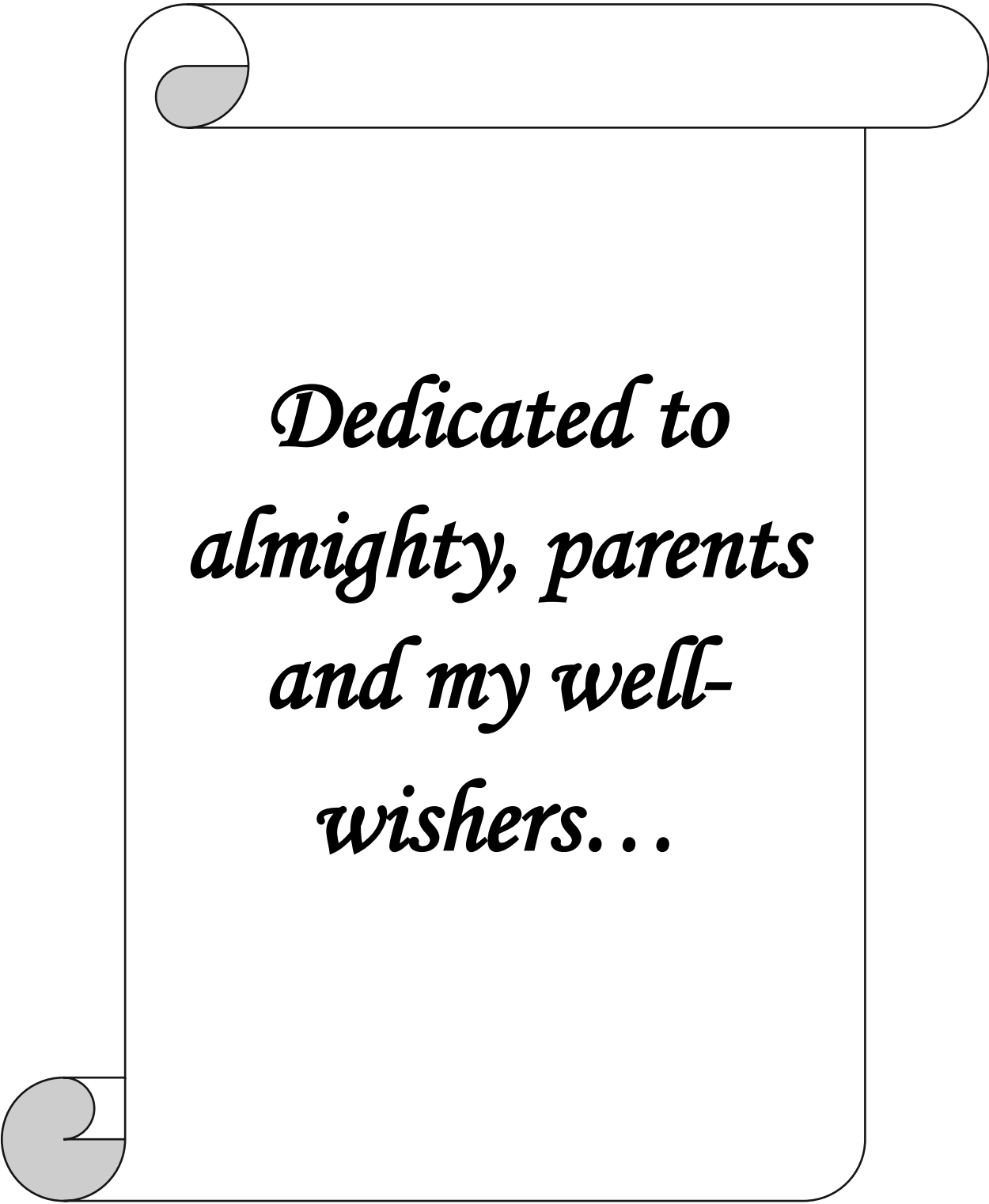
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*Dedicated to
almighty, parents
and my well-
wishers...*

Introduction

1. INTRODUCTION

Global warming and climate change associated heat stress (HS) is an emerging threat in the present scenario causing significant challenges to livestock production worldwide. As the global temperature continues to rise, understanding the adaptive responses of cattle to HS become imperative for sustainable livestock management. Incidence of extreme weather events are occurring due to greenhouse gases emission into the environment. These gases are derived from anthropogenic activities such as deforestation, burning of fossil fuels and increased release of pollutants from industrial activities that are causing harmful impacts on health and productivity of livestock.

Cattle are homeothermic animals that possess a comfort zone or thermo-neutral zone (TNZ) where there is no energy expenditure for thermoregulation. When the temperature exceeds the upper critical limit of TNZ, they experience heat stress. As per the fifth IPCC Assessment report, by the end of 2100 global surface temperature is expected to rise by 1-7 °C (IPCC 2013). Adaptation to prolonged HS may lead to production losses.

Cattle employ a range of adaptive thermoregulatory responses to maintain thermal homeostasis (Sejian *et al.*, 2018). Rectal temperature, respiratory rate and heart rate are the ideal physiological biomarkers for quantifying the impact of HS on livestock. Cattle rely on evaporative cooling mechanisms such as sweating and panting, to dissipate excess heat load and maintain a stable internal temperature.

Cattle also exhibit behavioural adaptations to cope with HS. The increased lying time under TNZ is considered as the welfare and comfort of the livestock (Polsky and von Keyserlingk, 2017). Increased standing time during HS will help the cattle in minimising heat gain *via* conduction and radiation from hot ground. These behavioural responses pave the way for designing optimal mitigation strategies to improve the well-being of livestock.

The neuro-endocrine response also plays a vital role in thermoregulation and metabolic adjustments in cattle. Hypothalamo-pituitary-adrenal axis (HPA axis) acts as the principal neuro-endocrine regulator of stress response in cattle. Heat stress in cattle will result in rise in serum cortisol level and decline in thyroid and growth hormone levels.

At the cellular levels, HS triggers a cascade of responses aimed at protecting cells from thermal damage. The primary biochemical response of cattle during HS is its endogenous antioxidant capacity to scavenge the reactive oxygen species (ROS) by the enzymatic antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx).

The upregulation of heat shock proteins (HSPs) in peripheral blood mononuclear cells (PBMCs) is the first line of molecular defense mechanism in cellular protection by assisting in protein folding, preventing protein aggregation and repair of damaged cellular structures during HS. The PBMC system is considered as a potential model for elucidating the heat shock responses in livestock. The most responsive and potential biomarker for quantifying HS response among various molecular chaperones is *HSP-70*. Heat stress can reduce the expression of insulin-like growth factor 1 (*IGF-1*) gene in cattle, which can subsequently affect growth and metabolism.

Holstein Friesian cattle evolved in the temperate climatic zone with high production capacity and increased metabolic rates are less heat tolerant. The temperature humidity index (THI) in Thrissur district during summer (March to May) may reach upto 95.52 which is far above the TNZ for crossbred cattle (Prasad, 2014). This high THI will impair the evaporative cooling mechanisms and increase the thermal load on animals.

The studies on impact of thermal stress in crossbred calves during summer season of Kerala evidenced the impact of multiple stressors than the lone effect of HS and none of the previous research were conducted under controlled climatic conditions using a climatic chamber to understand the exclusive effect of THI on crossbred cattle of Kerala. Climate-controlled chambers are the specialised enclosed spaces to evaluate the impact of specific environmental conditions on animals. Hence, the present study is undertaken exclusively with the following objectives:

1. Assess and compare the physiological, behavioural, metabolic, endocrine and antioxidant responses of crossbred calves under thermo-neutral and acute heat stressed conditions
2. Analysis of insulin like growth factor-1(*IGF1*) and heat shock protein- 70 (*HSP70*) gene expressions to evaluate growth and resistance to heat stress

Review of Literature

2. REVIEW OF LITERATURE

Heat stress (HS) is one of the major global challenges affecting the production, productivity and overall well-being of the livestock. To maintain the body temperature in heat-stressed animals, they initiate compensatory and adaptive thermoregulatory mechanisms like behavioural and physiological changes to re-establish homeothermy and homeostasis which promote welfare and favour survival in a specific stressed environment (Indu and Pareek, 2015). Understanding various adaptive mechanisms to cope with the changing climate paves the way for identification of various biological markers to quantify HS response and develop strategies to mitigate the adverse effects. The present review focuses on various adaptive responses to heat stress in cattle.

2.1 THERMO-NEUTRAL ZONE

Cattle are homeothermic animals that possess a thermo-neutral zone (TNZ) or thermal comfort zone, within which their physiological functions will be normal and there is no energy expenditure for thermoregulation. For the majority of farm animals, it is typically between 4 and 25°C. Lower critical temperature (LCT) is defined as the environmental temperature at which an animal needs to increase metabolic heat production to maintain body temperature. The environmental temperature at which the animal increases heat production due to a rise in body temperature that causes evaporative heat loss is known as upper critical temperature (UCT). Cattle are very sensitive to UCT and it has been established that even a 1°C rise in temperature from the UCT can bring down the production of livestock species (McManus *et al.*, 2009).

2.2 TEMPERATURE HUMIDITY INDEX

Elevated environmental temperature and humidity make the cattle more difficult to dissipate heat to the environment and achieve homeostasis. Temperature-humidity index (THI) is the well-described metric for thermal stress that combines temperature and relative humidity (RH). According to Bernabucci *et al.* (2010), cows were in the comfort zone at THI < 68, mildly stressed at THI between 69–78, moderately stressed at THI 79–89 and severely stressed at THI > 90. When the average THI was above 68, cows exhibited signs of HS including increased respiratory rate, increased rectal temperature and reduced production (Zimbelman *et al.*, 2009; Collier *et al.*, 2011).

2.3 MECHANISMS OF ADAPTATION TO HEAT STRESS IN CATTLE

The adaptive potential of an animal was enhanced by several phenotypic and genotypic characteristics, which allowed it to cope with the harsh environmental conditions. Animal adaptation involves the morphological, behavioural and genetic capacity of the animal for change. These adaptations might arise over generations through slow modifications as animals adapt to various environmental challenges. Increased heat dissipation, declined feed intake and increased water intake were the typical signs of homeostatic responses to HS (Collier *et al.*, 2019). When the temperature of the hypothalamus reached above TNZ, the heat loss mechanisms such as vasodilatation and sweating would be activated. The adaptive responses to HS include physiological, behavioural, neuro-endocrine, biochemical, molecular and cellular responses that combine to promote the survival of cattle in a stressed environment.

2.4 PHYSIOLOGICAL RESPONSES

Some of the physiological determinants of adaptations to HS in cattle were increased respiratory rate (RR), rectal temperature (RT), pulse rate (PR), skin temperature (ST) and sweating rate (SR) (Indu and Pareek, 2015).

2.4.1 Rectal Temperature (RT)

Rectal temperature was recognized as the ideal indicator for heat load in an animal's body as it represents the core body temperature of the animal as well as it is considered an important measure of physiological status.

Bernabucci *et al.* (2003) reported that in a climate – controlled chamber with high THI (76- 82), Holstein heifers experienced a significantly higher ($p<0.05$) RT value of 39.5°C than in a thermal comfort chamber (THI 68) where they evidenced 38.8°C.

According to Kendall *et al.* (2006), cattle experienced diurnal changes in body temperature and body temperature decreased during the early morning hours and increased in the afternoon.

Alameen and Abdelatif (2012) examined the impact of seasons on the thermoregulation of crossbred (Holstein Friesian x Zebu) dairy cows. Their study

evidenced a significantly greater RT value in the summer (THI=80.92) in comparison to winter (THI=65.75).

Hamzaoui *et al.* (2013) reported a higher RT in milking goats kept in the climatic chamber (THI = 77 – 85) compared to thermo-neutral conditions (THI= 60 – 65).

Garner *et al.* (2017) conducted study on 12 lactating Holstein-Friesian dairy cows for four days in climate-controlled chamber under moderate heat stress (THI between 74 and 84) and thermoneutral conditions (THI between 55 and 61) and concluded that mean rectal temperatures was significantly higher in cows under heat stress than under thermoneutral zone over the entire experimental period.

Wankar *et al.* (2020) reported a significant increase in rectal temperature at 35°C and 40°C as compared to 25°C and 30°C ($p<0.05$) in adult dry Murrah buffaloes kept at psychrometric chambers and they evidenced the highest RT on day 14 at 40°C (38.98 ± 0.05).

Hou *et al.* (2021) conducted a study by comparing the responses of dairy cows to short-term and long-term heat stress in the climate-controlled chamber and found that rectal temperature was significantly elevated in the HS group ($1.1\text{--}1.2^\circ\text{C}$, $p<0.05$) when compared to the cows in TNZ.

Woo *et al.* (2024) analysed the physiological responses of early-fattening Hanwoo steers to varying THI in climatic chambers and evidenced that the RT was significantly different in varying THI, for mild stress (THI 76 to 81) it was in the range of 38.5°C to 38.8°C , for moderate stress (THI 82 to 84), it was in the range of 38.9°C to 39.2°C and for severe level (THI 85 to 99) it was above 39.3°C respectively.

2.4.2 Respiratory Rate (RR)

Respiratory rate is one of the ideal biomarkers for quantifying HS response. Cattle employ respiratory evaporative cooling mechanisms through the process of a multi-fold increase in RR by dissipating heat load from the body to maintain homeostasis.

According to Bernabucci *et al.* (2003), Holstein calves housed in climatic chambers with high THI (76–82) had a higher ($p < 0.05$) respiration rate of 70/min compared to 43/min in comfort chambers (THI 68).

Banerjee and Ashutosh (2011) experimented on Tharparkar and Karan Fries heifers to determine the impact of temperature exposure on the diurnal cycles of physiological parameters and found a significant rise in RR in animals under heat stress (38–39°C and 60–70% RH) from 9:00 a.m. to 14:00 p.m. for seven days when compared to thermo-neutral controls.

Wankar *et al.* (2014) assessed the thermoregulatory and adaptive responses of adult buffaloes (*Bubalus bubalis*) during hyperthermia in an environmentally controlled chamber and found a significantly increased RR ($p < 0.001$) at 35°C and 40°C.

Respiratory rate is considered the practical and reliable measure of heat load and a RR higher than 80 breaths/min was found to be an indicator of a high quantum of HS in farm animals (Indu and Pareek, 2015).

Garner *et al.* (2017), in their research on 12 lactating Holstein-Friesian dairy cows for four days in climate-controlled chamber found that the mean respiration rate of the temperature-humidity-challenged cows was significantly higher on treatment days 1 ($p < 0.05$), 3 ($p < 0.05$) and 4 ($p < 0.10$) than that of thermo-neutral controls.

Wankar *et al.* (2020) reported that the respiratory rates of adult dry Murrah buffaloes kept at psychrometric chambers were significantly elevated during 35°C and 40°C as compared to 25°C ($p < 0.05$) and added that higher RR was observed at 40°C on days one and twenty-one (82.00 ± 3.46 & 58.00 ± 6.00).

Hou *et al.* (2021) compared the responses of dairy cows to short-term and long-term heat stress using a climate-controlled chamber and found a significantly increased RR in HS group (1.5-fold, $P < 0.05$) when compared to the cows in TNZ.

2.4.3 Heart Rate (HR)

Das *et al.* (2011) reported that the pulse rate was affected by HS and therefore it acted as a reliable indicator of livestock in harsh environmental conditions.

According to Pandey *et al.* (2017), heifers experienced an increased heart rate (tachycardia) during HS and this helped to maintain blood pressure as a response to the elevated vasodilation and increased blood flow during HS.

Wankar *et al.* (2020) found a significant increase in pulse rate at 40°C as compared to 25°C and 30°C ($p < 0.05$) in adult dry Murrah buffaloes kept at psychrometric chambers for analysing physio-metabolic responses.

Hou *et al.* (2021) evidenced a significantly increased ($p < 0.05$) heart rate of 5–7 beats per minute (bpm) in the heat-stressed group when compared to thermo-neutral control group in their experimentation on responses of dairy cows to short-term and long-term heat stress under controlled climatic conditions.

Yadav *et al.* (2021) found a non-significant increase in PR in crossbred cattle exposed to HS at 40°C (59 beats/min) than at 25°C (54 beats/min) and they reported that higher PR at 40°C might be due to higher secretion of catecholamines and higher expression of adrenergic receptors on cardiomyocytes.

Woo *et al.* (2024) experimented on the physiological responses of early-fattening Hanwoo steers to varying THI in a climate-controlled chamber and evidenced that the heart rate for the mild stress (THI 76 to 81) was in the range of 66 to 68 bpm, for the moderate stress (THI 82 to 84), it was in the range of 69 to 75 bpm and for the severe level (THI 85 to 99), it was above 75 bpm.

2.5 BEHAVIOURAL RESPONSES

Behavioural adaptation was recognised as the foremost and immediate adaptive response of cattle to reduce heat load (Shilja *et al.*, 2016).

2.5.1 Standing Time

Allen *et al.* (2015) reported that the duration of standing time was significantly increased in lactating dairy cows when their body temperature exceeded 38.6°C during exposure to HS.

An increase in standing time might render more evaporative and convective heat loss resulting in more heat elimination to the surroundings and minimised heat gain *via* conduction and radiation from hot ground (Kamal *et al.* 2016).

Kim *et al.* (2018) studied the correlation between haematological, physiological and behavioural responses of beef calves in a climate – controlled chamber and found a significantly increased standing time at THI of 74.22 to 87.72 (345 to 392 min/day) in comparison to THI of 70.01(236 min/day).

Kim *et al.* (2021) analysed the responses of beef calves to long-term heat exposure and documented that standing time was increased and lying time was decreased after exposure to HS (moderate and severe HS groups).

2.5.2 Lying Time

According to Ito *et al.* (2010), a typical dairy cow spent 11 to 14 h/day lying down in TNZ.

Goble (2012) reported that cows with lower core body temperature spent more time lying down than cows with higher core body temperature.

Lying down behaviour is an ideal indicator to ascertain dairy cattle health, welfare, reproduction and production performance (Solano *et al.*, 2016; Tullo *et al.*, 2019).

Kim *et al.* (2018) in their study on behavioural responses of beef calves using a climatic chamber found that the lying time was significantly decreased at THI of 74.22 to 87.72 ($p < 0.05$) compared to that at THI of 70.01.

According to Kim *et al.* (2018), as the severity of HS increased, cattle might have felt discomfort and prefer to stand for more heat dissipation *via* evaporation and convection and this might be the reason behind the gradual reduction in lying down duration with an increase in THI.

Tullo *et al.* (2019) reported that environmental variables such as high ambient temperature, relative humidity, solar radiation, wind velocity and rainfall affected the laying down behaviour of cattle.

2.5.3 Rumination Time

Soriani *et al.* (2013) reported that increased environmental temperature could reduce the rumination time of cattle and found that rumination was depressed during dehydration and heat stress.

According to Grant and Dann (2014), cattle preferred to ruminate while lying down rather than while standing and most of the rumination occurred at night and during the afternoon.

Moretti *et al.* (2017) analysed the impacts of heat stress on the rumination of Holstein dairy cows and found a strong correlation between THI and rumination time and they suggested that rumination could be a valuable parameter for evaluating the impact of heat stress on Holstein dairy cows.

Kim *et al.* (2018) reported that rumination time was decreased at high THI (87.72) compared to that at low THI (70.01) when beef calves were kept under controlled climatic conditions.

Park *et al.* (2019) investigated the effects of increase in THI on rumination time in lactating HF cows. They exposed cows to varying THI at 70–75 (T₁), 76–81 (T₂) and 82–87 (T₃) and evidenced a gradual reduction in rumination time from T₁ to T₃.

Antanaitis *et al.* (2024), in their study on the impacts of heat stress on rumination in fresh multiparous dairy cows found that the number of ruminations and rumination time (min/h) were decreased in cows exposed to high THI (>78) in comparison to moderate THI (<78).

2.5.4 Feed Intake and Feeding Time

Cook *et al.* (2007) found that there was no consistent reduction in total feeding time in lactating dairy cows with increasing THI.

Nonaka *et al.* (2008) reported that there was a reduction in dry matter intake (DMI) by nine per cent in Holstein Friesian heifers exposed to heat load at 33°C than at 28 or 20°C and that marked decrease in feed intake could be due to inhibition of appetite

centre located in hypothalamic arcuate and paraventricular nuclei which might relay a negative signal to lateral hypothalamic area.

Rhoads *et al.* (2013) reported that DMI was reduced by 40 per cent in lactating cows during exposure to 40°C ambient temperature.

Tripon *et al.* (2014), in their experiment on the effects of seasonal and climate variations on thermal comfort and behaviour of calves found that season had highly significant influence on feeding behaviour and feeding time was decreased in higher THI during summer.

Eslamizad *et al.* (2015) reported that early lactating Holstein cows when exposed to heat stress had significantly decreased feeding time ($p < 0.05$) compared to those in TNZ.

Min *et al.* (2015) reported higher DMI in cool lactating HF cows under low THI at 53.4 (24.45 kg/day) than under high THI at 81.7 (17.89 kg/day).

The important and well-documented behavioural response to enhanced heat load loss in ruminants was reduced feed intake and it was identified as an adaptive response to regulate internal metabolic heat production (Shilja *et al.*, 2016).

De Andrade Ferrazza *et al.* (2017) reported a significant reduction in DMI in heat-stressed cows (8.27 ± 0.33 kg/day) at 36.3°C in comparison with those cows under TNZ at 25.9°C (14.03 ± 0.29 kg/day).

According to Garner *et al.* (2017), cows altered their feeding pattern in response to varying THI. They documented that on the final day of the experimental period, cows exposed to high THI consumed a minimal per cent of their total daily DMI during the day and preferred to consume the majority during the cooler period at night.

Yadav *et al.* (2021) reported that in CB cattle, decline in DMI might lower the metabolic heat production to maintain thermal balance during HS and DMI was reported to be lowered during acute HS and revived during chronic HS.

2.5.5 Water Intake

West (2003) reported that water intake (WI) was elevated by 1.2 L/°C in dairy cattle during HS and that might be due to plasma hyperosmolarity resulted from evaporative heat loss via sweating.

Hamzaoui *et al.* (2013) found that water intake was highly significant in the heat-stressed goats when compared with the thermo-neutral goats (+5.50 L/d; $p < 0.001$) under controlled climatic conditions.

Yadav *et al.* (2016) evidenced a significantly increased water intake in crossbred cattle exposed to 40°C (25.01 L/day) than to 35°C (20.96 L/day), 30°C (16.65 L/day) and 25°C (16.48 L/day).

Kim *et al.* (2018) reported that beef calves when exposed to high THI in a climate-controlled chamber (THI>82) had a significantly increased water intake when compared to the calves exposed to comparatively lower THI.

Yadav *et al.* (2021) reported a significant increase in water intake in CB cattle subjected to heat stress at 40°C in contrast to TNZ at 25°C. Their study suggested that heat exposure at 40°C and 35°C would have caused excessive sweating which might have triggered the hypothalamic thirst centre which resulted in increased water intake. Another reason might be the direct activation of preoptic area and anterior hypothalamus.

Woo *et al.* (2024) evidenced a significantly increased water intake to varying THI in early-fattening Hanwoo steers kept in climate chamber.

2.6 BIOCHEMICAL RESPONSES

2.6.1. Antioxidant Status

Heat stress in cattle often lead to the production of reactive oxygen species (ROS) such as superoxide, peroxide, hydroxyl radical, singlet oxygen etc. Their accumulation due to overproduction or a decreased antioxidant defense mechanism lead to oxidative stress which damage the nucleic acids, proteins and lipid peroxidation of membranes and disruption of normal cell metabolism.

Antioxidant enzymes were synthesised in the body and protect from ROS generated during HS. Measuring these bio-markers played a significant role in evaluating the impact of high THI on oxidative stress levels of cows. The endogenous antioxidant capacity of the animal consisted of enzymatic antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). These were the first line of defense against reactive oxygen metabolites (ROM) such as superoxides and hydroperoxides. They scavenge both intracellular and extracellular superoxides and inhibit lipid peroxidation of plasma membranes (Zhang *et al.*, 2017).

2.6.1.1 Glutathione Peroxidase (GPx)

According to Sakatani *et al.* (2012), in Japanese black cows, activities of GPx and SOD were significantly decreased in summer (0.92 ± 0.04 mmol/L) compared to those in winter (1.49 ± 0.06 mmol/L).

Glutathione peroxidase (GPx), a selenium-dependent antioxidant enzyme converts hydrogen peroxide (H_2O_2) to water. The increased production of H_2O_2 might be due to increased activity of SOD during HS which results in a coordinated increase in activity of GSH-Px (Gupta *et al.*, 2013).

Lallawmkimi *et al.* (2013) experimented on the influence of season on the antioxidant status of calves, heifers and Murrah buffaloes. Their study evidenced a significantly elevated GSH-Px level in summer compared to winter in all the experimental groups.

Chaudhary *et al.* (2015) reported a significantly higher level of plasma malondialdehyde, SOD and GPx activities in Surti buffaloes during hot humid periods and hot dry periods indicating an increased free radical production during HS.

Chetia *et al.* (2017) reported that the mean GSH-Px concentration of Indian zebu cattle was significantly increased during hotter periods of the summer season (72.17 ± 0.75 unit/mg of protein) when compared to the winter season (44.48 ± 0.65 unit/mg of protein).

Rathwa *et al.* (2017) observed that plasma GPx levels in Indian indigenous sheep were significantly higher (1.29-fold; $p < 0.05$) during summer (THI = 82.55) when compared to winter (THI = 59.36).

Guo *et al.* (2018) studied the impacts of heat stress on blood amino acids profile in dairy cows and found a significantly decreased plasma GPx activity ($p < 0.05$) during heat stress when compared to the comfortable autumn period.

Maibam *et al.* (2018) analysed the effects of heat stress on the differential level of oxidative stress markers in the skin tissue of zebu and crossbred cattle and found a significantly increased level of GPx during summer ($p < 0.05$) compared to winter and spring seasons in both Tharparkar and Karan Fries.

Zeng *et al.* (2023) studied the effects of heat stress on the health status of dairy cows through blood oxygen availability and found that GPx concentration was significantly greater in the mild-HS group (55.9 U/mL; $p < 0.05$) in comparison to the no-HS group (40.9 U/mL).

2.6.1.2 Total Antioxidant Capacity (TAC)

Chaiyabutr *et al.* (2011) reported significantly higher total antioxidant status (TAS) values in a hot dry season in ruminants.

Turk *et al.* (2015), in their experiment on the impact of seasonal thermal stress on lipid mobilisation, antioxidant status and reproductive performance in dairy cows found that TAS value was significantly decreased in summer ($p < 0.001$) compared to winter.

Almoosavi *et al.* (2020) studied the impacts of late gestation heat stress in dairy cows and inferred that ferric reducing antioxidant power (FRAP) was highly significant ($p < 0.001$) in the heat-stressed cows with *ad-libitum* feed intake (2.12 mmol/L) in contrast to the cows under cooled conditions with *ad-libitum* feed intake (0.31 mmol/L).

Cecchini and Fazio (2020) assessed the TAC in hens and reported that ferric reducing ability of plasma (FRAP) was increased significantly at day seven and 14 in the stressed hens when compared to the control group ($p < 0.001$).

Tejaswi *et al.* (2020) experimented on impact of HS on antioxidant responses among native and crossbred cattle and found that TAC was significantly higher during summer ($p < 0.05$) in Tharparkar cattle followed by Sahiwal and crossbred cattle.

2.7 METABOLIC RESPONSES

Heat stress in cattle induces profound metabolic responses, including alterations in blood gas and electrolyte balance, often leading to respiratory alkalosis and dehydration. Blood glucose levels also can fluctuate, affecting energy metabolism and overall productivity.

Burhans *et al.* (2022) elucidated the underlying mechanisms of fatal conditions in dairy cattle to lethal HS. Their study suggested that blood electrolyte and acid-base imbalances arise due to sweating and hyperpnea for evaporative cooling mechanisms. This concurrently lowered the blood CO₂ levels, resulting in respiratory alkalosis.

2.7.1 Blood Gases

Hamzaoui *et al.* (2013) reported that milking goats kept in the climate controlled-chamber had significantly lower values of the partial pressure of CO₂ (pCO₂) and total CO₂ (THI = 77 – 85, $p < 0.01$) compared to those in thermo-neutral conditions (THI = 60 to 65). The significantly lower blood pCO₂ value during HS was attributed to hyperventilation of animals and the diminished ability of haemoglobin to bind with CO₂.

Garcia *et al.* (2015) investigated the metabolic responses of dairy cattle of Southern Brazil to varying THI (low, moderate and severe) and found that severe heat-stressed cows had lower blood pH than the control group. They also evidenced a significantly higher pO₂ values in heat stressed group. Their study also revealed that cows in comfortable THI showed higher blood bicarbonate and total CO₂ values than heat-stressed group. The significantly increased blood pO₂ values during HS was attributed to alveolar ventilation.

Pandey *et al.* (2017) analysed the impact of HS and hypercapnia in Tharparkar and Karan Fries cattle and evidenced that the values of blood pH and pO₂ were significantly increased with an increase in temperature and CO₂ exposure levels in both

the breeds. They also reported that $p\text{CO}_2$ significantly decreased with the increase in temperature and CO_2 levels in both the breeds.

Al-Qaisi *et al.* (2020) reported that HS lowered the $p\text{CO}_2$ and total CO_2 values in Holstein cows.

Joy *et al.* (2020) assessed the thermotolerance in lambs and found a significantly higher ($p < 0.01$) blood pH and significantly lower ($p < 0.01$) $p\text{CO}_2$ under HS at 40°C .

Yamin *et al.* (2022) analysed the biochemical responses of native and crossbred goats to summer stress and evidenced that the values of $p\text{O}_2$ increased and $p\text{CO}_2$ decreased when exposed to a high THI of 89.65.

Zeng *et al.* (2023) experimented with the impacts of heat stress on the health status of dairy cows through blood oxygen availability by categorising into mild (THI: <68), moderate (THI: 68 to 79) and severe (THI: 80 to 88) HS groups. The blood gas profile of their study revealed a significantly lower total CO_2 and pH ($p < 0.05$) in the moderate HS group when compared to the no HS group. Their study found no significant differences in $p\text{O}_2$ among different HS conditions.

2.7.2 Blood Electrolytes and Blood pH

West *et al.* (1999) reported that heat-stressed cows had compensated for higher blood pH due to respiratory alkalosis by increasing the urinary bicarbonate (HCO_3^-) excretion. They opined that acid-base status of heat-stressed cattle was altered when compared to normothermic cows.

According to Sivakumar *et al.* (2010), when goats were exposed to higher THI (40°C temperature and 30 per cent RH), blood HCO_3^- levels tremendously decreased. This was attributed to hyperventilation which increased the elimination of CO_2 dissociated from carbonic acid (H_2CO_3), which was formed by the combination of hydrogen ion and bicarbonate ion.

Hamzaoui *et al.* (2013) found that bicarbonate (HCO_3^-) level was significantly lower ($p < 0.001$) in the heat-stressed goats (37°C and 40 per cent RH) when compared with the thermo-neutral goats (15 to 20°C and 45 per cent RH) under controlled climatic

conditions. They also suggested that greater respiration rate and panting in heat-stressed goats contributed to significant loss of CO₂, diminishing the carbonic acid content of the blood. This consequently resulted in the transfer of HCO₃⁻ from the blood to urine *via* the renal pathway to maintain a constant blood pH. Their study also evidenced that HS had no effect on blood pH and blood electrolytes such as Na⁺ and K⁺ concentrations.

Wankar *et al.* (2014), in their study on thermoregulatory and adaptive responses of adult buffaloes during hyperthermia in an environmentally controlled chamber found a significantly increased value of Na⁺ at 40°C (139.51 mmol/L; p<0.05) than at 30°C (120.78 mmol/L). They also found no variation (p>0.05) in K⁺ and Cl⁻ ion values at different temperature exposures during the entire experimental period.

Chetia *et al.* (2016) studied the physiological and biochemical responses of crossbred cattle under the agroclimatic condition of Assam and found that serum electrolyte levels such as Na⁺, K⁺ and Cl⁻ were decreased in summer and significantly higher (p<0.01) in winter. They suggested that this might be due to acclimation and adaptation of crossbred cattle to heat stress.

Berian *et al.* (2019), in their study on the impact of heat stress on haemato-biochemical profile of cross bred dairy cattle found a significantly decreased (p<0.05) blood pH value in heat-stressed cattle when compared to the control group.

Wankar *et al.* (2020) reported that serum Na⁺ levels did not vary significantly at 25°C (p >0.05), but lowered significantly at 30°C as compared to 35°C and 40°C in adult dry Murrah buffaloes kept at climate – controlled chamber. They also reported a significantly higher (p<0.05) values for K⁺ at 40°C than at 25°C.

Meneses *et al.* (2021) studied the impacts of heat stress on Nellore cattle and found that blood HCO₃⁻ and blood pH levels were significantly decreased (p<0.05) when cattle were exposed to elevated environmental conditions.

Yamin *et al.* (2022) studied the biochemical responses of native and crossbred goats to summer stress and found a significantly increased K⁺ value at a THI of 89.65. They also reported a significantly decreased Na⁺, HCO₃⁻ and blood pH at that higher THI. They suggested that hyponatremia might be due to sodium loss *via* sweating, HCO₃⁻

loss due to hyperventilation and hyperkalaemia due to defects in the functioning of the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ pump during HS.

Zeng *et al.* (2023), in their study on the effects of heat stress on the health status of dairy cows through blood oxygen availability and found that blood pH was significantly decreased ($p < 0.05$) in the moderate-HS group when compared to the mild and no-HS group.

Antanaitis *et al.* (2024) experimented on the impacts of heat stress on acid-base balance in fresh multiparous dairy cows by classifying into low ($\text{THI} < 72$), medium (THI 72 to 78) and high HS ($\text{THI} > 78$) groups. In their study, they documented that there were no significant differences in blood pH and blood electrolyte levels of Na^+ , K^+ , HCO_3^- and Cl^- between different treatment groups of heat stress.

2.7.3 Blood Glucose

According to Baumgard and Rhoads (2013), blood glucose was significantly decreased in response to heat stress in dairy cows which might be attributed to glucose utilisation due to declined feed intake during HS.

Hamzaoui *et al.* (2013) in their study on physiological responses of late-lactation dairy goats to heat stress under controlled climatic conditions reported that heat stress did not affect blood glucose. They also found that at 8 a.m. heat stressed goats had a significantly lower glucose concentration than goats in TNZ ($p < 0.05$).

Garner *et al.* (2017) experimented on 12 lactating Holstein-Friesian dairy cows for four days in the climate-controlled chamber under moderate heat stress (THI between 74 and 84) and thermoneutral conditions (THI between 55 and 61) and reported that there was no significant difference in blood glucose values between heat stressed (4.49 mmol/L) and thermoneutral (4.29 mmol/L) groups.

Kim *et al.* (2018) in their study on the correlation between haematological, physiological and behavioural responses of beef calves in a climate – controlled chamber found a significantly ($p < 0.05$) decreased serum glucose level at higher THI of 82.92 (56.8 mg/dL) compared to that of 70.01 to 76.51. Their study also inferred that blood glucose level decreased with increasing THI .

Salama *et al.* (2021) in their study on metabolic responses of lactating goats to heat stress under controlled climatic conditions reported that there was no significant difference in blood glucose values of thermo-neutral (58.6 mg/dL) and heat-stressed (58.1 mg/dL) goats.

Woo *et al.* (2024) studied the physiological responses of early-fattening Hanwoo steers to varying THI in a climate-controlled chamber and evidenced significant differences ($p < 0.05$) in serum glucose levels of various treatment groups. They reported that it was the lowest in the severe THI (59.15 mg/dL) group and lower in the moderate THI (63.29 mg/dL) group when compared to the comfort THI (69.59 mg/dL) group. They also documented a decreased serum glucose concentration at a high THI of 83 (56.8 mg/dL) in contrast to low THI of 70 (73.5 mg/dL).

2.8 ENDOCRINE RESPONSES

The hypothalamo–pituitary–adrenal axis (HPA axis) acts as one of the principal endocrine regulators of stress responses. The activation of the HPA axis as a response to stress might lead to enhanced production and release of cortisol into circulation which is associated with immune suppression in cattle. Hence serum cortisol is a reliable and primary physiological index for determining stress response in animals (Binsiya *et al.*, 2017).

2.8.1 Cortisol

Ju *et al.* (2014) reported that cortisol secretion during acute stress acted as a stimulus for the immune system and its secretion during chronic stress was associated with immune suppression.

Yadav *et al.* (2015) concluded that the rise in serum cortisol concentrations was temperature-dependent after exposing crossbred cattle to different temperature levels.

Zarina (2016) in their study on the adaptability profile of male CB cattle and buffalo calves to varying THI in Kerala documented that there was no significant correlation between serum cortisol levels and THI.

Bharati *et al.* (2017) found that serum cortisol concentration was significantly elevated ($p < 0.05$) during short-term heat stress acclimation and it was gradually decreased during long-term heat stress acclimation (LTHSA) in Tharparkar cattle. The decline in cortisol levels during LHTSA was attributed to negative glucocorticoid feedback to HPA axis as an adaptation to prolonged HS in cattle.

Garner *et al.* (2017), in their experiment on 12 lactating Holstein-Friesian dairy cows for four days in climate-controlled chamber found that there was no significant difference in mean cortisol value between thermoneutral (3.7 ng/mL) and temperature challenged (3.2 ng/mL) animals.

Kim *et al.* (2018) reported a significantly higher serum cortisol level in Korean native calves exposed to THI of 87.72 (17.1 ng/mL) than those exposed to THI of 76.51 (4.6 ng/mL).

Tejaswi *et al.* (2020) studied the impact of summer HS on endocrine responses among native and crossbred cattle and found a significantly higher ($p < 0.05$) concentration of serum cortisol in crossbred cattle when compared to native breeds.

Yadav *et al.* (2021) evidenced a significantly higher serum cortisol level in crossbred cattle on day one of heat exposure at 40°C (32 nM/mL) than TNZ at 25°C (15.5 nM/mL), followed by a gradual dip of cortisol level from 6th day upto 21st day with a significant dip on 16th day of heat exposure at 40°C. They opined that the initial peak in cortisol level might be attributed to sudden activation of the HPA axis and gradual reduction from the 6th day to the 21st day followed by a significant reduction on the 16th day might be due to the cortisol acclimation pathway in heat-stressed crossbred cattle.

Woo *et al.* (2024) in their study on the physiological responses of early-fattening Hanwoo steers to varying THI in a climate-controlled chamber reported a significantly increased ($p < 0.05$) serum cortisol values with an increase in THI. Based on their study, serum cortisol values of comfort, mild, moderate, and severe stress groups were 11.96, 13.51, 15.57, and 17.17 ng/mL respectively.

2.9 MOLECULAR RESPONSES

Molecular responses serve as the most dominant adaptive response to HS in cattle. Collier *et al.* (2012) stated that those genes having a cellular adaptation function are considered as potential biomarkers for understanding adaptation mechanisms to heat stress. Gupta *et al.* (2013) reported that there were evident changes in the expression patterns of certain genes such as heat shock proteins (HSPs), NOD-like receptors (NLRs) and Toll-like receptors (TLRs) that are fundamental for thermo-tolerance at cellular level in livestock.

According to Hu *et al.* (2016), HS elicited a complex array of cellular and molecular responses in livestock. They also stated that HS altered several molecular mechanisms such as DNA synthesis, replication and repair, cellular division and functions of DNA polymerases.

2.9.1 Heat Shock Proteins

The first line of molecular defense against thermal stress in all mammals is the synthesis of heat shock proteins (HSPs). Borges *et al.* (2012) opined that these proteins exerted a protective role in cells by safeguarding cellular integrity, immune response, apoptosis and contributing to post-translational folding and transportation of proteins through cellular membranes. These proteins act as “molecular-chaperones” which facilitate the assembly and translocation of newly synthesized proteins within the cell and the repair and refolding of damaged proteins during HS (Sejian *et al.*, 2019).

Heat shock proteins were classified according to their molecular weight into small HSPs (<40 kDa) and large HSPs (>40 kDa). Under HS conditions, the mRNA expression of both small and large HSPs was significantly upregulated (Kim *et al.*, 2020). According to Shandilya *et al.* (2020) and Mishra (2021), almost all the cellular systems express heat shock proteins as cellular adaptations to heat stress.

Mishra (2021) reported that tremendous expression of HSPs was evidenced in peripheral blood mononuclear cells (PBMCs). Hence these cells were considered as the standard model to evaluate the differential expression of various HSPs during HS.

2.9.1.1 Heat Shock Protein-70 gene

Guerriero and Raynes (1990) found that *HSP-70* transcripts were expressed in cattle lymphocytes after several minutes of heat stress exposure.

Muhammed (2014) conducted thermotolerance and gene expression studies in different cattle breeds of Kerala and found that crossbred cattle showed significantly increased fold ($p \leq 0.05$) in *HSP-70* expression to increased heat exposures.

Mishra *et al.* (2011) studied the effects of induced heat stress on *HSP-70* in buffalo lymphocytes and found a 200-fold increase in serum *HSP-70* levels.

Dangi *et al.* (2012) analysed the impacts of seasonal variation on *HSP* expression in caprine PBMCs and found a non – significant elevation of *HSP-70* expression during peak summer when compared to peak winter.

Kishore *et al.* (2014) conducted comparative analysis of the changes in the gene expression profile of *HSP* genes (*HSP-40*, *HSP-60*, *HSP-70* and *HSP-90*) using quantitative PCR (qPCR) in heat-stressed PBMCs of Sahiwal cattle, HF cattle and Murrah buffalo, post 1-hour sublethal heat stress at 42°C in culture and found that *HSP-70* was the most responsive gene and a potential bio-marker for quantifying the heat stress response compared to other *HSP* chaperones. They also opined that the PBMC system is a potential model for elucidating the differential heat shock responses in cattle and buffaloes.

Manjari *et al.* (2015) found that the relative expression values of *HSP-70* in Tarai buffalo were significantly higher ($p < 0.05$) in summer (2.37 ± 0.12) compared to winter (0.29 ± 0.04). They also inferred that the expression was positively correlated with changes in physiological variables such as respiratory rate (RR), pulse rate (PR) and rectal temperature (RT).

Bhanuprakash *et al.* (2016) documented higher expression of *HSP-70* mRNA in PBMCs of Sahiwal cattle under heat stress (44°C) as well as cold stress (10 °C).

Hu *et al.* (2016) analysed the effect of heat stress on gene expression and synthesis of heat-shock and milk proteins in bovine mammary epithelial cells and reported that

HSP-70 transcript was gradually increased up to 5 h and then decreased at 12 h of heat exposure at 42° C.

Archana *et al.* (2017) reported that intracellular HSPs like *HSP-70* and *HSP-90* had their role in antigen processing and presentation despite their functions as molecular chaperones. They also inferred that among various HSPs studied, *HSP-70* was the ideal biological marker for thermo-tolerance in farm animals.

Kim *et al.* (2020) conducted an *in vivo* experiment on *HSP* gene expression in PBMCs to heat stress in beef calves in a climate-controlled chamber and found that the *HSP-70* gene expression was significantly increased ($p < 0.05$) after sudden exposure to HS (severe THI of 88 to 90). They also opined that *HSP-70* served an important role in cellular protection by inhibiting caspase-3 activity in response to acute HS.

2.9.2 Insulin like Growth Factor 1 Gene

Insulin-like growth factor 1 (*IGF-1*), also called Somatomedin, is a hormone similar in molecular structure to insulin but has a much higher growth-promoting activity. The *IGF-1* gene in cattle is located on chromosome five and is expressed in various tissues including the liver, muscle and mammary gland, influencing growth traits and milk production. Heat stress can reduce *IGF-1* expression in cattle, which can subsequently affect growth and lactation performance.

Satrapa *et al.* (2013) studied the differential expressions of the *IGF* gene family in heat stressed embryos produced *in-vitro* from ovum pick-up derived oocytes of Nellore and Holstein cows and reported a significantly higher ($p < 0.05$) mRNA expression of *IGF-1* in pools of blastocysts under HS (41°C) when compared to control (39°C) in both Nellore and Holstein cows. This greater bioavailability of *IGF-1* was attributed to improved oocyte quality and embryo development.

Pragna *et al.* (2018) studied the impact of summer heat stress on growth performance of three different indigenous goat breeds and found a significantly ($p < 0.05$) lower expression of *IGF-1* mRNA in PBMCs of Osmanabadi goats exposed to HS when compared to control goats. Their study also opined that plasma growth hormone (GH)

level and *IGF-1* gene could be considered as ideal biomarkers for assessing the impact of HS on growth performance.

Fabris *et al.* (2020) experimented on the effect of HS during the early and late dry period on mammary gland development of Holstein cows and evidenced that cows exposed to HS had downregulation of *IGF-1* ($p \leq 0.10$) when compared to cows provided with cooling.

Kim and Kim (2023) analysed the impact of heat stress on skeletal muscle hypertrophy in bovine myocytes and found that extreme heat stress of 41°C upregulated ($p < 0.01$) the expression of *IGF-1* when compared to the control group (38°C).

Shashank *et al.* (2023) conducted a comparative assessment of growth performance of indigenous and cross-bred calves subjected to combined stressors and found that hepatic mRNA expression of *IGF-1* and growth hormone receptor significantly ($p < 0.05$) varied between control and combined stressor groups in both the breeds.

2.9.3 *GAPDH* – A House Keeping Gene

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene is commonly chosen as reference or house-keeping gene for accurate qRT-PCR analysis (Parmar *et al.*, 2015). This gene is consistently expressed at relatively stable levels across different tissues and varying experimental conditions. This stability made it a reliable reference point for normalizing the expression levels of other genes of interest.

The *GAPDH* gene, owing to its stability and less altered by external factors was widely used by many researchers as the house-keeping gene for analysing the expression of HSPs in response to HS (Manjari *et al.*, 2015; Parmar *et al.*, 2015; Pires *et al.*, 2019; Kaushik *et al.*, 2022).

Materials and *Methods*

3. MATERIALS AND METHODS

3.1 LOCATION OF STUDY

The research work was conducted at Climate Controlled Research Complex (CCRC) of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS) of Kerala Veterinary and Animal Sciences University. The analysis part of the research was conducted at Department of Veterinary Physiology, Central Instruments Laboratory and School of Applied Animal Production and Biotechnology (SAAPBT) of College of Veterinary and Animal Sciences, Mannuthy.

The climate-controlled animal chamber of CCRC was 600 feet wide and 1200 feet long (concreted floor) provided with automatic waterer facilities for six large animals. The chamber temperature and humidity could be adjusted in a climate control panel according to the research protocol. Evaporators (air-conditioners) were two in numbers inside the chamber. The chamber was also provided with various sensor probes which could sense the ammonia, methane and carbon- dioxide levels. Dehumidifier would be automatically turned on if the humidity exceeded the critical level. Exhaust fans were provided for proper ventilation inside the chamber. The chamber was provided with continuous closed-circuit television (CCTV) surveillance for instantaneous monitoring of all the experimental animals.

3.2 EXPERIMENTAL LAYOUT

The experiment was conducted following the approval of Committee for the Control and Supervision of Experimentation on Animals (CCSEA), New Delhi. Six apparently healthy crossbred (CB) female calves of 8 to 12 months of age were selected randomly from University Livestock Farm and Fodder Research Development Scheme (ULF and FRDS), Mannuthy. All the animals were maintained as per standard feeding and management practices followed at the farm. The research consisted of two experimental groups: thermo-neutral zone (TNZ) or Control group and acute heat stress or Treatment group. Same six animals were used for experimentation in both the groups. The experiment in the CAADECCS involved the following phases:

- Acclimatisation and gentling period of 7 days in a shed near the chamber
- Acclimatisation period of 10 days in comfort chamber

- Experimental period of 10 days in ‘comfort chamber’ (Control)
- Experimental period of 10 days in ‘climatic chamber’ (Treatment) for acute heat stress study

Table 3.1: Experimental layout

Groups	Treatments	Number of animals
THERMO-NEUTRAL ZONE (CONTROL)	Animals housed under thermo-neutral zone, in comfort chamber	6
ACUTE HEAT STRESS (TREATMENT)	Animals kept at climatic chamber for acute HS study	6

3.3 CALCULATION OF TEMPERATURE HUMIDITY INDEX

Temperature Humidity Index (THI) for the experiment in climate – controlled chamber was fixed based on the microclimatic data obtained from CAADECCS. It was calculated based on the formula of THI given by Mader *et al.* (2006).

$$THI = (0.8 \times T_{db}) + [(RH/100) \times (T_{db} - 14.4)] + 46.4$$

Where, RH = Relative Humidity (%), T_{db} = Dry bulb temperature (°C)

3.4 ACCLIMATISATION PERIOD FOR ANIMALS

Acclimatisation and gentling period of 10 days was given for the experimental animals in a shed near the CCRC once they were taken from ULF&FRDS, Mannuthy to CAADECCS. This was followed by acclimatisation period inside the comfort chamber for 3 hours per day for 10 days to make the animals get acclimatised to the chamber conditions.

3.5 EXPERIMENTAL PERIOD IN CLIMATE – CONTROLLED CHAMBER

3.5.1 Comfort Chamber Study

The experimental animals were housed under thermo-neutral zone (TNZ) in comfort chamber for a period of 10 days from 6 a.m. to 6 p.m. maintained at a temperature of 27°C and relative humidity of 45 – 55 per cent after the acclimatisation period.

3.5.2 Climatic Chamber Study

After experimentation under TNZ, the next day same animals were shifted to the climatic chamber for acute HS study. Maximum temperature of 40°C and relative humidity of 55-65 per cent was simulated for three hours a day for 10 days. In the climatic chamber, HS was applied as follows:

Table 3.2: Fixing of THI in the climatic chamber for acute heat stress study

Time	Temperature (°C)	Relative Humidity (%)	THI
6 a.m.	25	55 to 65	72.23 to 73.29
7 a.m.	28	55 to 65	76.28 to 77.64
8 a.m.	31	55 to 65	80.33 to 81.99
9 a.m.	34	55 to 65	84.38 to 86.34
10 a.m.	37	55 to 65	88.43 to 90.69
11 a.m.	40	55 to 65	92.48 to 95.04
2.15 p.m.	37	55 to 65	88.43 to 90.69
3 p.m.	34	55 to 65	84.38 to 86.34
4 p.m.	31	55 to 65	80.33 to 81.99
5 p.m.	28	55 to 65	76.28 to 77.64
6 p.m.	25	55 to 65	72.23 to 73.29

Every day at 6 a.m. the animals were taken from the shed into the climatic chamber maintained at 25°C. The temperature inside the climatic chamber was increased @ 3°C/h from 6 a.m. to 11 a.m. and the animals were subjected to a maximum temperature of 40°C from 11 a.m. to 2 p.m. The temperature was again reduced @ 3°C/h from 2.15 p.m. to 6 p.m. The relative humidity within the chamber was maintained at 55

to 65 per cent throughout the study period. The animals were taken out at 6 p.m. and kept in the shed located near the climatic chamber.

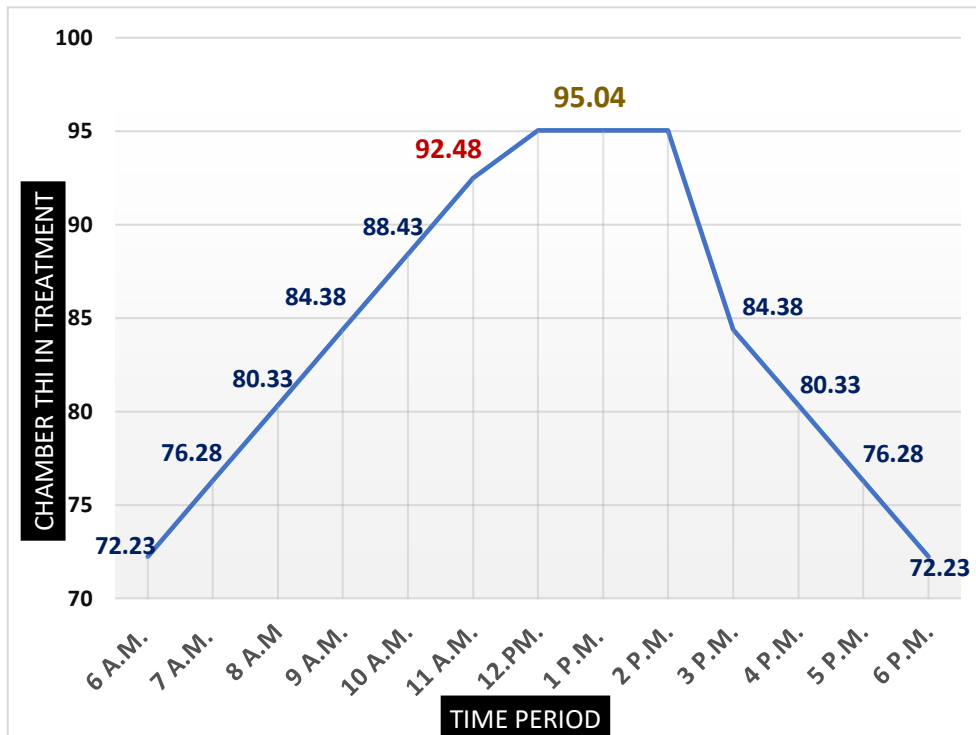


Fig. 3.1. Fixing of THI in climatic chamber for acute heat stress study.

3.6 RECORDING OF PHYSIOLOGICAL PARAMETERS

The physiological parameters of the experimental animals were measured on all alternate days at 8.00 a.m., 11.00 a.m., 2.00 p.m. and 5.00 p.m. during the entire period of study.

3.6.1 Rectal Temperature (RT)

Rectal temperatures were recorded at 8.00 a.m., 11.00 a.m., 2.00 p.m. and 5.00 p.m. by using a clinical thermometer by inserting into the rectum for one minute.

3.6.2 Respiratory Rate (RR)

Respiratory rates were measured at 8.00 a.m., 11.00 a.m., 2.00 p.m. and 5.00 p.m. by counting the flank movements for one minute, from a distance of 2 m without disturbing the animals.

3.6.3 Heart Rate (HR)

Heart rates were recorded at 8.00 a.m., 11.00 a.m., 2.00 p.m. and 5.00 p.m. by using stethoscope.

3.7 COLLECTION OF BLOOD SAMPLES

Blood samples (5 mL) were collected in both the experimental groups on days zero, one, five and 10 of the experiment into clot activator vacutainers between 1 p.m. and 2 p.m. through jugular venipuncture. Blood clotting was ascertained after one hour of incubation at a controlled temperature of 5°C to ensure optimal serum preparation and were subjected for centrifugation at 2700×g at 4°C for 15 minutes. Sera were separated in Eppendorf tubes and were stored at –20°C for cortisol estimation. Blood samples were also collected in lithium heparin coated vacutainers and aliquoted into two. One aliquot was used for instantaneous estimation of blood gases and electrolytes analysis and for the preparation of haemolysate to assess GPx activity. Another aliquot was centrifuged at 2700×g at 4 °C for 15 minutes for separation of plasma. Plasma samples were stored at –80°C for assessment of total antioxidant capacity. On final day of the experiment in both the groups, whole blood samples were drawn into 15 mL centrifuge tubes containing ethylene diamine tetra acetic acid salt (EDTA) at dose rate of 1.5mg/mL of blood for gene expression studies.

3.8 BLOOD GASES, ELECTROLYTES AND BLOOD GLUCOSE ANALYSIS

Venous blood samples were collected in heparin containing vials for analysis of blood gases (pO₂ and pCO₂), electrolytes (Na⁺, K⁺, HCO₃⁻) and blood glucose level using EPOC blood gas analyser (Epocal Inc., Ottawa, ON Canada).

Principle

The [®]Epoc system consists of test cards containing the sensors, a wireless card reader, and a personal data assistant (PDA) for data analysis. The test cards use selective electrode potentiometry to measure pH, pCO₂, sodium, potassium, and ionised calcium. Glucose, pO₂, and lactate level were measured amperometrically. Hematocrit is determined conductometrically. Bicarbonate, total carbon dioxide, base excess, oxygen saturation and haemoglobin are calculated. The [®]Epoc card reader is a battery-powered portable device with a barcode scanner. Insertion of a test card into the reader releases

calibration fluid over the sensors. After completion of a 190 s calibration cycle, the user has seven min to apply a patient sample to the card. The reader then sends the sensor data via Bluetooth to the PDA. The PDA is a handheld computer that calculates analytical values from the data sent by the reader.

Procedure

After proper calibration with the new [®]Epoc card for 190 s, held the syringe barrel vertically between fingertips and thumb. Then by using a slight downward pressure, secured the syringe's luer tip into the center recess of the blood sample entry port of the Test Card. Rotated the syringe up to 1/4 turn to ensure a good seal. While maintaining downward pressure, used index finger of the other hand to steadily depress the syringe plunger with a single, smooth, continuous motion until prompted to stop. After some seconds of analysis, the result was displayed in the screen.

3.9 ENDOCRINE PARAMETER

3.9.1 Serum Cortisol

Cortisol levels were estimated in serum by using Cortisol C. T. Radio immuno assay (RIA) kit (M/s Beckman Coulter, United States) supplied by Anand Brothers, Delhi, India, using gamma counter (Perkin Elmer model Wizard).

Principle

Serum, the control and calibrators are incubated in monoclonal antibody-coated tubes with ¹²⁵I-labeled cortisol tracer. After incubation, the liquid contents of the tube are aspirated and bound radioactivity is measured. A calibration curve is established and unknown values are determined by interpolation from the standard curve.

Reagents

Anti-cortisol monoclonal antibody-coated tubes (ready to use)

¹²⁵I-labeled cortisol tracer (ready to use)

Calibrators (0, 20, 70, 200, 700 and 2000 nM/L) (ready to use)

Procedure

Assay procedure is summarized in Table 3.3.

Table 3.3: Assay procedure for cortisol estimation

Steps	Calibrators	Serum	Tubes for total count (T)
1. Additions To antibody-coated tubes, added successively			
- Calibrators	50 μ L	-	-
- Serum samples	-	50 μ L	-
- Tracer	500 μ L	500 μ L	500 μ L
2. Incubation One hour at 18-25°C with shaking (400 rpm)			
3. Count Aspirated carefully contents of tubes except two tubes for total count.			

The tubes were placed in the gamma counter for counting radiations.

Calculation of results

Results were obtained from standard curve by interpolation. The results were calculated using a semi-logarithmic curve fit with B (bound) / T (total %) on vertical axis and the cortisol concentration of the calibrators on the horizontal axis (nM/L). The sample values were directly read from the standard curve as nM/L and converted into ng/mL (1 nM/L = 0.3627 ng/mL).

3.10 ANTIOXIDANTS ESTIMATION

3.10.1 Estimation of Glutathione Peroxidase Activity (Gpx)

Glutathione peroxidase activity was determined by Perkin Elmer Lambda UV – VIS spectrophotometer using glutathione peroxidase (GSH-Px/GPX) activity assay kit of Origin supplied by Origin Diagnostics and research Kerala.

3.10.1.1 Principle

Glutathione peroxidase (GSH-Px) can promote the reaction of hydrogen peroxide (H_2O_2) to reduced glutathione to produce H_2O and oxidised glutathione (GSSG). The activity of glutathione can be calculated by measuring the consumption of reduced glutathione. Hydrogen peroxide (H_2O_2) and reduced glutathione can react without catalysis of GSH-PX, so the portion of GSH reduction by non-enzymatic reaction should be subtracted. GSH can react with dinitrobenzoic acid to produce 5-thio-dirutrobenzoic acid anion, which showed a stable yellow colour. Measure the absorbance at 412 nm. Calculate the amount of GSH.

Table 3.4: Kit components of GPx kit

Reagent	Volume	Storage
Extract Solution	60mL × 1	4°C
Reagent I	Powder × 2	4°C
Added 3.3 mL of diluent to dissolve the contents before use.		
Reagent II	10μL × 1	4°C
Mixed 2μl Reagent II and 10 mL distilled water before use. Prepared in a micro centrifuge tube.		
Reagent III	30mL × 1	4°C
If crystals are found in the bottom of the bottle, warm in a 50 °C water bath till it dissolves. If crystals persist after heating, the supernatant can be used for the assay.		
Reagent IV	15mL × 1	4°C
Reagent V	5mL × 1	4°C
Standard (10mg reduced glutathione)	Powder × 1	4°C
Added 0.405mL diluent to get a standard concentration of 80 μmol/mL.		
Diluent	20mL	4°C

3.10.1.2 Protocol

Haemolysate samples were prepared and used for the assay.

3.10.1.3 Preparation of Haemolysate

1. Blood sample was collected using lithium heparin coated vacutainer.
2. RBC was spin down by centrifugation at 3000 rpm for 10 min at 4°C.
3. Plasma was removed from the cells by drawing it off from the top.
4. Buffy coat (the white interface between the pelleted RBCs and the plasma) was removed and discarded.
5. RBC pellet was washed with saline at 4°C and centrifuged at 3000 rpm for 10 min at 4°C. The clear saline from top was discarded and repeated once.
6. To an aliquot of RBC, an equal volume of deionised water was added and vortexed well for complete lysis of the cells.
7. Estimation of hemoglobin concentration of the 1:2 diluted RBC hemolysate was performed using Drabkin's solution (Agappe). Unit was converted from g/dl to g/L.
8. Hemolysate sample aliquots were stored at < -65°C freezer until analysis.
9. Frozen samples were thawed out and vortexed well before analysis.
10. Thawed out red blood cell lysate was diluted in deionised water to 6-7 g/L of hemoglobin. Mixed well and left the diluted samples on ice before analysis.

3.10.1.4 Assay Procedure

1. Preheated the spectrophotometer for 30 min Adjusted the wavelength to 412 nm and set zero with distilled water.
2. Diluted the standard solution (80 μL mol/mL) to 0.08 $\mu\text{mol/mL}$ with diluent.
3. Performed the subsequent assay in a 1.5 mL microcentrifuge tube. Added the reagents in the order as mentioned in the Table 4.2.

Table 3.4.1: GPx assay procedure 1

Reagent	Test tube (T) (μL)	Control tube (C) (μL)
Sample (Haemolysate)	20 μL	-
Reagent I	20 μL	20 μL
Incubated for 5 min at 37°C		
Reagent II	10 μL	10 μL
Incubated for 5 min at 37 °C		

Reagent III	200µL	200µL
Sample (Haemolysate)	-	20µL

4. Centrifuged at 4000 rpm at room temperature for 5 min and transferred the supernatant into a new 1.5 mL microcentrifuge tube or 96 well plate.
5. Continued the assay as given in the Table 4.3.

Table 3.4.2: GPx assay procedure 2

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

6. Mixed well and placed at room temperature for 15 min and the absorbance at 412 nm was measured.

The absorbance was recorded as A_T , A_C , A_S and A_B respectively.

$$\Delta A_T = A_C - A_T$$

$$\Delta A_S = A_S - A_B$$

3.10.1.5 Calculation

Liquids (Haemolysate/Serum/Plasma) unit definition: One unit of enzyme is defined as the amount of enzyme which catalyzes the oxidation of 1nmol of GSH in the reaction system per minute for every milliliters of liquid.

$$\text{GPx (U/mL)} = \Delta A_T \div (\Delta A_S \div C_S) \times 1000 \times V_{EV} \div V_S \div T = 200 \times \Delta A_T \div \Delta A_S$$

$$\text{Hence, GPx (U/mL)} = 200 \times \Delta A_T \div \Delta A_S$$

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

$$\text{Actual value} = \text{GPx (U/mL)} \times 10$$

$$\text{GPx (U/L of Haemolysate)} = \frac{\text{Actual value}}{100}$$

3.10.2 Estimation of Total Antioxidant Capacity

Total antioxidant capacity (TAC) was measured by using Ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996).

Principle

At low pH, reduction of ferric tripyridyl triazine ($\text{Fe}^{3+}\text{TPTZ}$) complex to the ferrous form occurs, which has an intense blue colour. This colour can be monitored by measuring the change in absorbance at 593 nm. The reaction is non-specific, in that any half reaction that has a lower redox potential, under reaction conditions, than that of the ferric or ferrous half reaction will drive the ferric (Fe^{3+}) to ferrous (Fe^{2+}) reaction. The change in absorbance therefore, is directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture.

Reagents

1. Acetate buffer 300mM pH 3.6: Weigh 3.1 g sodium acetate trihydrate and add 16 mL glacial acetic acid and make volume to 1 L with distilled water.
2. TPTZ (2, 4, 6 - tripyridyl-s-triazine) (M.W.312.34) 10 mM in 40mM HCl (M. W.36.46)
3. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (M. W. 270.30) 20 mM: The working FRAP reagent was prepared by mixing 1, 2, 3 in the ratio 10:1:1 at the time of use.
4. Standard: Ascorbic acid (M. W.176.13) 1000 μM

Procedure

Three mL of working FRAP reagent was mixed with 100 μL test sample or standard in a test tube. Then it was mixed and absorbance at 593 nm was measured against a reagent blank for 5 min in Perkin Elmer Lambda 25 UV-Vis Spectrophotometer. The

change in absorbance was translated into FRAP value (μM) by relating ΔA_{593} of test sample with that of standard solution of known FRAP value using formula.

Calculation

$$\frac{0 - 5 \text{ min } \Delta A_{593} \text{ test sample}}{0 - 5 \text{ min } \Delta A_{593} \text{ standard}} \times \text{FRAP value of standard}$$

FRAP value of 1000 μM ascorbic acid (standard) is 2000 $\mu\text{moles/L}$.

3.11 BEHAVIOURAL STUDY

3.11.1 Recording of Feed Intake

The feed intake of the individual animals including roughage (15kg/animal/day) and concentrate (2.25kg/animal/day) was measured by weighing the left-over feed using a weighing machine.

$$\text{Feed intake (kg/day)} = \text{Total feed given per day} - \text{Left over feed}$$

3.11.2 CCTV Camera Surveillance

The major behavioural responses of the crossbred calves under thermo-neutral and acute heat stressed conditions in the climate-controlled chamber was monitored in the entire study period through closed circuit television (CCTV) camera. The recorded data were extracted in a hard drive and the following parameters were studied by randomly selecting different days and time periods of the experiment.

3.11.2.1 Standing Time (ST)

The standing time of the animals were measured on days zero, one, five and ten of the experiment from 7 a.m. to 5 p.m. through CCTV recordings in both thermo-neutral and acute heat stressed groups. The standing time was recorded as min/10 h.

3.11.2.2 Lying Time (LT)

The lying time of the animals were also measured on days zero, one, five and ten of the experiment from 7 a.m. to 5 p.m. through CCTV recordings in both the control and treatment groups. The lying time was recorded as min/10 h.

3.11.2.3 Water Intake Frequency (WIF)

The water intake frequency of the calves was studied on days zero, one, five and ten of the experiment from 7 a.m. to 5 p.m. through CCTV recordings in both the groups. The water intake frequency was recorded as number of times/10h.

3.11.2.4 Rumination Time

The rumination time of the animals in the present study was measured on alternate days of the study from 11 a.m. to 2 p.m. through CCTV camera recordings. The rumination time was recorded as min/3 h. This time was selected because of the maximum exposure at that time during heat exposure.

3.11.2.5 Feeding Time

The time spent by animals for taking feed was recorded as feeding time on days zero, one, five and ten of the experiment in both the control and treatment groups from 8 a.m. to 2 p.m. through CCTV recordings. The animals were fed thrice when they kept inside the chamber. The feeding time was recorded as min/6h.

3.12 MOLECULAR GENE EXPRESSION STUDIES

3.12.1 Isolation of RNA from Blood

Fifteen mL of venous blood sample was collected in a 15 mL centrifuge tube using EDTA (1mg/mL of blood) as anticoagulant. The collected blood was centrifuged at 3000 rpm for 8 min and plasma is discarded. Two to three volumes of ice-cold RBC lysis buffer were then added to the remaining erythrocytes and leukocytes in centrifuge tube and incubated the mixture in ice with occasional shaking for 10 min for the complete lysis of erythrocytes. After ten min it was centrifuged at 6000 rpm for eight min to pellet the contents. Pellet of leukocytes was recovered after decanting the red coloured supernatant, containing the lysed erythrocytes and haemoglobin. This procedure was repeated until pellet is clear without any unlysed erythrocytes. After centrifugation one mL of TRI reagent was added and mixed well by pipetting. This was then transferred into a two mL RNase free Eppendorf tube and again mixed by pipetting and vortexing. Vortexed sample was then incubated for five min at room temperature to permit complete dissociation of

nucleoprotein complex. After incubation 200 μ L of chloroform per mL of TRI Reagent was added for homogenization and vortexed vigorously for five min and incubated again for 10 min at room temperature. The sample was then centrifuged at 12000 rpm for 15 min at 4°C. The mixture separates into a lower red phenol chloroform phase, an interphase and a colourless upper aqueous phase in which RNA is present. Aqueous phase was removed carefully by angling the tube at 45° and pipetting the solution out into a new RNase free spin column provided in the total RNA isolation kit (ORionX Total RNA Isolation Kit, Origin). 500 μ L of Buffer RD is added to the RNase-Free spin column and centrifuged at 12,000 rpm for 1 min at 4°C. Flow-through was discarded and 700 μ L Buffer RW was added to the RNase-Free spin column and centrifuged again at 12,000 rpm for one min at 4°C. Flow-through was discarded and 500 μ L Buffer RW was added to the RNase-Free spin column and centrifuged at 12,000 rpm for one min at 4°C. Flow-through was discarded and dry spinning was done at 12,000 rpm for 2 min at 4°C. After dry spinning RNase-Free spin column was placed in a new 1.5mL RNase- Free Eppendorf tube and 35 μ L of RNase-Free water directly to the spin column membrane. It was then incubated in room temperature for two min and Centrifuge at 12,000 rpm for two min at 4°C to elute the RNA. Concentration and purity of RNA was determined immediately for complementary DNA (cDNA) synthesis.

3.12.2 Determination of Concentration and Purity of Total RNA

The quantity and purity of total RNA were estimated by Nanodrop (Thermo-Scientific NanoDrop™2000 Spectrophotometer) method spectrophotometrically. The concentration of total RNA per microliter of the sample was quantified by placing 1 μ L of the sample in the Nano-Drop spectrophotometer after calibrating it with the blank solution, nuclease-free water. The purity of total RNA was checked by A_{260}/A_{280} and A_{260}/A_{230} ratio using Nano-Drop spectrophotometer. A ratio of 1.8 or above for both A_{260}/A_{280} and A_{260}/A_{230} was preferred.

3.12.3 Checking the Quality of RNA by an Electrophoretic Method

The quality and integrity of total RNA were analysed electrophoretically using agarose gel (0.8%) electrophoresis. Agarose gel (0.8%) was prepared in 1X Tris Acetate EDTA buffer (TAE), which was prepared using diethyl pyrocarbonate (DEPC) treated

water. Ethidium bromide (to a final concentration of 0.5g/mL) was added into the molten agarose, mixed thoroughly and poured into gel casting tray to polymerise. After solidification, the comb and the seal were removed. The gel with the tray was immersed in 1X TAE buffer in the electrophoresis tank. RNA sample (5 μ L) was mixed with 1 μ L of RNA gel loading dye in a 0.25mL microcentrifuge tube and denatured at 70°C for 10 min, snap chilled in ice and loaded into the wells along with a molecular size ladder. Horizontal electrophoresis unit was set at 70 V for 50 min and the gel was visualized under gel documentation system (BioRad, Gel Doc 2000TM). After analysing the quantity and integrity, the RNA samples were taken for cDNA synthesis.

3.12.4 Preparation of complementary DNA (cDNA)

Complementary DNA is the DNA copy of RNA molecules, synthesized by the enzyme RNA dependent DNA polymerase (Reverse transcriptase). The total RNA was reverse transcribed into DNA using Origin cDNA synthesis kit with RNase inhibitor (Origin Diagnostics and Laboratory, Kerala) as per manufacturer's protocol using oligo (dT)₁₈ and random hexamer primers. For cDNA synthesis, 500ng of RNA was reverse transcribed. The RNA samples were set up as per the standard protocol of the cDNA synthesis kit.

Protocol

First strand cDNA synthesis (reaction volume 20 μ L)

1. Prepared the following mixture in a PCR tube

Table 3.5: Master mix for cDNA synthesis

Components	Volume (μ L)
Template RNA	To get 500 ng
Primer	0.5 μ L oligo (dT) ₁₈ and 0.5 μ L Random hexamer
dNTP Mix (10 mM)	1
Nuclease-free water	Added to make 14.5 μ L of the total mix

2. Heated the mixture at 65°C for 5 min and cooled down immediately on ice for 1 min Spun down the tube by centrifugation briefly.
3. Added the following components to the same tube.

Reagents	Volume
5X RT Reaction buffer	4 μ L
RTase	0.5 μ L
RNase inhibitor	1 μ L

4. Mixed gently.

5. Incubated the combined reaction mixture (Priming) at 25°C for 5 min.

6. Incubated at 50°C for 60 min.

7. Inactivated the reaction mixture by heating at 95°C for 5 min in the PCR machine (Biorad, Thermal cycler)

The product of first strand cDNA synthesis was stored at -80°C until use. The 1 μ L of cDNA was used for further qRT-PCR experiments.

3.12.5 Polymerase Chain Reaction (PCR) and quantitative Real-Time PCR (qRT-PCR)

3.12.5.1 Designing and Selection of Primers

Exon-spanning primers (Table 3.6) were designed using online NCBI primer design software (Primer3, <http://bioinfo.ut.ee/primer3/>) and specificity of the primers was checked using BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Designed primers were assessed for quality using PCR primer stats (Bioinformatics.org). The primers purchased in the lyophilized form was reconstituted with nuclease-free water to make a concentration of 100pM/ μ L for the stock and then stored at -20°C. Working solution of each primer was prepared by diluting the stock to 10pM/ μ L using nuclease free water.

Table 3.6: Description of primers

GENE	GENE ACCESSION NO. (NCBI):	PRIMER NAME	PRIMER SEQUENCE (5'-----3')	PRIMER LENGTH (bp)	PRODUCT SIZE (bp)
HSP-70	Genbank: JN604432.1	<i>HSP-70</i> F	CCTTCCCTGGATTGCTCATGTT	22	96
		<i>HSP-70</i> R	TCATCAGCCATCAGCTATGC	20	
IGF-1	NCBI reference sequence NM_001077828.1	<i>IGF-1</i> F	CAACAAGCCCCACGGGGGTAT	19	99
		<i>IGF-1</i> R	TCCAGCCTCCTCAGATCACA	20	
GAPDH	NCBI reference sequence NM_001034034.2	<i>GAPDH</i> F	TGGAGAAACCTGCCAAGTATG	21	127
		<i>GAPDH</i> R	TGAGTGTCGCTGTTGAAGTC	20	

3.12.5.2 Standardization of PCR Conditions

Complementary DNA samples were used as templates for PCR. PCR was carried out to amplify *HSP-70*, *IGF-1* and *GAPDH* genes. The conditions were optimized by setting different temperatures for annealing processes that was calculated by using Thermal melting temperatures (T_m°) of forward and reverse primers. The optimum annealing temperature for the primers was determined by gradient PCR (Biorad, Thermal cycler) and in quantitative real-time PCR system using SYBR green chemistry. The PCR reaction mix and cyclic conditions used are given in Table 3.7.

Table 3.7 Reaction mix for standard PCR reactions

Components	Volume (μ L)
Taq PCR Smart mix (2x) w/o dye	25.00
Forward primer (10 pM/ μ L)	2.00
Reverse primer (10 pM/ μ L)	2.00
Template DNA	1.00
Nuclease-free water	20.00
Total	50.00

The tubes were spun briefly and placed in a thermal cycler. The details of PCR conditions used are given in Table 3.8.

Table 3.8: PCR conditions for amplification

Steps		Temperature (°C)	Time
Initial denaturation		95	2 min
35 cycles of	Denaturation	95	20 sec
	Annealing*	61.1°C for <i>HSP – 70</i> 63.3°C for <i>IGF – 1</i> 60.5°C for <i>GAPDH</i>	40 sec
	Extension	72	1 min
Final extension		72	5 min

* Based on gradient PCR results, temperature set for all the 3 genes.

The PCR amplified products were stored at -20°C till further processing. The amplified products were used in 2% agarose gel [1X Tris-Borate-EDTA buffer (TBE)] electrophoresis. Five microlitre of the product was mixed with one microliter of 6 X gel loading buffer and loaded into the wells carefully. As a molecular size standard, 1 µL of 100 bp DNA ladder (Thermo scientific, USA) was also loaded into one of the wells. Electrophoresis was then carried out at 70 V for 50 min or until bromophenol blue dye migrated more than 4 cm length of gel in 1X TBE buffer. The gel was documented in a gel documentation system (BioRad, Gel Doc 2000TM).

After detecting the bands by electrophoresis, the product size of *HSP-70*, *IGF-1* and *GAPDH* was confirmed as 96, 99, 127 bp respectively. Annealing temperature at good dark bands for each target genes were standardised for a further real-time PCR reaction.

3.12.5.3 Quantitative Real-Time PCR Amplification of Target Genes

After validation of primer annealing temperature, the cDNA of target gene along with the reference gene was studied for mRNA expression by Real Time PCR (Biorad, USA) and analysed using CFX Maestro software.

The expression of *HSP-70*, *IGF-1* and *GAPDH* was studied using iTaq™ Universal SYBR Green Supermix, Bio-Rad Laboratories, USA. It is a 2x concentrated, ready-to-

use reaction master mix optimized for dye-based quantitative PCR (qPCR) on any real-time PCR instrument. It contains antibody-mediated hot-start iTaq DNA polymerase, dNTPs, MgCl₂, SYBR® Green I dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein).

The reaction was carried out in triplicates. Besides, one non-template control (NTC) for each gene and a negative control (with only nuclease free water) were also included in the reaction. A suitable plate setting was done prior to the start of the experiment. Master Mix with template cDNA was loaded into the designated PCR strips of 8 tubes and sealed with caps. The strips were centrifuged at 2500×g for 30 sec and were placed in Bio-Rad real-time Thermal cycler. The details of reaction mix are given in Table 3.9. The reaction was carried out in thermal cycler (Bio-Rad, USA) according to pre-programmed temperature, cycling conditions for each gene as specified in Table 3.10. The results were expressed as threshold cycle values (C_T). This value is the cycle number when the fluorescence of the reporter dye is appreciably higher than the background fluorescence. The threshold automatically adjusted by the instrument was used for the generation of C_T values. The expression of target genes (*candidate genes*) was compared with a reference gene (*GAPDH*) to calculate ΔC_T and the ΔC_T in the treatment sample versus control samples was used to calculate $\Delta\Delta C_T$. The method used is referred to as the comparative C_T method (Livak and Schmittgen, 2001).

Table 3.9: Optimized concentrations of qRT-PCR Master Mix (10 μ L) for *HSP-70*, *IGF-1* and *GAPDH* genes

Components	Volume (μ L)
Template (cDNA)	1.00
iTaq™ Universal SYBR Green Supermix (2 x)	5.00
Forward Primer (10pM/ ul)	0.50
Reverse primer (10pM/ul)	0.50
Nuclease-free water	3.00

Table 3.10: qRT-PCR conditions for *HSP-70*, *IGF-1* and *GAPDH* genes

Steps		Temperature	Time
Polymerase activation		95°C	2 min
35-40 cycles of	Denaturation	95°C	5 sec
	Combined Annealing & Extension *	61.1°C for <i>HSP-70</i> 63.3°C for <i>IGF-1</i> 60.5°C for <i>GAPDH</i>	30 sec
Melt curve		95°C	5 sec
		65°C	5 sec
		95°C	5sec

* Temperature varies depending on primer

Fluorescence signals were measured in each cycle. For each sample, the curve was generated after the completion of amplification. Data acquisition was performed during the final denaturation step.

3.12.5.4 Melt Curve Analysis

A melt curve analysis was performed after the reaction for checking the specificity of amplification. The program for melt curve analysis was done at 65-95°C for 5 sec. Data acquisition was performed at the final denaturation step.

3.12.5.5 Relative Quantification of Genes

Relative quantification describes the change in expression of the target sample relative to some reference group such as an untreated control. *HSP-70* and *IGF-1* genes quantification was done by C_T (Cycle Threshold) comparative method and was expressed as 'n' fold up-regulation of the transcribed gene in relation to the calibrator (Untreated control). For relative quantification by comparative method, the values were expressed relative to control sample called calibrator. *GAPDH* was used as an internal control. The C_T of the target gene and C_T of control gene were determined for each sample and calibrated ($\Delta\Delta C_T$ method) (Livak and Schmittgen, 2001).

$$\Delta C_T = C_{T(\text{target gene})} - C_{T(\text{reference gene})}$$

$$\Delta\Delta C_T = \Delta C_{T(\text{test sample})} - \Delta C_{T(\text{reference sample})}$$

$$RQ = 2^{-\Delta\Delta C_T}$$

3.13 STATISTICAL ANALYSIS

Results of the study were expressed in Mean $\pm$ Standard error. The statistical analysis of the obtained data was done using the software SPSS version 24.0. 'Linear mixed model' ANOVA was performed for the data of all the recorded parameters. The statistical significance of gene expression studies were analysed using independent t-test. Correlation studies were done using Pearson's correlation and Spearman's rho correlation co-efficient methods.



Fig. 3.2. Climate controlled research complex (CCRC) of CAADECCS, KVASU.



Fig. 3.3. Animal holding facility in CAADECCS for acclimatisation.



Fig. 3.4. Experimental animals kept in the climate-controlled chamber.



Fig. 3.5. CCTV camera surveillance of experimental animals.

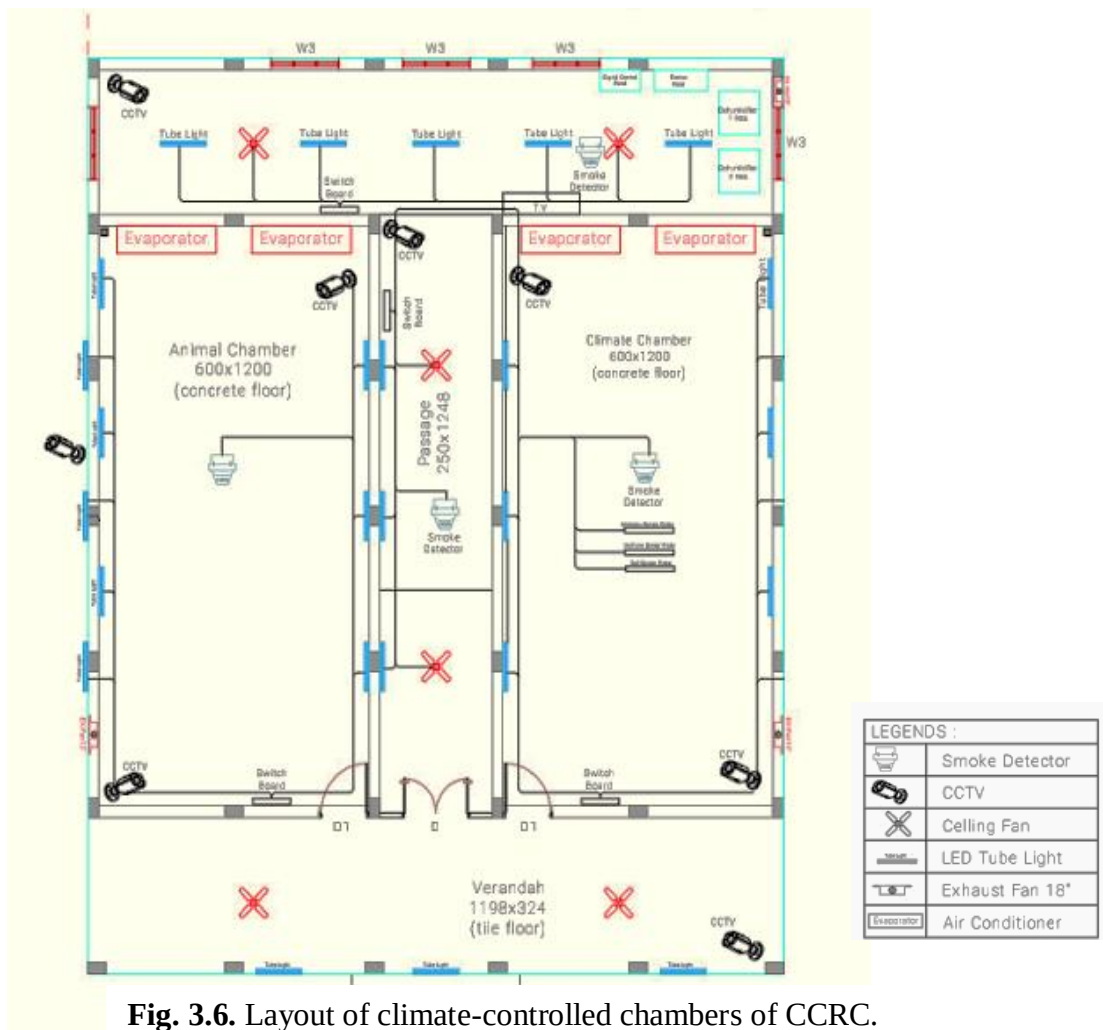


Fig. 3.6. Layout of climate-controlled chambers of CCRC.



Fig. 3.7. Gamma counter for the estimation of serum cortisol by radio-immunoassay.



Fig. 3.8. EPOC blood analyser for blood gases and electrolytes analysis.



Fig. 3.9. Perkin Elmer Lambda 35 UV-Vis Spectrophotometer for the estimation of antioxidant status.

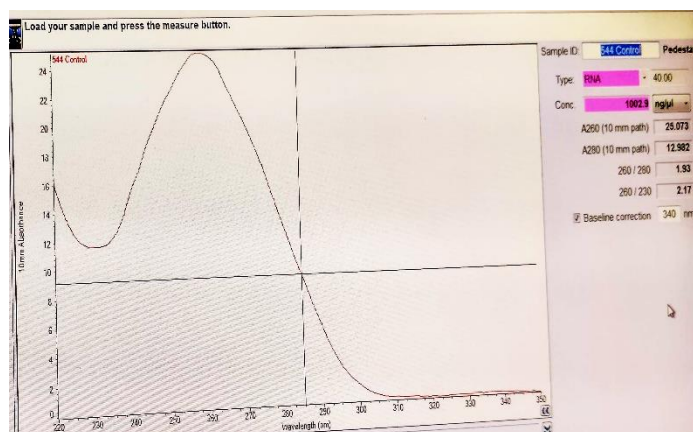


Fig. 3.10. Purity and concentration of RNA determined by Nanodrop 2000 spectrophotometer.

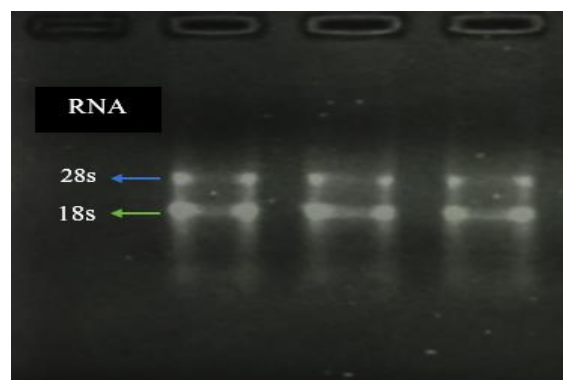


Fig. 3.11. Quality of RNA determined by 0.8% agarose gel electrophoresis.



Fig. 3.12. Thermal cycler (Bio-Rad) for performing cDNA synthesis and gradient PCR.

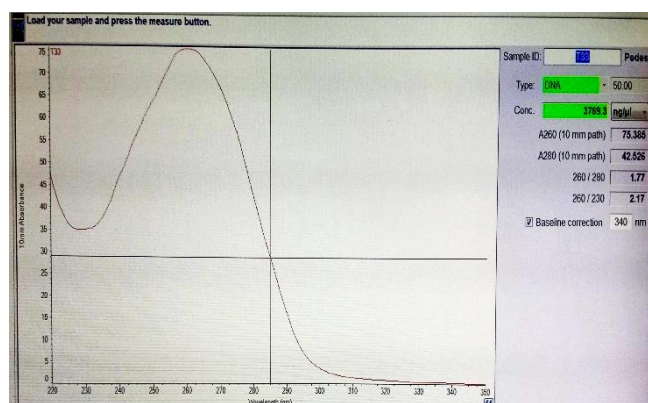


Fig. 3.13. Purity and concentration of cDNA determined by Nanodrop spectrophotometer.



Fig. 3.14. Gel documentation system for standardisation of PCR (Bio-Rad).

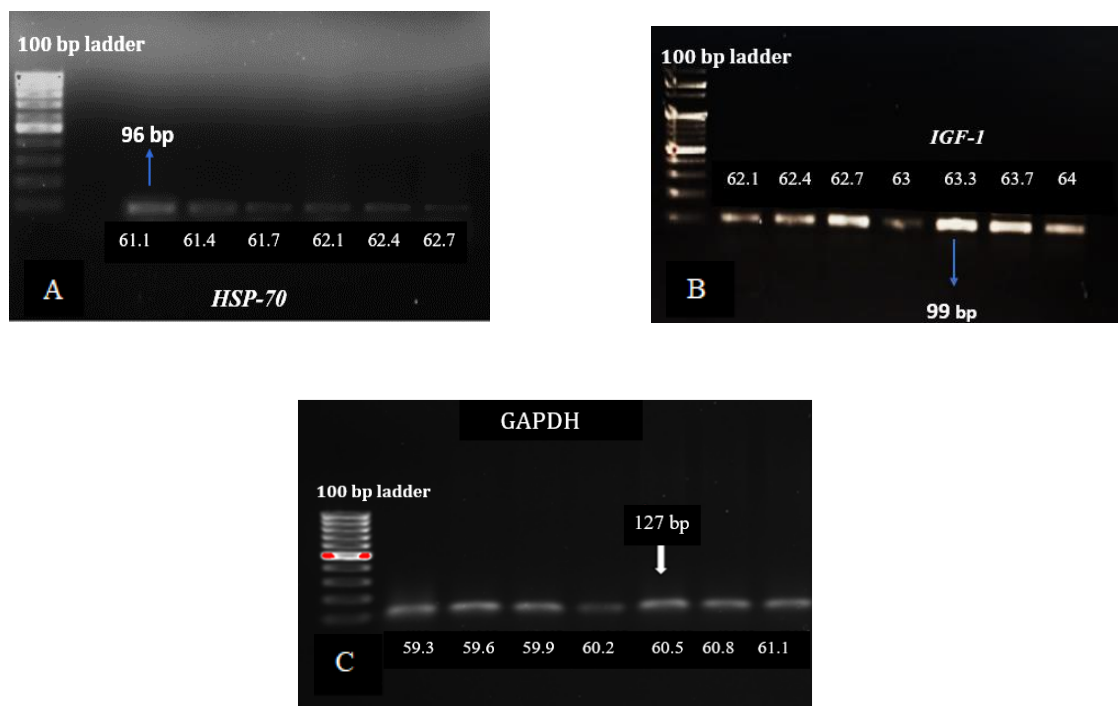


Fig. 3.15. Gel documentation images of standardisation of annealing temperature for target genes (A. *HSP-70* & B. *IGF-1*) and reference gene (C. *GAPDH*).

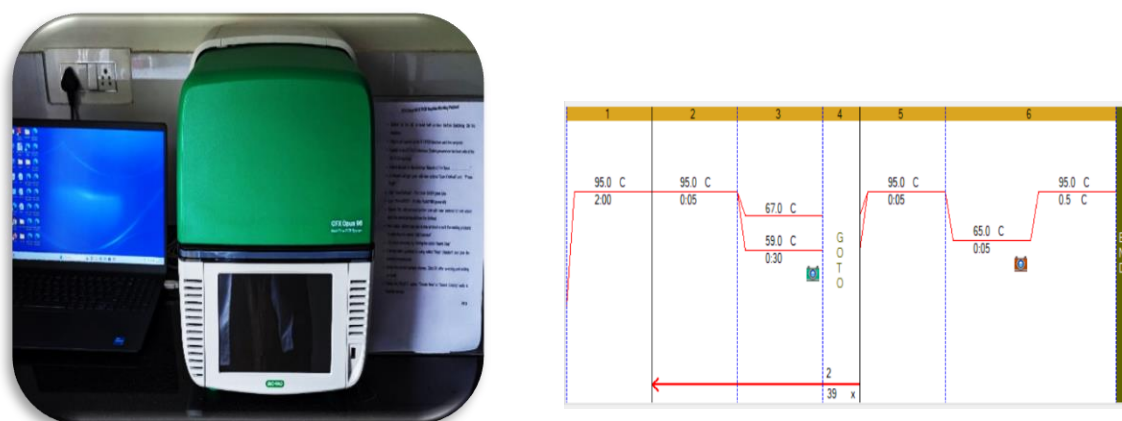


Fig. 3.16. Quantitative Real Time PCR (qRT-PCR) for amplification of target genes.

Results

4. RESULTS

The present study was conducted with the objective of comparing the physiological, behavioural, metabolic, endocrine, antioxidant and molecular responses of crossbred calves under thermo-neutral zone and acute heat stressed conditions using a climate – controlled chamber. The results of the current study are detailed in this context.

4.1 PHYSIOLOGICAL RESPONSES

The mean values of physiological variables such as rectal temperature, respiratory rate and heart rate in both the groups are represented in Table 4.1. All the physiological parameters were significantly increased in the treatment group ($p<0.001$) than the control group.

Table 4.1. Overall physiological responses of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

PARAMETERS \ GROUPS	CONTROL (TNZ)	TREATMENT (HS)	p – value
Rectal temperature (°C)	37.87 ^a $\pm$ 0.038	38.99 ^b $\pm$ 0.03	$p<0.001$
Respiratory rate (breaths/min)	21.48 ^a $\pm$ 0.37	37.01 ^b $\pm$ 0.37	$p<0.001$
Heart rate (beats/min)	64.04 ^a $\pm$ 1.74	83.50 ^b $\pm$ 1.74	$p<0.001$

Means bearing different superscripts within row (a-b) differ significantly ($p<0.01$)

4.1.1 Rectal Temperature (RT)

The mean values RT of the animals under study between groups are shown in Table 4.1. In thermo-neutral zone, the value was found to be 37.87 $\pm$ 0.03 °C. In acute heat stressed condition, the mean RT observed was 38.99 $\pm$ 0.03 °C. The mean value of RT in the treatment group was significantly ($p<0.001$) higher when compared to control group. The mean values of RT on alternate days of study and different time intervals of both the control and treatment groups are represented in Table 4.2a and 4.2b and pictorially in Fig.4.1a and Fig 4.1b.

Table 4.2a. Day wise comparison of rectal temperature (°C) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9
CONTROL (TNZ)	38.15 ^{aA} ±0.06	37.84 ^{bA} ±0.06	37.825 ^{bA} ±0.06	37.92 ^{bA} ±0.06	37.77 ^{bA} ±0.06	37.75 ^{bA} ±0.06
TREATMENT (HS)	38.59 ^{aB} ±0.06	38.99 ^{bB} ±0.06	39.02 ^{bB} ±0.06	38.92 ^{bB} ±0.06	38.94 ^{bB} ±0.06	39.25 ^{cB} ±0.06
p-value	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly (p<0.05)

Table 4.2b. Time wise comparison of rectal temperature (°C) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

GROUP \ TIME	8 a.m.	11 a.m.	2 p.m.	5 p.m.
CONTROL (TNZ)	37.94 ^{aA} ±0.05	37.85 ^{aA} ±0.05	37.83 ^{aA} ±0.05	37.88 ^{aA} ±0.05
TREATMENT (HS)	38.40 ^{aB} ±0.05	39.27 ^{bB} ±0.05	39.81 ^{cB} ±0.05	38.33 ^{aB} ±0.05
p-value	p<0.001	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly (p<0.05)

In the present study, there was significant difference ($p < 0.001$) between the mean value of RT of day zero and other days of both thermo-neutral zone and acute heat stressed conditions which is represented in Table 4.2a. The mean RT was significantly decreased in different days of the control group when compared to day zero and significantly increased on different days of heat exposure in the treatment group. The mean RT was found maximum on ninth day of heat exposure. Also, the mean RT values of each days increased significantly in the treatment group.

The mean RT of the animals did not differ significantly in different time intervals of the control group. In the treatment group, mean RT values differed significantly ($p < 0.001$) in different time intervals where the highest mean RT was noticed at 2 p.m. followed by 11 a.m. where maximum exposure of 40 °C and 55 per cent relative humidity (THI=92.48 to 95.04) was simulated (Table 4.2b).

4.1.2 Respiratory Rate (RR)

The mean values RR of the animals under study between groups are presented in Table 4.1. The value was found to be 21.48 ± 0.37 breaths per minute in thermo-neutral zone whereas in acute heat stressed condition, the mean RR observed was 37.01 ± 0.37 breaths per minute. In this study, the mean value of RR in the treatment group was significantly ($p < 0.001$) higher than the control group.

The mean values of RR of alternate days of study and different time intervals of both the control and treatment groups are represented in Table 4.3a and 4.3b. and pictorially represented in Fig.4.2a and 4.2b.

In the present study, the mean value of RR significantly differed ($p < 0.001$) between different days of both thermo-neutral zone and acute heat stressed conditions which is represented in Table 4.3a. The mean RR varied significantly higher between each day in the treatment group where the highest value was evidenced on 7th day (45.25 ± 0.79 breaths per minute). But there was no significant difference in RR evidenced between day zero of groups. The mean RR of the animals did not differ significantly in different time intervals of the thermo-neutral zone. But the mean RR values differ significantly ($p < 0.001$) in different time

intervals in the treatment group where the highest mean RR was noticed at 2 p.m. (53.5 ± 0.663 breaths per minute) where the chamber THI was 92.48 to 95.04.

4.1.3 Heart Rate (HR)

The mean values of HR between groups are shown in Table 4.1. It was found to be 64.04 ± 1.74 beats per minute in control group and 83.50 ± 1.74 beats per minute in the treatment group. The mean value of HR in the treatment group was significantly ($p < 0.001$) higher than the control group.

The mean values of HR of alternate days of study and different time intervals of animals in both the control and treatment groups are displayed in Table 4.4a and 4.4b. and pictorially in Fig. 4.3a and 4.3b.

Furthermore, the mean values of HR had significantly differed ($p < 0.001$) between different days of both the groups which was represented in Table 4.4a. The mean HR varied significantly higher between each day in the treatment group where the highest value was evidenced on 9th day (88.75 ± 1.94 beats per minute).

By comparing different time intervals of the control group, the mean HR of the animals did not differ significantly. But the mean HR values differed significantly ($p < 0.001$) in different time intervals in the treatment group where the highest mean RR was noticed at 2 p.m. (91.89 ± 1.86 beats per minute; THI of 92.48 to 95.04) and the lowest at 8 a.m. (76.44 ± 1.86 beats per minute; THI of 80.33).

4.2 BEHAVIOURAL RESPONSES

The mean values of behavioural parameters such as standing time, lying time, feeding time, water intake frequency, rumination time and feed intake in the control and treatment groups are presented in Table 4.5.

The recorded behavioural responses such as standing time, feeding time and water intake frequency were significantly increased in the treatment group ($p < 0.001$) in contrast to the control group. The animals in thermo-neutral zone had significantly higher ($p < 0.001$) lying time and feed intake. Hence the feed intake and lying time was significantly decreased ($p < 0.001$) in acute heat stressed conditions.

Table 4.3a. Day wise comparison of respiratory rate (breaths/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9
CONTROL (TNZ)	25.67 ^{aA} $\pm$ 0.79	24.417 ^{aA} $\pm$ 0.79	17.75 ^{bA} $\pm$ 0.79	19.5 ^{bA} $\pm$ 0.79	21.00 ^{bA} $\pm$ 0.79	20.58 ^{bA} $\pm$ 0.79
TREATMENT (HS)	25.58 ^{aA} $\pm$ 0.79	30.75 ^{bB} $\pm$ 0.79	37.58 ^{cB} $\pm$ 0.79	41.00 ^{dB} $\pm$ 0.79	45.25 ^{eB} $\pm$ 0.79	41.90 ^{fB} $\pm$ 0.79
p-value	p=0.93 ^{ns}	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p < 0.05$), ns – non- significant at 0.05 level

Table 4.3b. Time wise comparison of respiratory rate (breaths/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ TIME	8 a.m.	11 a.m.	2 p.m.	5 p.m.
CONTROL (TNZ)	21.56 ^{aA} $\pm$ 0.66	21.67 ^{aA} $\pm$ 0.66	21.50 ^{aA} $\pm$ 0.66	21.222 ^{aA} $\pm$ 0.66
TREATMENT (HS)	23.72 ^{aB} $\pm$ 0.66	42.11 ^{bB} $\pm$ 0.66	53.5 ^{cB} $\pm$ 0.66	28.72 ^{dB} $\pm$ 0.66
p-value	p<0.05 (p=0.016)	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p < 0.05$)

Table 4.4a. Day wise comparison of heart rate (beats/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

DAYS GROUP	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9
CONTROL (TNZ)	68.41 ^{aA} ±1.94	60.75 ^{bA} ±1.94	63.33 ^{bA} ±1.94	66.06 ^{cA} ±1.94	63.50 ^{cA} ±1.94	62.17 ^{cA} ±1.94
TREATMENT (HS)	70.67 ^{aB} ±1.94	85.00 ^{bB} ±1.94	87.25 ^{bB} ±1.94	81.8 ^{cB} ±1.94	85.50 ^{dB} ±1.94	88.75 ^{eB} ±1.94
p-value	p<0.01 (p=0.004)	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-d) and columns (A-B) differ significantly (p<0.05)

Table 4.4b. Time wise comparison of heart rate (beats/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

TIME GROUP	8 a.m.	11 a.m.	2 p.m.	5 p.m.
CONTROL (TNZ)	64.72 ^{aA} ±1.87	63.5 ^{aA} ±1.87	64.39 ^{aA} ±1.87	63.5 ^{aA} ±1.87
TREATMENT (HS)	76.44 ^{aB} ±1.87	85.94 ^{bB} ±1.87	91.89 ^{cB} ±1.87	79.72 ^{dB} ±1.87
p-value	p<0.001	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-d) and columns (A-B) differ significantly (p<0.05)

Table 4.5. Overall behavioural responses of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

PARAMETERS	GROUP		p – value
	CONTROL (TNZ)	TREATMENT (HS)	
Standing time (min/10 h)	165.25 ^a $\pm$ 3.28	255.83 ^b $\pm$ 5.25	p<0.001
Lying time (min/10 h)	434.58 ^a $\pm$ 3.26	339.50 ^b $\pm$ 6.59	p<0.001
Water intake frequency (number of times/ 10 h)	28.50 ^a $\pm$ 0.60	38.63 ^b $\pm$ 0.60	p<0.001
Rumination time (min/3 h)	99.36 ^a $\pm$ 0.95	72.91 ^b $\pm$ 1.002	p<0.001
Feed intake (kg/day)	16.81 ^a $\pm$ 0.05	15.21 ^b $\pm$ 0.11	p<0.001
Feeding time (min/5 h)	42.75 ^a $\pm$ 0.18	54.54 ^b $\pm$ 0.75	p<0.001

Means bearing different superscripts within row (a-b) differ significantly (p<0.01)

4.2.1 Standing Time (ST)

The mean values of standing time of animals in each group are represented in Table 4.5. In the treatment group, standing time was observed as 255.83 $\pm$ 5.25 min/10 h which was significantly higher (p<0.001) than the control group where it was 165.25 $\pm$ 3.28 min/10 h.

The mean values of standing time of animals on different days of study in both the control and treatment groups are presented in Table 4.5a and Fig.4.4c.

In the present study, there was significant difference (p<0.001) between the mean values of ST of day zero and other days of both control and treatment groups which is represented in Table 4.5a. The mean ST was significantly lower (p<0.001) in different days of the control group when compared to day zero and vice versa in

Table 4.5a. Day wise comparison of standing time (min/10 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

DAYS GROUP	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	211.50 ^{aA} $\pm$ 10.08	152.50 ^{bA} $\pm$ 3.12	153.33 ^{bA} $\pm$ 6.41	143.67 ^{bA} $\pm$ 4.46
TREATMENT (HS)	203.33 ^{aA} $\pm$ 6.48	243.67 ^{bB} $\pm$ 11.60	274.67 ^{bB} $\pm$ 13.23	301.67 ^{cB} $\pm$ 9.44
p-value	p=0.514 ^{ns}	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p<0.05$), ns – non- significant at 0.05 level

Table 4.5b. Day wise comparison of lying time (min/10 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

DAYS GROUP	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	388.50 ^{aA} $\pm$ 10.07	447.50 ^{bA} $\pm$ 3.13	446.67 ^{bA} $\pm$ 6.41	455.67 ^{bA} $\pm$ 4.24
TREATMENT (HS)	378.00 ^{aA} $\pm$ 17.19	356.33 ^{aB} $\pm$ 11.60	325.33 ^{bB} $\pm$ 13.23	298.33 ^{cB} $\pm$ 9.44
p-value	p=0.612 ^{ns}	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p<0.05$), ns – non- significant at 0.05 level

the treatment group. The ST was maximum on the 10th day of acute heat stress treatment whereas minimum on the 10th day of thermo-neutral condition. Hence the standing time was accelerating from day zero to 10 in the treatment group whereas it was vice versa in the control group.

There was also significant difference ($p<0.001$) between mean standing time of each day between groups except day zero of the experiment.

4.2.2 Lying Time (LT)

In the present study, mean values of LT were significantly increased ($p<0.001$) in the animals in thermo-neutral zone when compared to acute heat stressed conditions which are presented in Table 4.5. The mean LT values observed was 434.58 ± 3.26 min/10 h and 339.50 ± 6.59 min/10 h for the control and treatment groups respectively.

The mean values of LT of animals on different days of study in both the control and treatment groups are represented in Table 4.5b and Fig. 4.4d.

In the present study, there was significant difference ($p<0.001$) between the mean values of LT of day zero and other days of both control and treatment groups which is represented in Table 4.5b. The mean LT was significantly increased ($p<0.001$) in different days of the control group when compared to day zero and it was significantly decreased in different days of heat exposure in the treatment group. The LT of animals was found to be maximum on the 10th day of thermo-neutral condition whereas minimum on the 10th day of acute heat stress group. Hence the lying time was increasing day by day in the thermo-neutral zone whereas it was decreasing in acute heat stressed conditions. Further there was significant difference ($p<0.001$) between mean lying time of each day between groups except day zero of study.

4.2.3 Water Intake Frequency (WIF)

The overall mean values of WIF were significantly increased ($p<0.001$) in the animals in acute heat stressed conditions when compared to thermo-neutral zone which are presented in Table 4.5. The mean WIF values observed was 28.5 ± 0.60

times/10 h and 38.63 ± 0.60 times/10 h for the control and treatment groups respectively.

The mean values of WIF on different days of study in both the control and treatment groups are represented in Table 4.5c and Fig. 4.5.

There was highly significant difference ($p < 0.001$) found between the mean values of WIF of day zero and other days of control group which is represented in Table 4.5b. The WIF of animals was found to be declined in the thermo-neutral condition whereas remained constant in the acute heat stressed condition. There was also significant difference ($p < 0.001$) between mean WIF of each day between groups except day zero of the study period.

4.2.4 Rumination Time

The overall mean values of rumination time of animals in each group were represented in Table 4.5. In the control group, rumination time was observed as 99.36 ± 0.95 min/3h which was significantly higher ($p < 0.001$) than the treatment group where it was 72.91 ± 1.00 min/3h.

The mean values of rumination time of animals on alternate days of study in both the control and treatment groups are presented in Table 4.5d and Fig.4.6.

In the present study, there was no significant difference between the mean values of rumination time of different days of the control group. Whereas in the treatment group there was high significant difference between mean values of different days. The mean rumination time was significantly lower ($p < 0.001$) on different days of the treatment group when compared to day zero. The rumination time was maximum on the day zero (102.16 ± 2.57 min/3h) of acute heat stress treatment whereas minimum on the 10th day (60.33 ± 2.33 min/3h). Hence the rumination time was decreasing from day zero to 10 in the treatment group whereas it remained constant in the control group. Further there was significant difference ($p < 0.001$) between mean rumination time of each day between groups except day zero of study.

Table 4.5c. Day wise comparison of water intake frequency (number of times/10 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	37.17 ^{aA} ±1.01	25 ^{bA} ±0.86	25.67 ^{bA} ±1.28	26.17 ^{bA} ±1.53
TREATMENT (HS)	39.0 ^{aA} ±0.82	38.0 ^{aB} ±1.46	38.67 ^{aB} ±1.14	38.83 ^{aB} ±1.30
p-value	p=0.191 ^{ns}	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly ($p<0.05$), ** Significant at 0.01 level, ns – non- significant at 0.05 level

Table 4.5d. Day wise comparison of rumination time (min/3h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9
CONTROL (TNZ)	99.33 ^{aA} ±1.33	98.83 ^{aA} ±2.06	97.33 ^{aA} ±1.43	101 ^{aA} ±2.46	99.33 ^{aA} ±4.0	100.33 ^{aA} ±1.70
TREATMENT (HS)	102.16 ^{aA} ±2.57	73.67 ^{bB} ±2.23	70.33 ^{bB} ±2.70	70 ^{bB} ±2.88	61 ^{cB} ±1.86	60.33 ^{cB} ±2.33
p-value	p=0.359 ^{ns}	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p<0.05$), ns – non- significant at 0.05 level

4.2.5 Feed Intake

The overall mean values of feed intake (both roughage and concentrate) of animals in each group are represented in Table 4.5. The feed intake in acute heat stress group was 15.21 ± 0.11 kg/day which was significantly lower ($p < 0.001$) than the thermo-neutral group where it was 16.81 ± 0.05 kg/day.

The mean values of feed intake of animals on different days of study in both the control and treatment groups are presented in Table 4.5e and Fig. 4.7.

In the present study, there was no significant difference between the mean values of feed intake in different days of the control group. In the treatment group, there was no significant difference between mean values of day zero and one but there was high significant difference between day zero and other different days. The mean feed intake remained constant with no significant difference on different (0,1,5 and 10) days of the control group. The feed intake was maximum on the day zero (16.81 ± 0.052 kg/day) of the treatment group whereas minimum on the 10th day (15.21 ± 0.114 kg/day). Hence the feed intake was declining from day zero to 10 in the treatment group whereas it remained constant in the control group. Further there was significant difference ($p < 0.001$) between mean feed intake of each day between groups except day zero and three of the experimental period.

4.2.6 Feeding Time

The mean values of feeding time of animals in each group were represented in Table 4.5. In the treatment group, the mean feeding time observed was 54.54 ± 0.754 min/6h. which was significantly higher ($p < 0.001$) than the control group where it was 42.75 ± 0.18 min/6h.

The mean values of feeding time of animals on different days of the experiment in both the control and treatment groups are presented in Table 4.5f and in Fig. 4.8.

There was no significant difference between the mean values of feeding time on different days of the thermo-neutral group. Whereas in the treatment group there was high significant difference ($p < 0.001$) between mean values of different

Table 4.5e. Day wise comparison of feed intake (kg/day) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9
CONTROL (TNZ)	16.75 ^{aA} ±0.16	16.96 ^{aA} ±0.05	16.55 ^{aA} ±0.17	16.98 ^{aA} ±0.09	16.74 ^{aA} ±0.16	16.91 ^{aA} ±0.04
TREATMENT (HS)	16.81 ^{aA} ±0.111	16.53 ^{aB} ±0.09	16.42 ^{bA} ±0.09	14.68 ^{cB} ±0.26	14.205 ^{cB} ±0.50	12.65 ^{dB} ±0.34
p-value	p=0.769 ^{ns}	p<0.01 (p=0.004)	p=0.502 ^{ns}	p<0.001	p<0.01 (p=0.003)	p<0.001

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly (p<0.05), ns – non- significant at 0.05 level

Table 4.5f. Day wise comparison of feeding time (min/6 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean±SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	45.16 ^{aA} ±2.21	44.5 ^{aA} ±1.02	41.17 ^{aA} ±1.42	40.17 ^{aA} ±1.66
TREATMENT (HS)	43.67 ^{aA} ±1.38	58.0 ^{bB} ±1.55	57.00 ^{bB} ±1.69	59.50 ^{bB} ±1.38
p-value	p=0.58 ^{ns}	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly (p<0.05), ns – non- significant at 0.05 level

days of heat stress. The mean feeding time was significantly higher ($p<0.001$) on different days of the treatment group when compared to day zero. The feeding time was maximum on the 10th day (59.5 ± 1.38 min/6h) of acute heat stress treatment whereas minimum on the day zero (43.67 ± 1.38 min/6h). Therefore, the feeding time was gradually increasing from day zero to 10 in the treatment group. Furthermore, there was significant difference ($p<0.001$) between mean feeding time of each day between groups except day zero of study.

4.3 METABOLIC RESPONSES

The overall mean values of metabolic parameters in both groups such as blood gases, electrolytes and blood glucose levels are represented in Table 4.6 and pictorially in Fig. 4.9a and 4.9b.

Table 4.6. Overall metabolic responses of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

PARAMETERS \ GROUP	CONTROL (TNZ)	TREATMENT (HS)	p – value
	BLOOD GASES		
pO ₂ (mm Hg)	37.58 ^a ±0.51	38.95 ^b ±0.51	p<0.05 (p=0.02)
pCO ₂ (mm Hg)	42.71 ^a ±0.56	40.29 ^b ±0.56	p<0.001
BLOOD ELECTROLYTES			
HCO ₃ ⁻ (mEq/L)	27.77 ^a ±0.20	25.70 ^b ±0.20	p<0.001
Na ⁺ (mEq/L)	138.79 ^a ±0.48	133.87 ^b ±0.48	p<0.001
K ⁺ (mEq/L)	3.97 ^a ±0.05	4.07 ^a ±0.05	p=0.10 ^{ns}
BLOOD GLUCOSE (mg/dL)	66.96 ^a ±0.79	63.91 ^b ±0.79	p<0.01 (p=0.004)

Means bearing different superscripts within a row (a-b) differ significantly ($p<0.05$), ns – non- significant at 0.05 level

4.3.1 Blood Gases

4.3.1.1 Partial Pressure of Oxygen (pO_2)

The observed mean value of pO_2 in the venous blood of treatment group was 38.95 ± 0.51 mm Hg which was significantly higher ($p < 0.05$) than the control group pO_2 value that was 37.58 ± 0.51 mm Hg.

The mean values of pO_2 on different days of study period in both the control and treatment groups are represented in Table 4.6a and Fig.4.10. The mean values differed significantly between different days of both control and treatment groups. In control group, the highest mean value was noticed on day zero (45.55 ± 1.65 mm Hg) and the lowest value on the 10th day (33.47 ± 0.68 mm Hg). Highly significant difference in pO_2 values ($p < 0.01$) was also observed on different days of the treatment group. The mean pO_2 was significantly higher ($p < 0.01$) on the day one of heat exposure when compared to day zero which was 36.65 ± 0.34 mm Hg and 42.32 ± 1.11 mm Hg respectively.

In the current study, high significant differences ($p < 0.01$) in pO_2 values of each day between the groups was also observed.

Table 4.6a. Day wise comparison of partial pressure of oxygen (mm Hg) in venous blood of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

DAYS GROUP	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	45.55^{aA} ± 1.65	36.62^{bA} ± 0.53	34.72^{cA} ± 0.70	33.47^{cA} ± 0.68
TREATMENT (HS)	36.65^{aB} ± 0.34	42.32^{bB} ± 1.11	38.68^{cB} ± 0.56	38.15^{cB} ± 0.59
p-value	$p < 0.01$ ($p = 0.002$)	$p < 0.01$ ($p = 0.002$)	$p < 0.01$ ($p = 0.008$)	$p < 0.01$ ($p = 0.003$)

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p < 0.05$)

4.3.1.2 Partial Pressure of Carbon dioxide ($p\text{CO}_2$)

In the treatment group, the observed mean value of $p\text{CO}_2$ in the venous blood was 40.29 ± 0.56 mm Hg which was significantly lower ($p < 0.01$) than the control group $p\text{CO}_2$ value of 42.71 ± 0.56 mm Hg.

The mean values of $p\text{CO}_2$ on different days of study in both the control and treatment groups are represented in Table 4.6b and pictorially in Fig. 4.11. The mean values of $p\text{CO}_2$ differed significantly between different days of the control group ($p < 0.05$) as well as of the treatment group ($p < 0.001$). In control group, the highest value was noticed on 10th day (46.57 ± 1.976 mm Hg) and the lowest value on the day zero (40.033 ± 0.658 mm Hg).

Highly significant differences ($p < 0.01$) in mean values were also observed on different days of the treatment group. The mean $p\text{CO}_2$ was significantly lower ($p < 0.01$ on the day one of heat exposure when compared to day zero which was 38.32 ± 0.63 mm Hg and 43.88 ± 0.75 mm Hg respectively.

Furthermore, mean $p\text{CO}_2$ values of each day between the groups were also highly significant ($p < 0.01$).

Table 4.6b. Day wise comparison of partial pressure of carbon dioxide (mm Hg) in venous blood of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	$40.033^{aA} \pm 0.65$	$41.18^{aA} \pm 0.64$	$43.08^{bA} \pm 0.64$	$46.57^{bA} \pm 1.97$
TREATMENT (HS)	$43.88^{aB} \pm 0.75$	$38.32^{bB} \pm 0.63$	$40.78^{cB} \pm 0.66$	$38.18^{aB} \pm 0.74$
p-value	$p < 0.01$ ($p = 0.009$)	$p < 0.01$ ($p = 0.009$)	$p < 0.01$ ($p = 0.054$)	$p < 0.01$ ($p = 0.007$)

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p < 0.05$)

4.3.2 Blood Electrolytes

4.3.2.1 Bicarbonate Ion (HCO_3^-) Concentration

In the treatment group, the observed mean value of HCO_3^- level in the blood was 25.70 ± 0.20 mEq/L which was significantly lower ($p < 0.01$) than the HCO_3^- level of 27.77 ± 0.20 mEq/L in the control group, represented in Table 4.6.

The mean values of HCO_3^- on different days of study in both the control and treatment groups are represented in Table 4.7a and pictorially in Fig. 4.12. The mean values of HCO_3^- differed significantly between different days of both the control group and treatment group ($p < 0.001$). In treatment group, there was high significant difference between day zero and day one of heat exposure where the mean HCO_3^- levels were 27.75 ± 0.42 mEq/L and 24.65 ± 0.48 mEq/L respectively.

High significant differences ($p < 0.01$) in mean values were also observed between day zero and other days of the treatment group. Furthermore, mean HCO_3^- levels of each day of the study period between the groups were also highly significant ($p < 0.01$).

Table 4.7a. Day wise comparison of bicarbonate ion concentration (mEq/L) in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

DAYS GROUP	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	26.28 ^{aA} ± 0.24	28.87 ^{bA} ± 0.45	26.86 ^{aA} ± 0.49	29.10 ^{bA} ± 0.82
TREATMENT (HS)	27.75 ^{aB} ± 0.42	24.65 ^{bB} ± 0.48	25.11 ^{bB} ± 0.32	25.28 ^{bB} ± 0.58
p-value	$p < 0.01$ ($p = 0.003$)	$p < 0.001$	$p < 0.01$ ($p = 0.005$)	$p < 0.01$ ($p = 0.002$)

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly ($p < 0.05$)

4.3.2.2 Sodium Ion (Na^+) Concentration

The overall Na^+ level in the treatment group was 133.87 ± 0.48 mEq/L that was significantly lower ($p < 0.01$) than the Na^+ level of the control group which was 138.79 ± 0.48 mEq/L.

The mean values of Na^+ ion concentration on different days of study in both the control and treatment groups are represented in Table 4.7b and pictorially in Fig. 4.13.

Table 4.7b. Day wise comparison of sodium ion concentration (mEq/L) in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	137.50 ^{aA} ± 0.62	142.00 ^{aA} ± 1.59	138.17 ^{aA} ± 0.79	137.5 ^{aA} ± 0.563
TREATMENT (HS)	133.67 ^{aB} ± 0.55	134.88 ^{aB} ± 0.87	133.5 ^{aB} ± 0.76	133.5 ^{aB} ± 1.14
p-value	$p < 0.01$ ($p = 0.003$)	$p < 0.01$ ($p = 0.002$)	$p < 0.01$ ($p = 0.008$)	$p < 0.01$ ($p = 0.003$)

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly ($p < 0.05$)

The mean values of Na^+ did not differ significantly between different days in both the control group and treatment group. But the mean levels of Na^+ ion concentration on each day of the study period between the groups were highly significant ($p < 0.01$). Among those observations, lower value of Na^+ ion concentration was noticed on day one of heat exposure when compared to day one of thermo-neutral or comfort zone.

4.3.2.3 Potassium Ion (K^+) Concentration

There was no significant difference observed between the mean K^+ ion concentration of the control and treatment groups, represented in the Table 4.6. The observed value was 3.97 ± 0.05 mEq/L and 4.07 ± 0.05 mEq/L for the control and treatment groups respectively.

The mean values of K^+ observed on different days of study in both the control and treatment groups are represented in Table 4.7c and Fig. 4.14. Though there was no significant difference in the overall mean value of K^+ between thermo-neutral and heat stressed groups, the mean values of K^+ differed significantly between different days of both the control group and treatment group ($p < 0.05$). There was also high significant difference ($p < 0.01$) in the mean values found on 10th day between groups.

Table 4.7c. Day wise comparison of potassium ion concentration (mEq/L) in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	3.8 ^{aa} ± 0.07	4.03 ^{ba} ± 0.04	3.95 ^{ba} ± 0.02	4.13 ^{ba} ± 0.06
TREATMENT (HS)	4.18 ^{ab} ± 0.12	4.25 ^{aA} ± 0.11	4.08 ^{aA} ± 0.07	3.77 ^{bB} ± 0.02
p-value	$p < 0.05$ ($p = 0.018$)	$p = 0.206^{ns}$	$p = 0.121^{ns}$	$p < 0.01$ ($p = 0.003$)

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly ($p < 0.05$), ns – non- significant at 0.05 level

4.3.3 Blood Glucose Level

The overall mean blood glucose value in the acute heat stressed group was significantly lowered ($p < 0.001$) compared to the control group. The observed values were 63.91 ± 0.79 mg/dL and 66.96 ± 0.79 mg/dL for the treatment and control groups respectively which was displayed in Table 4.6.

The mean values of blood glucose on different days of study period in both the control and treatment groups are represented in Table 4.7d and pictorially in Fig. 4.15. The mean values differed significantly between different days of both control ($p < 0.05$) and treatment ($p < 0.001$) groups. In control group, the highest mean value was noticed on 10th day (70.67 ± 0.95 mg/dL) and the lowest value on day zero (64.00 ± 1.53 mg/dL). Hence it was observed that the blood glucose level was increased after ten days of exposure of crossbred calves to thermo-neutral zone.

Highly significant reduction ($p<0.01$) in blood glucose levels were also observed on different days of the treatment group. The mean glucose level was significantly lower ($p<0.01$) on the day one, five and 10 of heat exposure when compared to day zero.

In the present study, highly significant difference ($p<0.01$) was observed in blood glucose values on day zero, five and 10 between the groups with significantly declined level in the treatment group.

Table 4.7d. Day wise comparison of blood glucose level (mg/dL) in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	64.00 ^{aA} ± 1.53	66.33 ^{aA} ± 1.02	66.83 ^{aA} ± 1.25	70.67 ^{bA} ± 0.95
TREATMENT (HS)	70.00 ^{aB} ± 0.57	62.33 ^{bA} ± 2.60	61.33 ^{bB} ± 0.61	62.00 ^{bB} ± 0.51
p-value	$p<0.01$ ($p=0.003$)	$p=0.123^{ns}$	$p<0.05$ ($p=0.011$)	$p<0.001$

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly ($p<0.05$), ns – non- significant at 0.05 level

4.3.4 Blood pH

In the treatment group, the observed mean value of blood pH was 7.29 ± 0.02 which was significantly lower ($p<0.01$) than the control group pH value of 7.42 ± 0.004 .

The mean values of blood pH on different days of study in both the control and treatment groups are represented in Table 4.7e and pictorially in Fig. 4.12b. The mean values of blood pH differed significantly between day zero and different days of the treatment group ($p<0.05$).

Table 4.7e. Day wise comparison of blood pH in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10	Overall Mean
CONTROL (TNZ)	7.36 ^{aA} ± 0.02	7.44 ^{aA} ± 0.01	7.42 ^{aA} ± 0.005	7.45 ^{aA} ± 0.01	7.42 ^A ± 0.004
TREATMENT (HS)	7.40 ^{aA} ± 0.01	7.29 ^{bB} ± 0.01	7.26 ^{bB} ± 0.04	7.27 ^{bB} ± 0.02	7.29 ^B ± 0.02
p-value	p=0.063 ^{ns}	p<0.01 (p=0.002)	p<0.01 (p=0.008)	p<0.01 (p=0.002)	p<0.01 (p=0.005)

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly ($p < 0.05$), ns – non- significant at 0.05 level

In the present study, high significant difference ($p < 0.01$) was also observed in blood pH values on day one, five and 10 between the groups.

4.4 ENDOCRINE RESPONSE

4.4.1 Serum Cortisol

The overall mean value of serum cortisol level between thermo-neutral and acute heat stressed groups are presented in Table 4.8 and in Fig. 4.16a. The serum cortisol level of acute heat stressed condition was significantly higher ($p < 0.001$) when compared to thermo-neutral condition. The observed values are 8.28 ± 0.38 ng/mL and 14.64 ± 0.78 ng/mL for the control and treatment groups respectively.

Table 4.8. Overall serum cortisol levels (ng/mL) of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ PARAMETER	CONTROL (TNZ)	TREATMENT (HS)	p – value
Serum Cortisol (ng/mL)	8.28 ^a ± 0.38	14.64 ^b ± 0.78	p<0.001

Means bearing different superscripts within a row (a-b) differ significantly ($p < 0.01$)

The mean values of serum cortisol on different days of the study period in both the control and treatment groups are represented in Table 4.8a and pictorially displayed in Fig. 4.16b.

Table 4.8a. Day wise comparison of serum cortisol level (ng/mL) in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	16.53 ^{aA} ± 0.83	9.008 ^{bA} ± 0.48	3.61 ^{cA} ± 0.77	3.98 ^{cA} ± 0.60
TREATMENT (HS)	5.89 ^{aB} ± 1.56	21.89 ^{bB} ± 1.93	23.28 ^{bB} ± 0.96	7.48 ^{cA} ± 1.61
p-value	p<0.01 (p=0.001)	p<0.01 (p=0.002)	p<0.001	p=0.097 ^{ns}

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

The mean values differed significantly between different days of both control and treatment groups. In thermo-neutral group, the highest mean value of serum cortisol was noticed on day zero (16.53 $\pm$ 0.83 ng/mL). There was significant decline in serum cortisol levels throughout the experimental days of TNZ.

High significant difference (p<0.01) was also observed on different days of the treatment group. The mean serum cortisol level was significantly higher (p<0.01) on day one of sudden heat exposure when compared to day zero. It was further increased on day five of heat stress. There was a significant dip (p<0.01) in serum cortisol level on 10th day of HS when compared to all other days of treatment. The observed mean values of serum cortisol in the treatment group on day zero, one, five and ten were 5.89 $\pm$ 1.56 ng/mL, 21.89 $\pm$ 1.93 ng/mL, 23.28 $\pm$ 0.96 ng/mL and 7.48 $\pm$ 1.614 ng/mL respectively.

Furthermore, high significant difference ($p<0.01$) was observed in serum cortisol values of day zero, one and five between the groups. There was no significant difference found between the 10th day of the groups.

4.5 ANTIOXIDANT STATUS

The antioxidant status was interpreted by analysing glutathione peroxidase (GPx) activity and estimation of total antioxidant capacity (TAC) by ferric reducing ability of plasma (FRAP) assay. The overall mean values of antioxidant status parameters in both the groups are presented in Table 4.9 and shown pictorially in Fig. 4.17a and 4.17b.

Table 4.9. Overall antioxidant status of crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP STATUS	CONTROL (TNZ)	TREATMENT (HS)	p – value
GPx (U/L of Haemolysate)	9.23 ^a $\pm$ 0.23	15.28 ^b $\pm$ 0.641	$p<0.001$
FRAP (μ M)	1068.67 ^a $\pm$ 24.65	2212.77 ^b $\pm$ 89.59	$p<0.001$

Means bearing different superscripts within a row (a-b) differ significantly ($p<0.01$)

4.5.1 Glutathione Peroxidase Activity (GPx)

The overall mean GPx values observed in the control and treatment group were presented in Table 4.9. The mean GPx value was significantly higher ($p<0.001$) in the acute heat stressed group when compared to the control group.

The mean values of GPx on different days of the study period in both the groups are represented in Table 4.9a and Fig. 4.18.

The mean GPx values did not differ significantly between different days of the control group. Whereas in heat stressed group, the mean values differed significantly between different days. The GPx value was gradually increased from day one of heat exposure to day ten. The highest GPx value of 21.33 $\pm$ 2.13 U/L of

Haemolysate was recorded on the 10th day of heat exposure and the lowest value of 9.17 ± 0.26 U/L of Haemolysate on day zero or before exposure.

Table 4.9a. Day wise comparison of glutathione peroxidase activity (U/L of Haemolysate) in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	9.13 ^{aA} ± 0.33	9.37 ^{aA} ± 0.63	9.25 ^{aA} ± 0.44	9.158 ^{aA} ± 0.32
TREATMENT (HS)	9.17 ^{aA} ± 0.263	13.83 ^{bB} ± 0.95	16.80 ^{bB} ± 1.014	21.33 ^{cB} ± 2.13
p-value	p=0.917 ^{ns}	p<0.01 (p=0.004)	p<0.001	p<0.01 (p=0.002)

Means bearing different superscripts within a row (a-c) and columns (A-B) differ significantly ($p < 0.05$), ns – non- significant at 0.05 level

There were also high significant differences ($p < 0.01$) found in mean GPx values of days one, five and ten between the groups. There was no significant difference ($p > 0.05$) found between the day zero of the groups.

4.5.2 Total Antioxidant Capacity (TAC)

The overall mean TAC value measured as ferric reducing ability of plasma (FRAP) in thermo-neutral and heat stressed groups are presented in Table 4.9. The mean FRAP value was significantly higher ($p < 0.001$) in the treatment group when compared to the control group. The observed mean FRAP values of the control and treatment groups were $1068.67 \pm 24.65 \mu\text{M}$ and $2212.77 \pm 89.59 \mu\text{M}$ respectively.

The mean FRAP values on different days of the study period in both the groups are represented in Table 4.9b and Fig. 4.19.

The mean FRAP values differ significantly between day zero and 10 of the control group. Whereas in heat stressed group, the mean values differed significantly between different days of heat exposure.

Table 4.9b. Day wise comparison of Ferric Reducing Ability of Plasma (μM) in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUP \ DAYS	Day 0	Day 1	Day 5	Day 10
CONTROL (TNZ)	971.145 ^{aA} ± 25.85	1028.83 ^{aA} ± 28.07	1043.81 ^{aA} ± 54.76	1230.86 ^{bA} ± 72.57
TREATMENT (HS)	1221.89 ^{aA} ± 134.89	1764.92 ^{aB} ± 212.1	2675.79 ^{bB} ± 237.06	3188.45 ^{bB} ± 95.15
p-value	p=0.123 ^{ns}	p<0.001	p<0.001	p<0.001

Means bearing different superscripts within a row (a-b) and columns (A-B) differ significantly ($p < 0.05$), ns – non- significant at 0.05 level

The FRAP value was gradually increased from day one of heat exposure to day ten. The highest mean FRAP value of $3188.45 \pm 95.15 \mu\text{M}$ was recorded on the 10th day of heat stress and the lowest value of $1221.89 \pm 134.89 \mu\text{M}$ on day zero of the treatment group. Hence the TAC was increasing day by day of heat exposure whereas it remained constant from days one to 10 in thermo-neutral conditions.

There were also high significant differences ($p < 0.01$) found in FRAP values of days one, five and ten between the groups. There was no significant difference ($p > 0.05$) found in day zero between the groups.

4.6 MOLECULAR RESPONSES

4.6.1 Relative Expression of Heat Shock Protein 70 (HSP-70) Gene

The mean values of C_T , ΔC_T , $\Delta\Delta C_T$ and fold change ($2^{-\Delta\Delta C_T}$) in *HSP-70* gene expression of crossbred calves in both the control and treatment groups are presented in Table 4.10. The fold change of *HSP-70* gene expression is presented pictorially in Fig. 4.20. Relative quantification of *HSP-70* gene expression in peripheral blood leukocytes showed a significant ($p < 0.05$) 2.98 fold upregulation in crossbred calves under acute heat stress when compared to thermo-neutral condition.

Table 4.10. Cycle threshold values and fold change in *HSP-70* expression in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUPS	Mean C _T $\pm$ SE		Δ C _T	$\Delta\Delta$ C _T	Fold change (2 ^{-$\Delta\Delta$C_T})	p-value
	<i>HSP-70</i>	<i>GAPDH</i>				
CONTROL (TNZ)	20.78 $\pm$ 0.28	23.07 $\pm$ 0.38	-2.28 $\pm$ 0.25	0.00 $\pm$ 0.25	1.00 ^A	p<0.05 (p=0.015)
TREATMENT (HS)	19.24 $\pm$ 0.24	23.11 $\pm$ 0.35	-3.86 $\pm$ 0.33	-1.58 $\pm$ 0.33	2.98 ^B	

Means bearing different superscripts within columns (A-B) differ significantly (p<0.05)

Table 4.11. Cycle threshold values and fold change in *IGF-1* expression in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, n=6).

GROUPS	Mean C _T $\pm$ SE		Δ C _T	$\Delta\Delta$ C _T	Fold change (2 ^{-$\Delta\Delta$C_T})	p-value
	<i>IGF-1</i>	<i>GAPDH</i>				
CONTROL (TNZ)	30.48 $\pm$ 0.21	22.75 $\pm$ 0.25	7.40 $\pm$ 0.16	0.00 $\pm$ 0.16	1.00 ^A	p=0.077 ^{ns}
TREATMENT (HS)	30.10 $\pm$ 0.44	22.18 $\pm$ 0.37	7.92 $\pm$ 0.51	0.48 $\pm$ 0.51	0.71 ^A	

Means bearing same superscript within columns did not differ significantly, ns - Significant at 0.05 level

4.6.2 Relative Expression of Insulin like Growth Factor 1 (*IGF-1*) Gene

The mean values of C_T , ΔC_T , $\Delta\Delta C_T$ and fold change ($2^{-\Delta\Delta C_T}$) in *IGF-1* gene expression of crossbred calves in thermo-neutral zone and acute heat stressed conditions are presented in Table 4.10. The fold change of *IGF-1* gene expression is presented pictorially in Fig. 4.20. Relative quantification of *IGF-1* gene expression in peripheral blood leukocytes shown a non-significant ($p>0.05$) 0.71fold downregulation in crossbred calves exposed to heat stress when compared to thermo-neutral group.

4.7 CORRELATION STUDIES

4.7.1 Correlation between Physiological Variables in Heat Stress

The Spearman's correlation coefficient of rectal temperature (RT) with respiratory rate (RR) and heart rate (HR) under acute heat stress are presented in Table 4.13. There was a strong positive correlation (significant at 0.01 level) between various physiological variables studied such as RT, RR and HR.

4.7.2 Correlation of Respiratory Rate with Blood Gases and Bicarbonate Level in Heat Stress

The Spearman's correlation coefficient of respiratory rate (RR) with blood gases and bicarbonate level under acute heat stress are represented in Table 4.14. There was a strong positive correlation between respiratory rate and partial pressure of oxygen (pO_2) in heat stress (significant at 0.01 level). Also, there was strong negative correlation between RR and partial pressure of carbon dioxide (pCO_2). Furthermore, there was strong negative correlation between RR and bicarbonate level under acute heat-stressed conditions.

4.7.3 Correlation of Rectal Temperature with Antioxidant Status in Heat Stress

Pearson's correlation coefficient of rectal temperature with antioxidant status parameters such as glutathione peroxidase (GPx) level and total antioxidant capacity measured as FRAP values are presented in Table 4.12. There was strong positive correlation of RT with GPx and FRAP values (significant at 0.01 level) under heat stress in the climatic chamber.

Table 4.12. Spearman's correlation coefficient of physiological variables in crossbred calves in heat stress.

PHYSIOLOGICAL VARIABLES			Rectal temperature (°C)	Respiratory rate (breaths/min)	Heart rate (beats/min)
Spearman's correlation coefficient	Rectal temperature	Correlation Coefficient	1.000	0.655**	0.634**
	Respiratory rate	Correlation Coefficient	0.655**	1.000	0.533**
	Heart rate	Correlation Coefficient	0.634**	0.533**	1.000

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

Table 4.13. Spearman's correlation coefficient of respiratory rate with blood gases and bicarbonate level under heat stress.

Spearman's correlation coefficient		pO ₂	pCO ₂	HCO ₃ ⁻
Respiratory rate (breaths/min)	Correlation Coefficient	0.596**	-0.617**	-0.621**
	Sig. (2-tailed)	0.000	0.000	0.000

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

Table 4.14. Pearson's correlation coefficient of rectal temperature with antioxidant status under heat stress.

Pearson's correlation coefficient		GPx	FRAP
Rectal temperature	Correlation Coefficient	0.635**	0.638**
	Sig. (2-tailed)	0.001	0.001

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

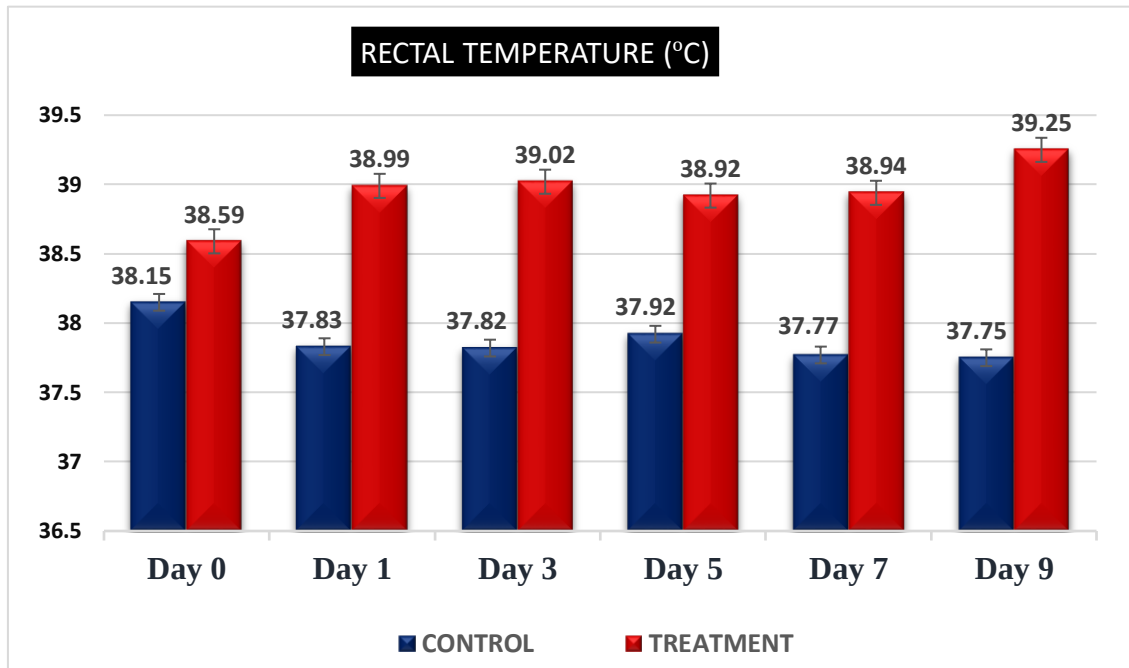


Fig. 4.1a. Day wise comparison of rectal temperature (°C) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

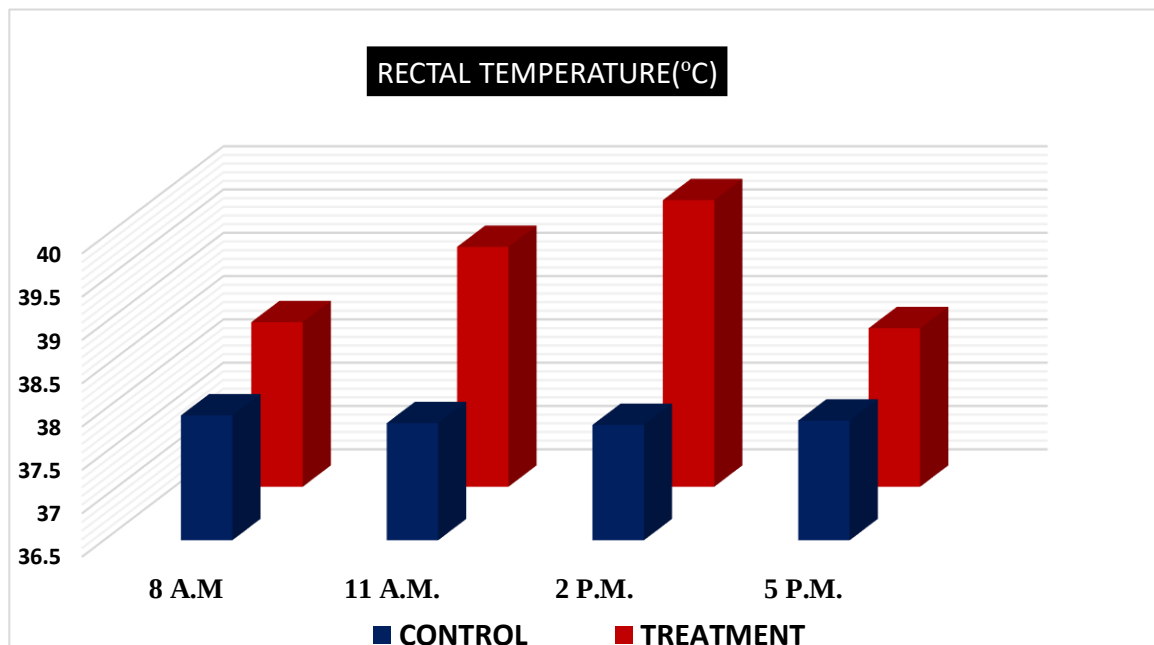


Fig. 4.1b. Time wise comparison of rectal temperature (°C) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

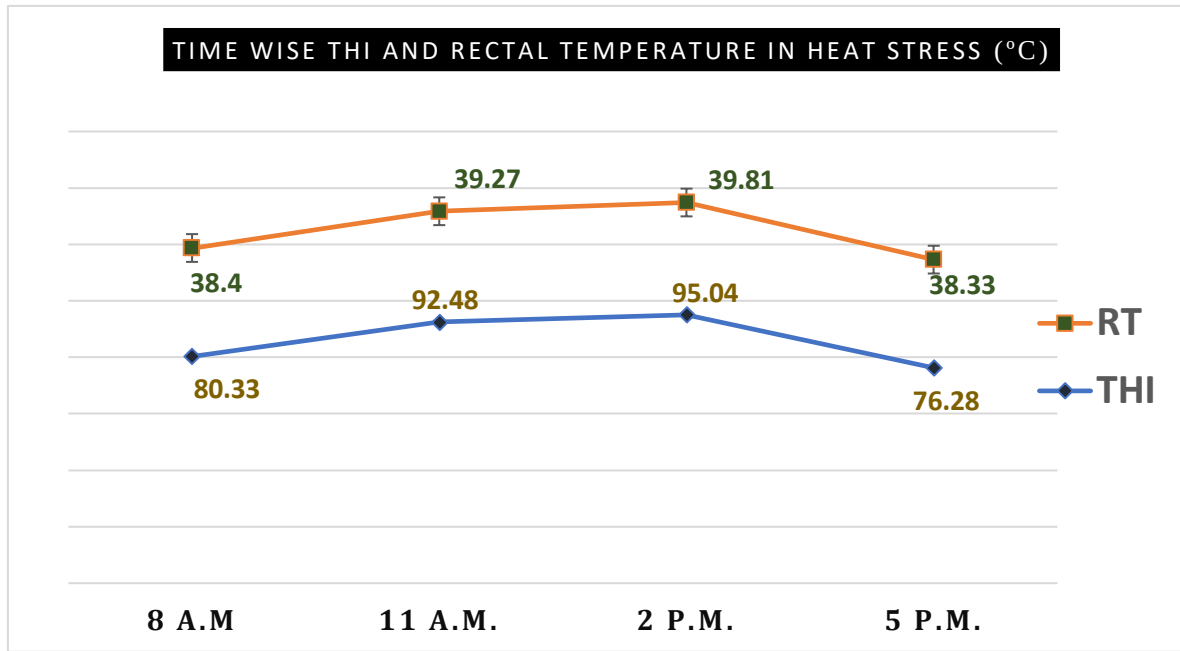


Fig. 4.1c. Time wise recordings of rectal temperature (°C) of crossbred calves to varying THI under acute heat stress.

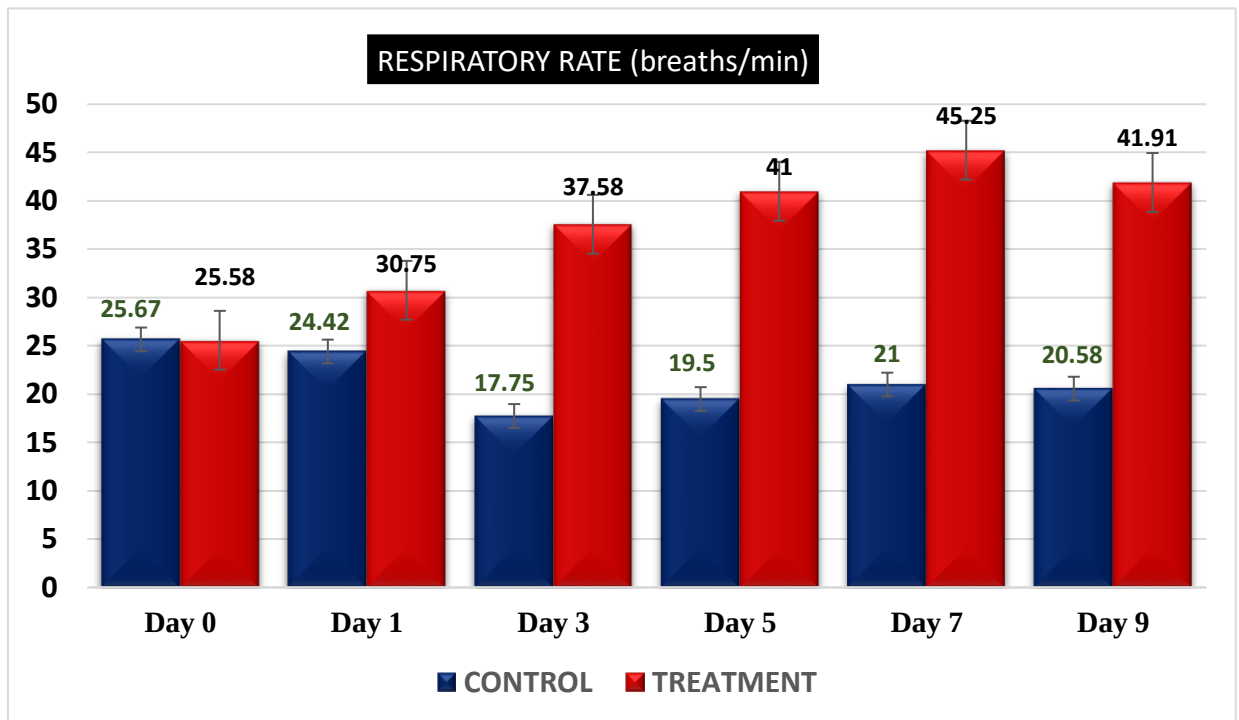


Fig. 4.2a. Day wise comparison of respiratory rate (breaths/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

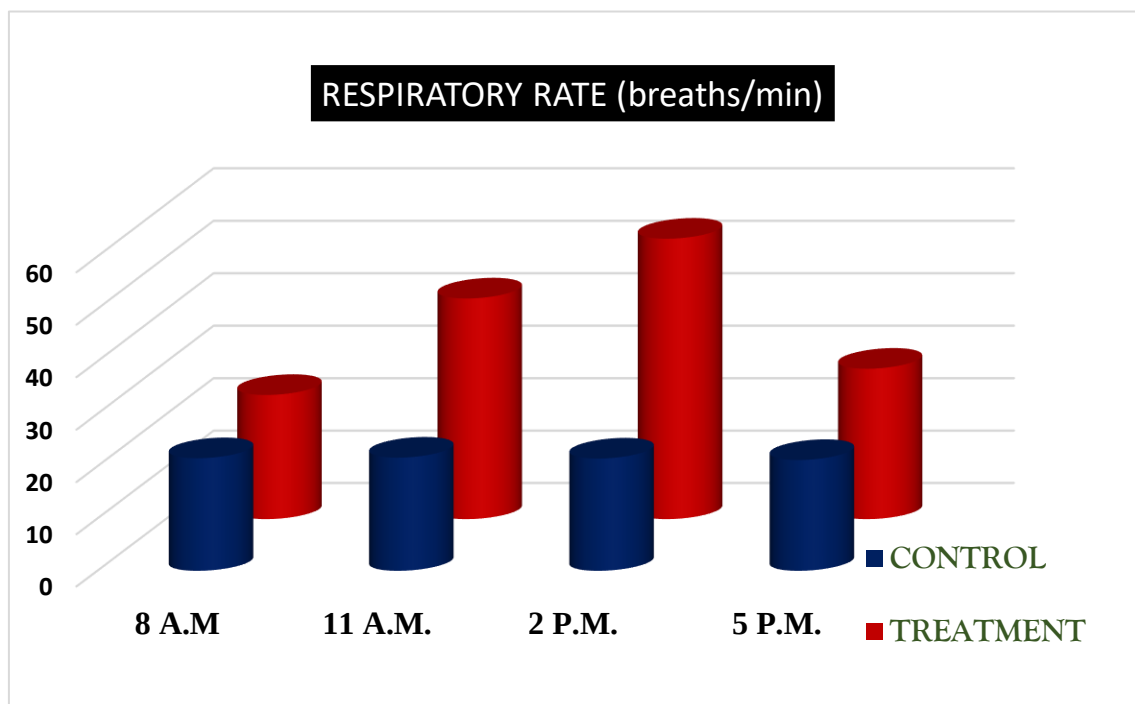


Fig. 4.2b. Time wise comparison of respiratory rate (breaths/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

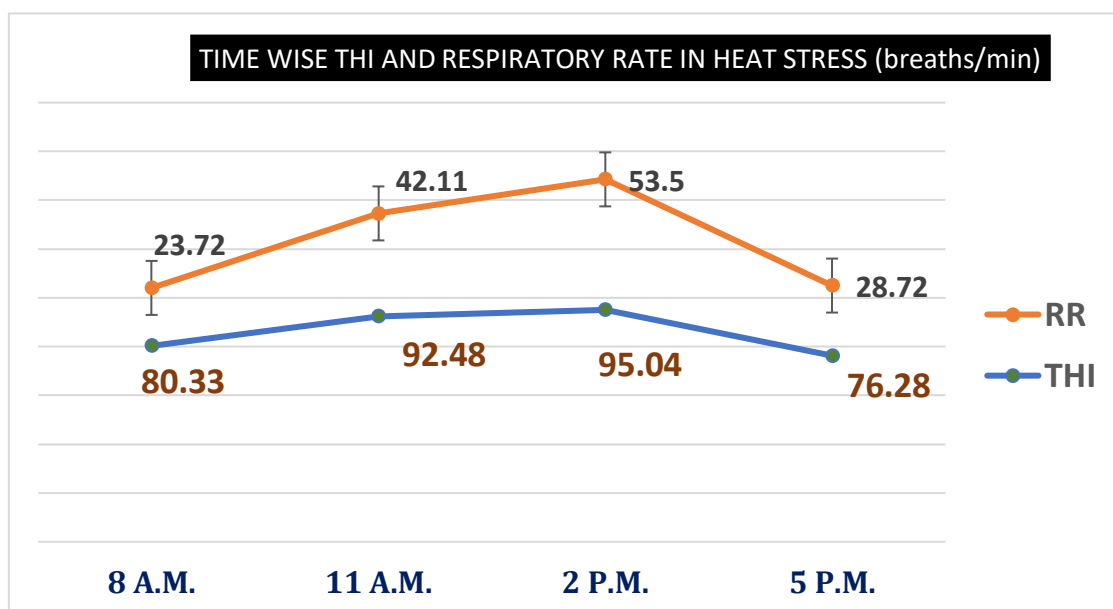


Fig. 4.2c. Time wise recordings of respiratory rate of crossbred calves to varying THI under acute heat stress.

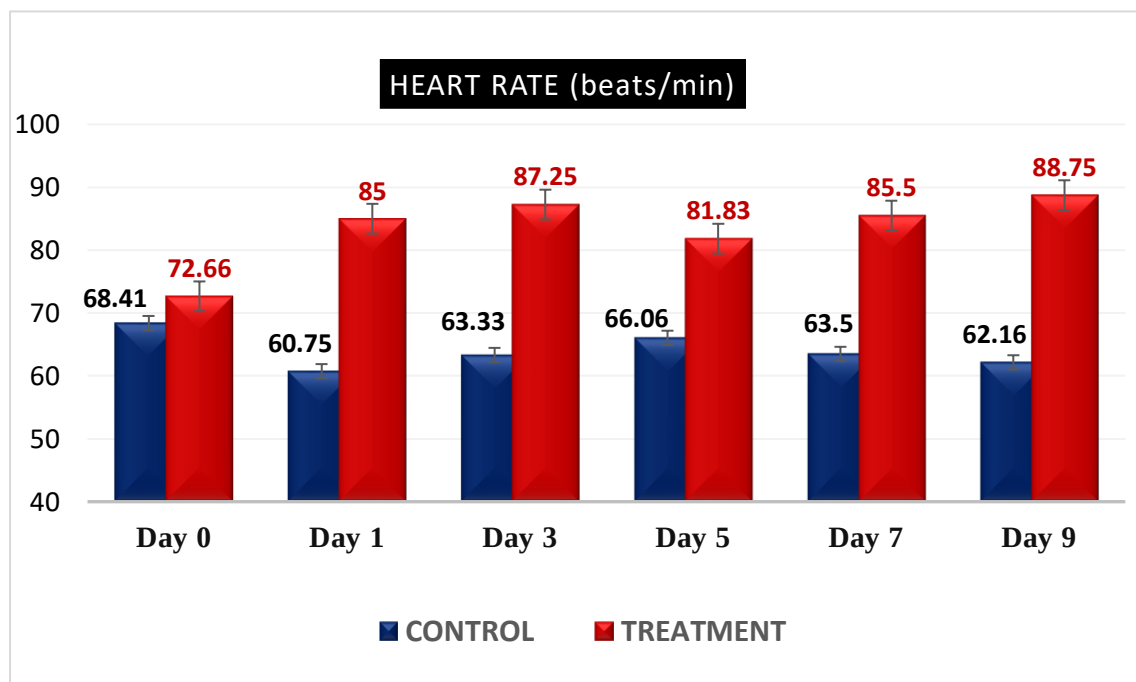


Fig. 4.3a. Day wise comparison of heart rate (beats/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

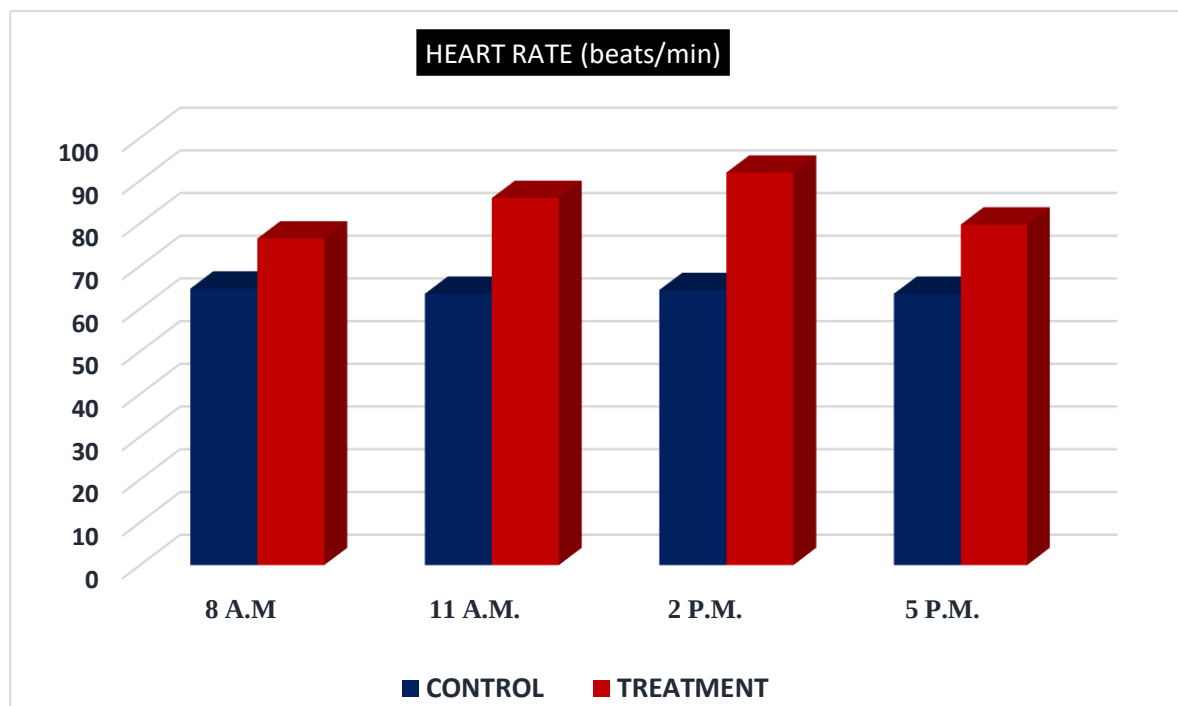


Fig. 4.3b. Time wise comparison of heart rate (beats/min) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

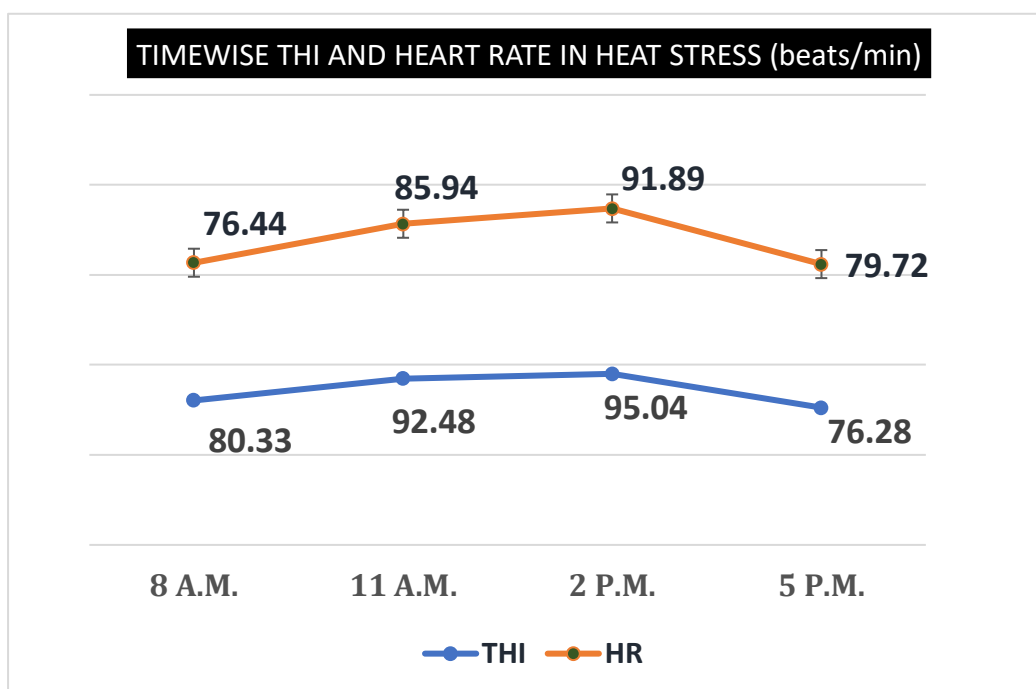


Fig. 4.3c. Time wise recordings of heart rate of crossbred calves to varying THI under acute heat stress.

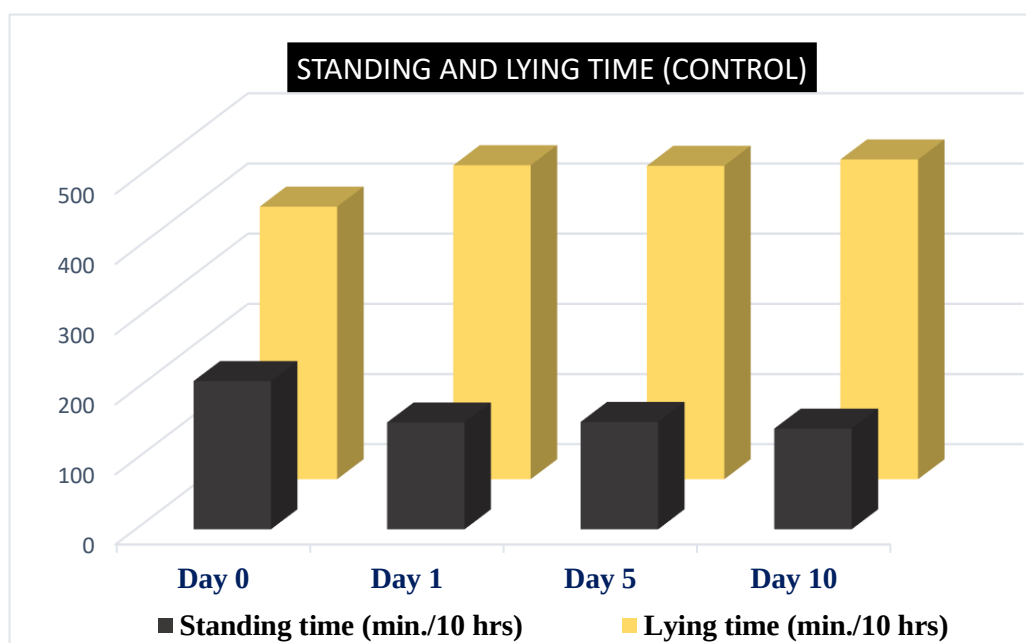


Fig. 4.4a. Comparison of standing time and lying time of crossbred calves under thermo-neutral zone.

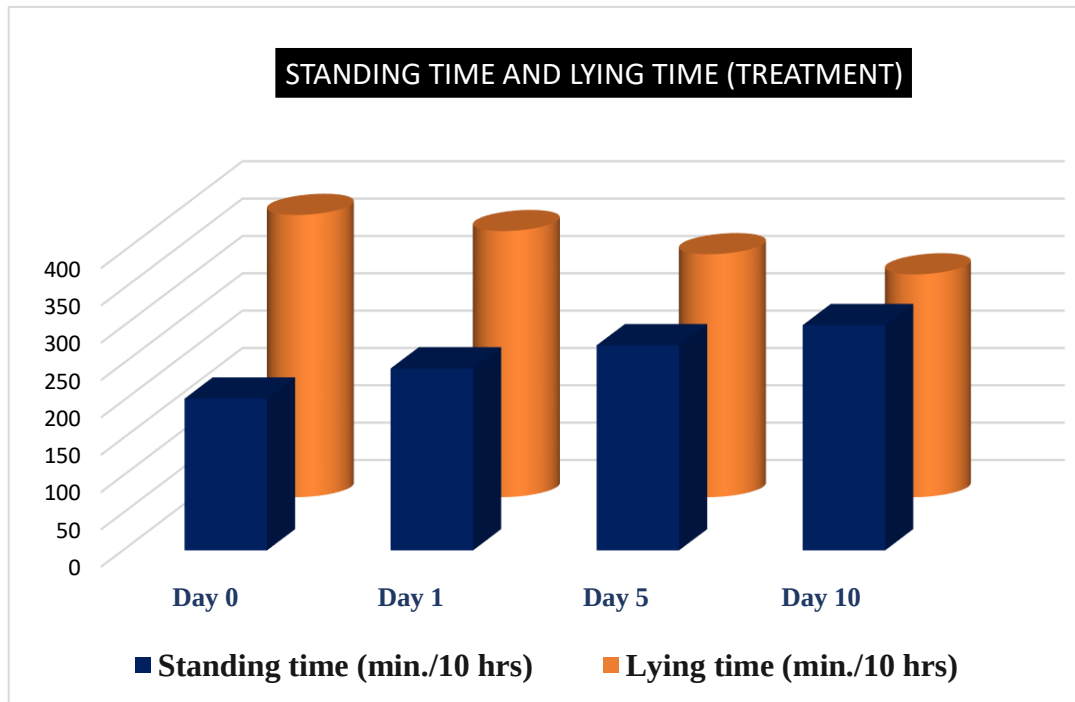


Fig. 4.4b. Comparison of standing time and lying time of crossbred calves under acute heat stress.

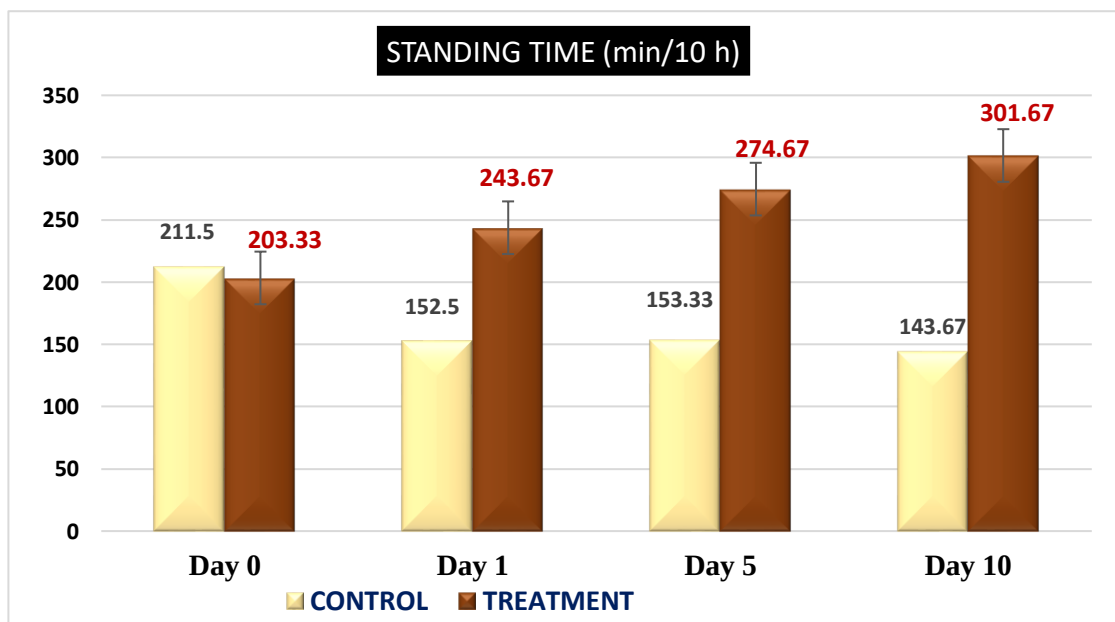


Fig. 4.4c. Day wise comparison of standing time (min/10 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

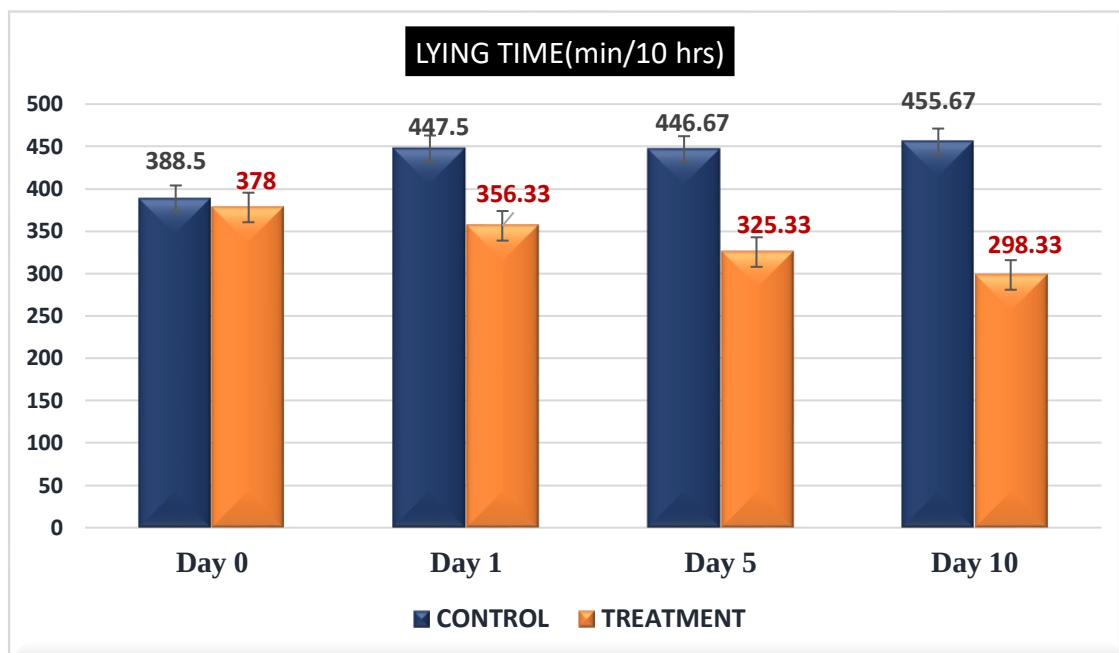


Fig. 4.4d. Day wise comparison of lying time (min/10 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

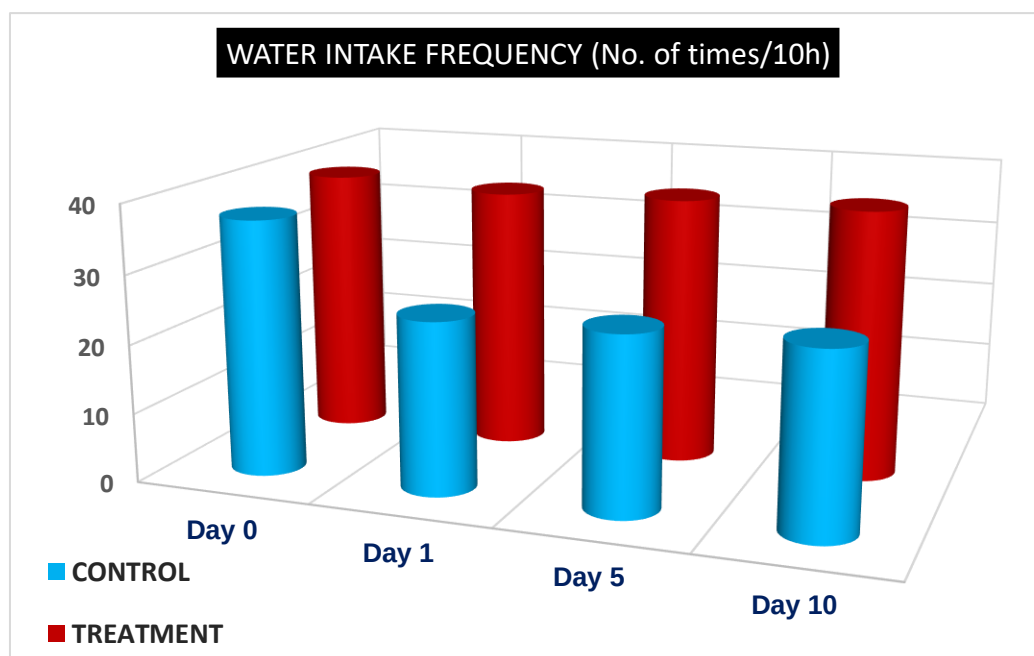


Fig. 4.5. Day wise comparison of water intake frequency (No. of times/10 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

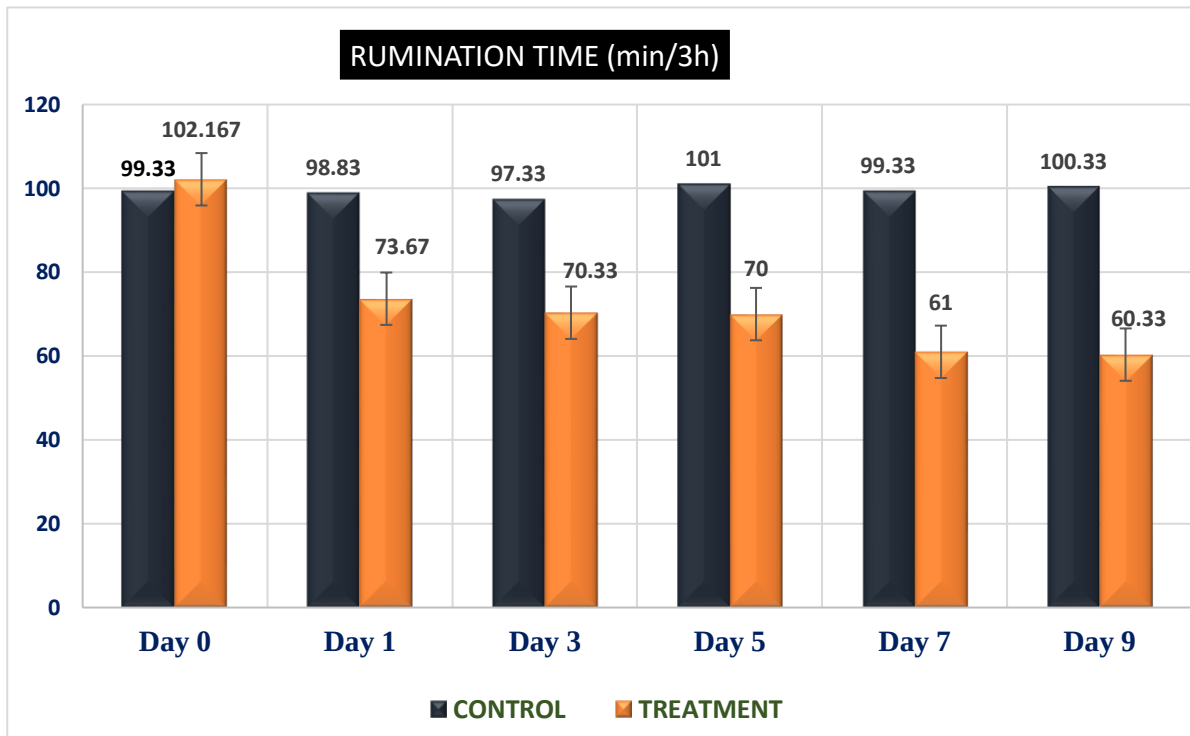


Fig. 4.6. Day wise comparison of rumination time (min/3 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

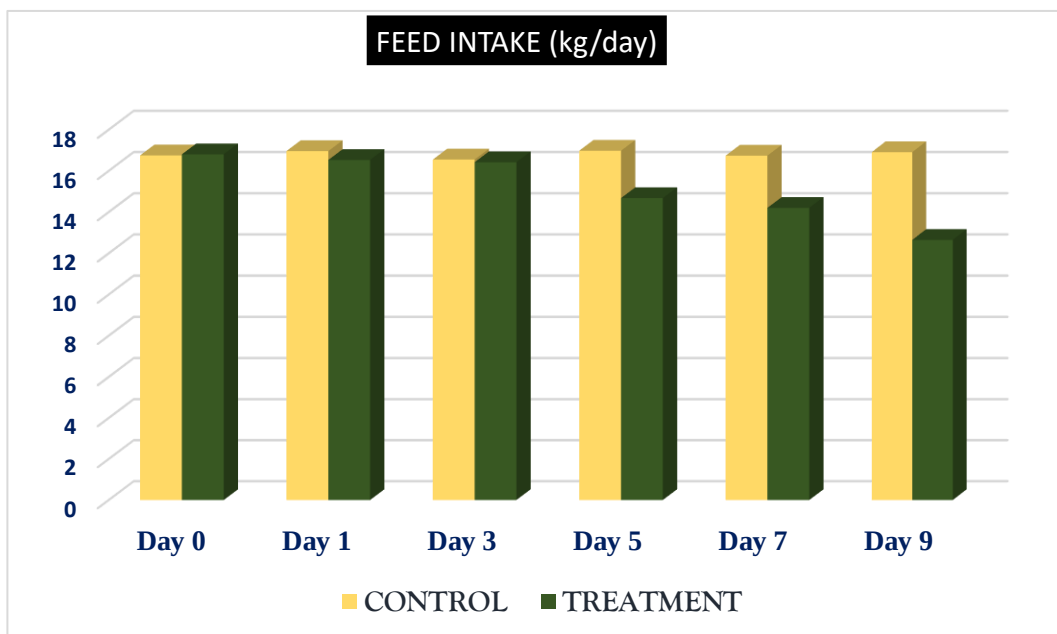


Fig. 4.7. Day wise comparison of feed intake (kg/day) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

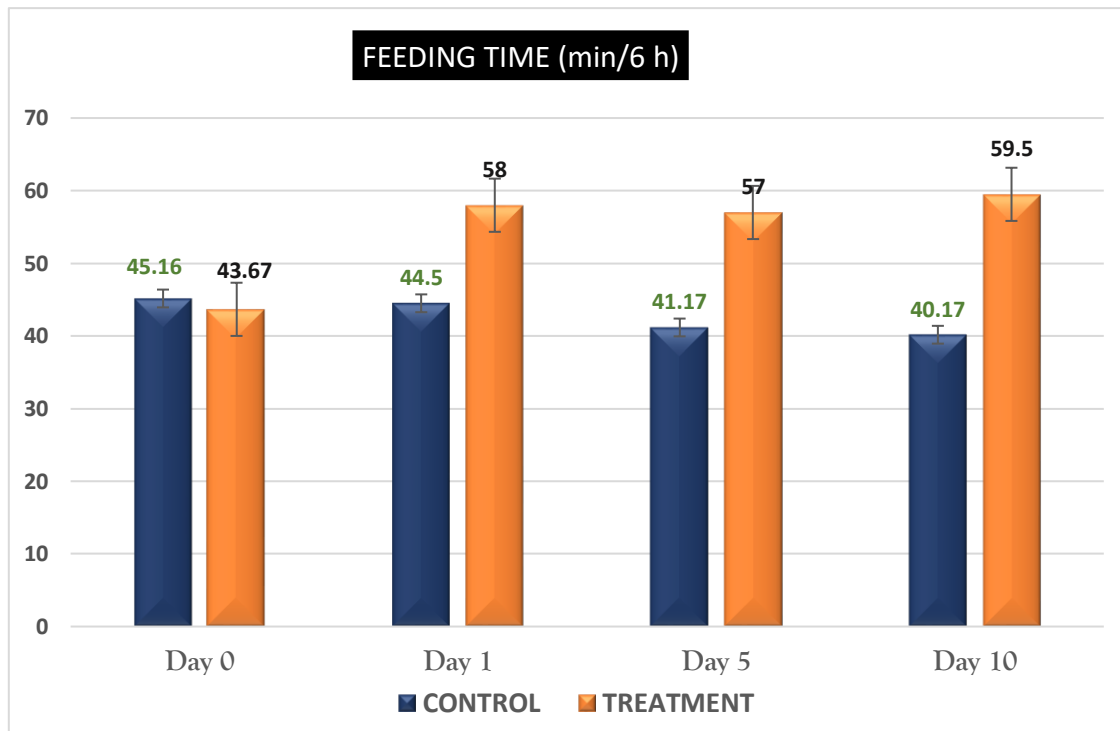


Fig. 4.8. Day wise comparison of feeding time (min/6 h) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

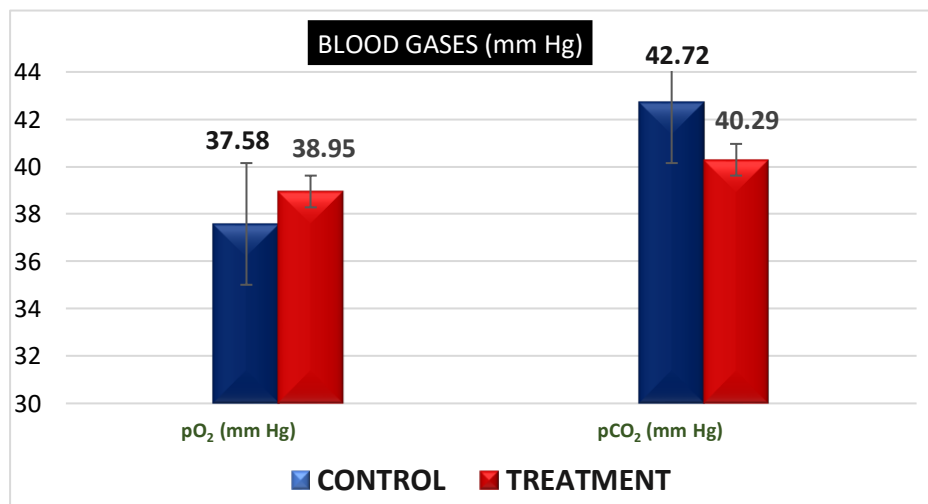


Fig. 4.9a. Blood gas level of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

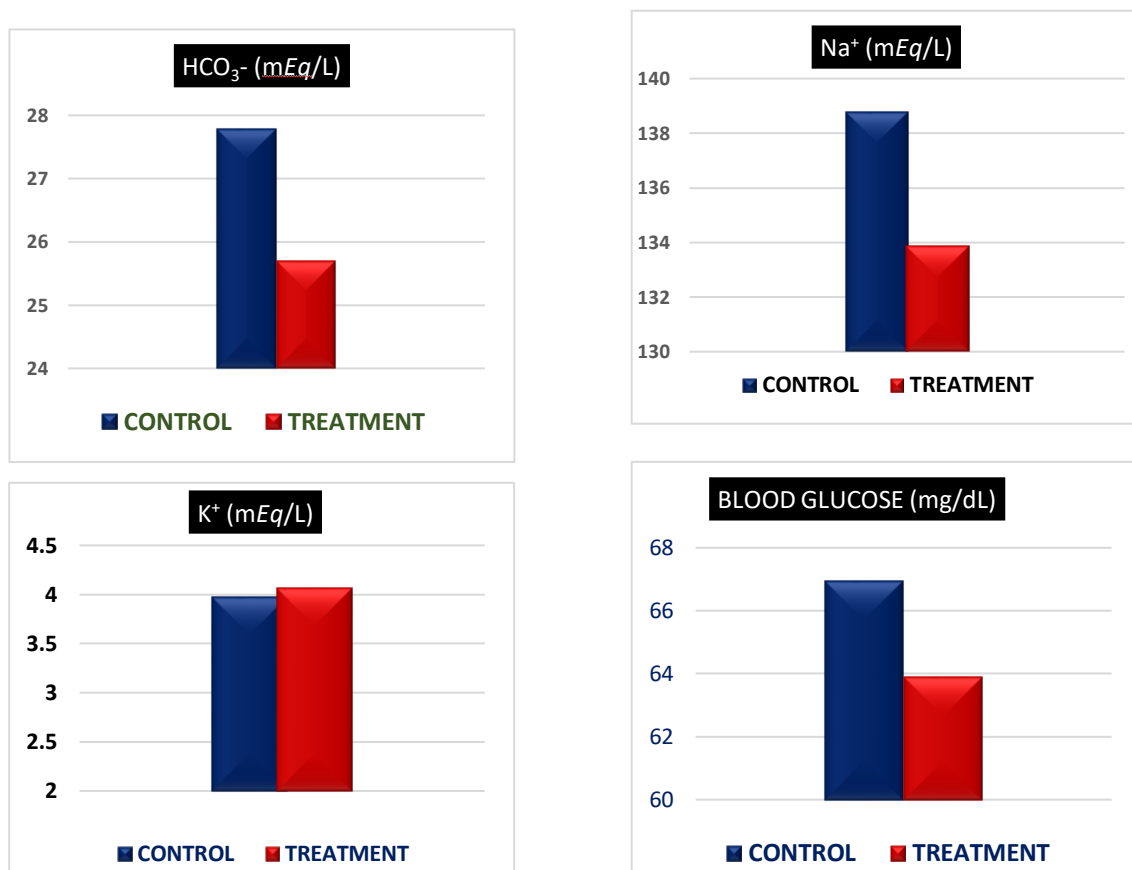


Fig. 4.9b. Blood electrolytes level of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

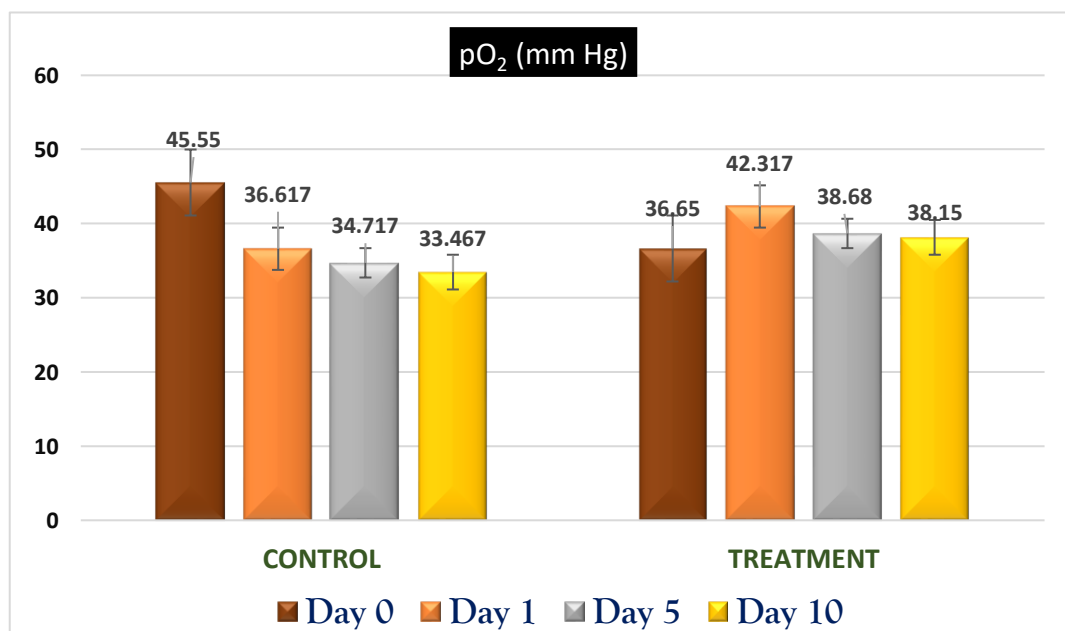


Fig. 4.10. Day wise comparison of partial pressure of oxygen (mm Hg) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

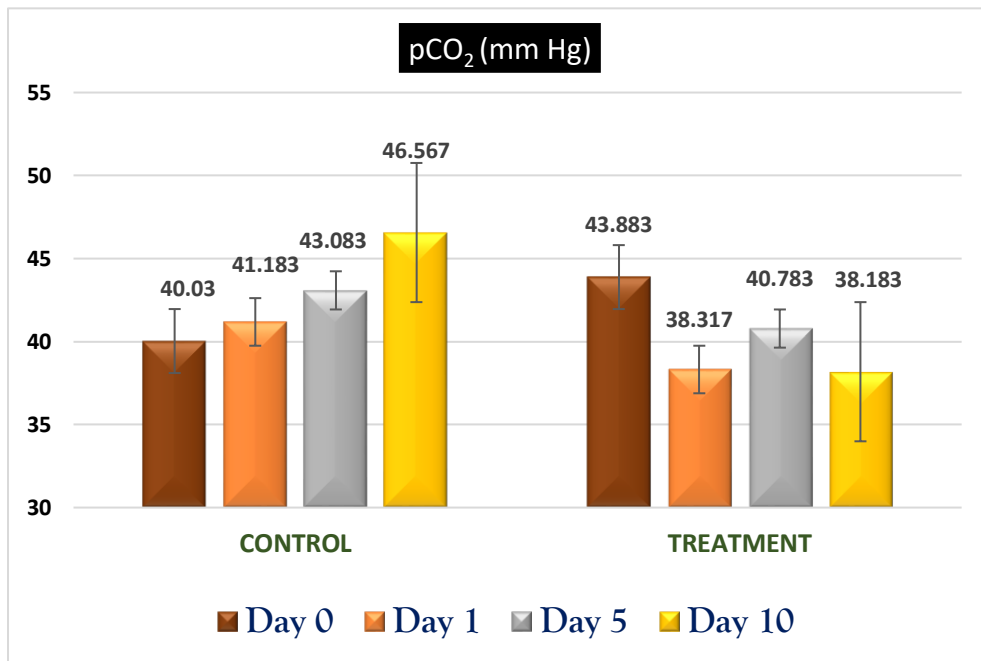


Fig. 4.11. Day wise comparison of partial pressure of carbon dioxide (mm Hg) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

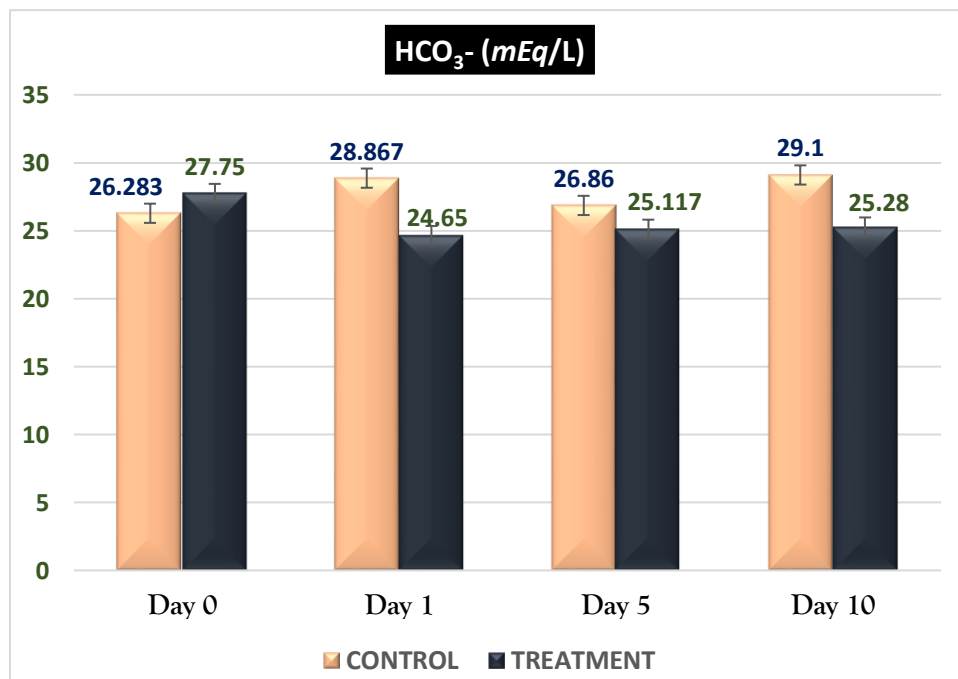


Fig. 4.12a. Day wise comparison of bicarbonate ion concentration (mEq/L) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

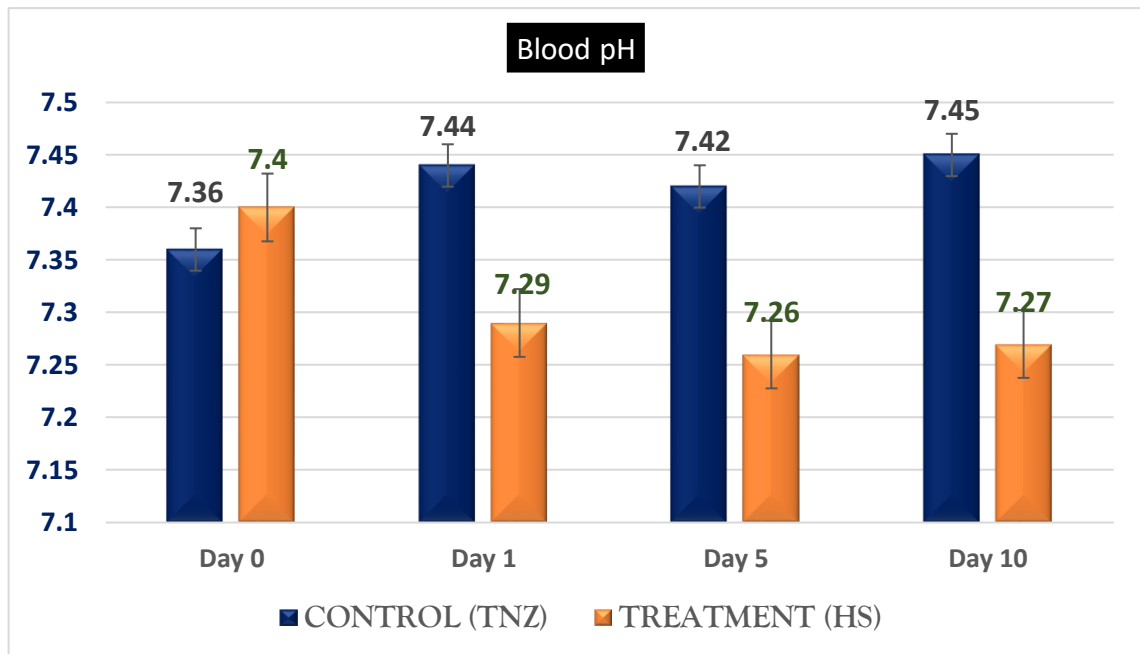


Fig. 4.12b. Day wise comparison of blood pH of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

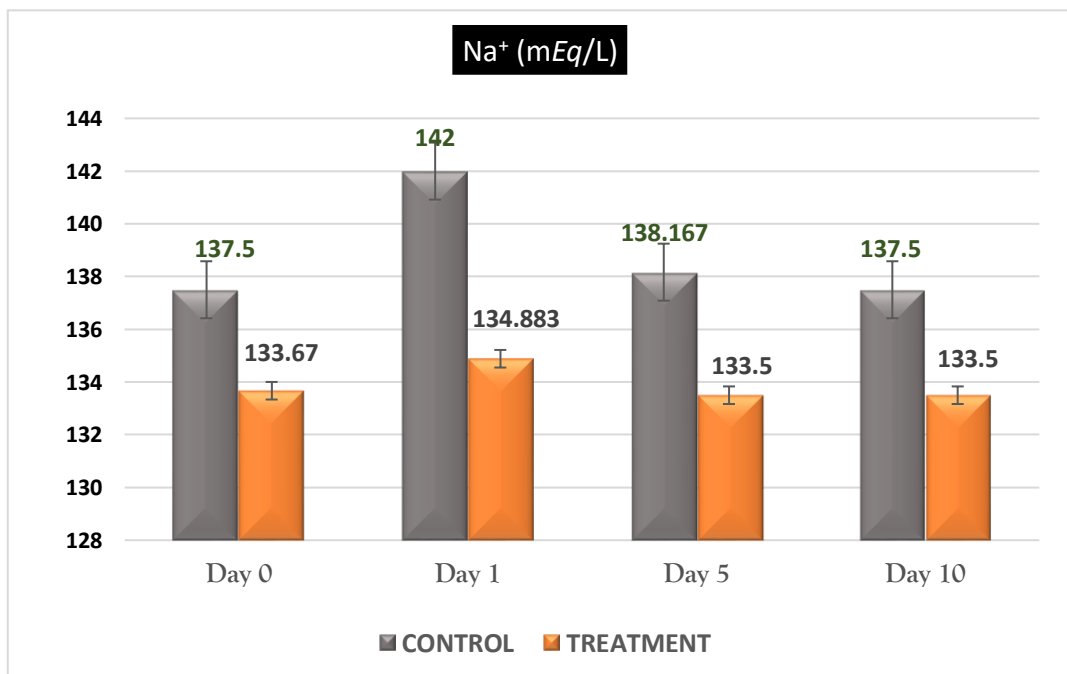


Fig. 4.13. Day wise comparison of sodium ion concentration (mEq/L) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

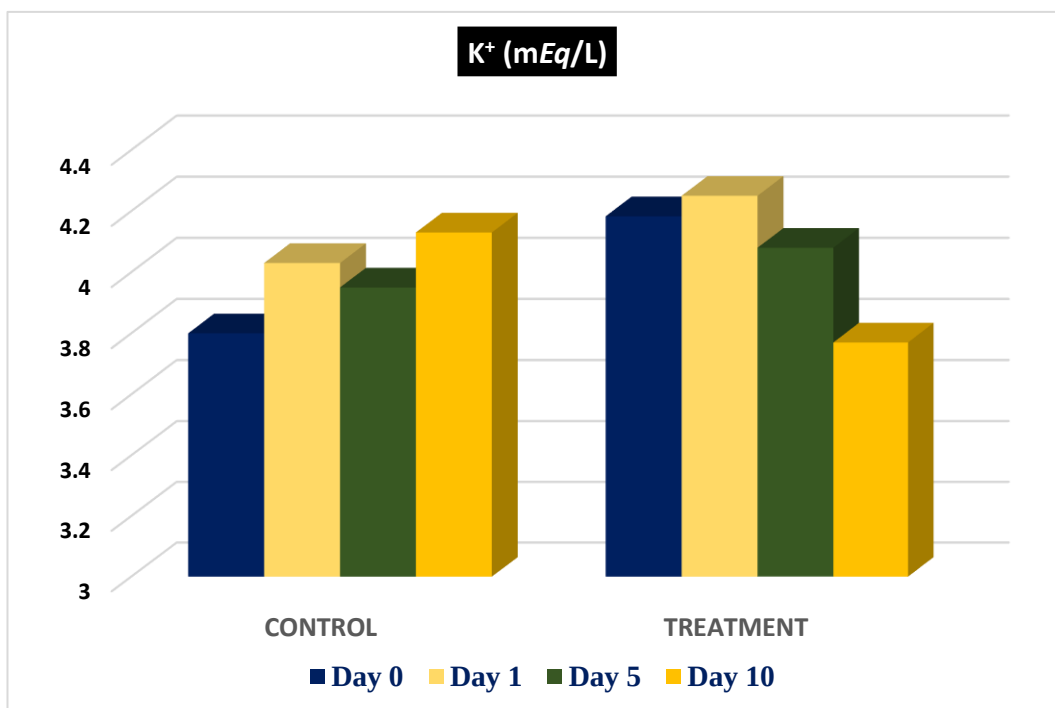


Fig. 4.14. Day wise comparison of potassium ion concentration (mEq/L) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

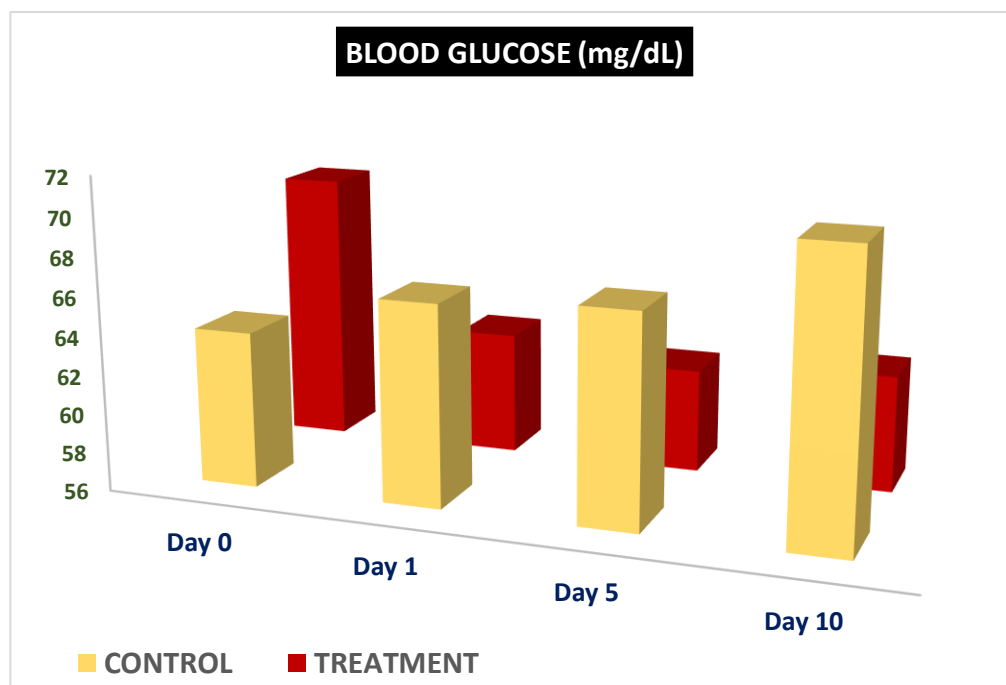


Fig. 4.15. Day wise comparison of blood glucose level (mg/dL) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

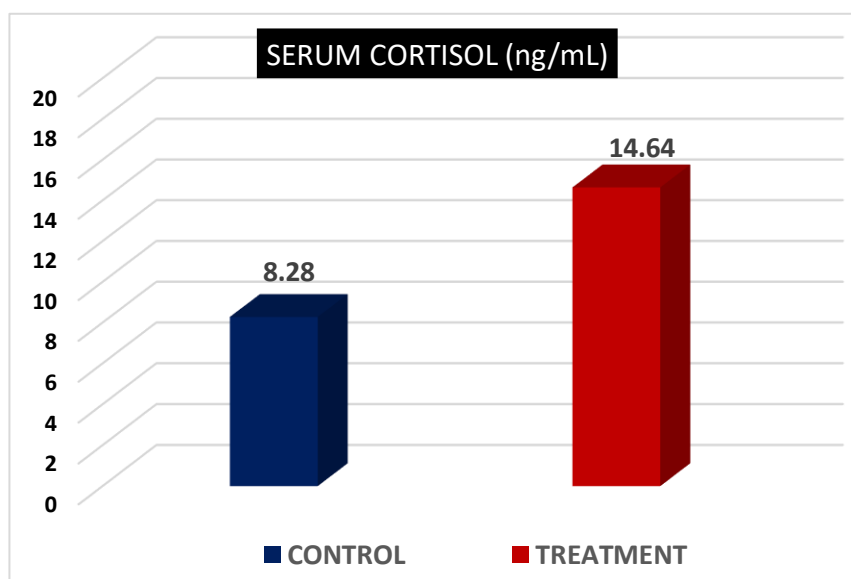


Fig. 4.16a. Serum cortisol level of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

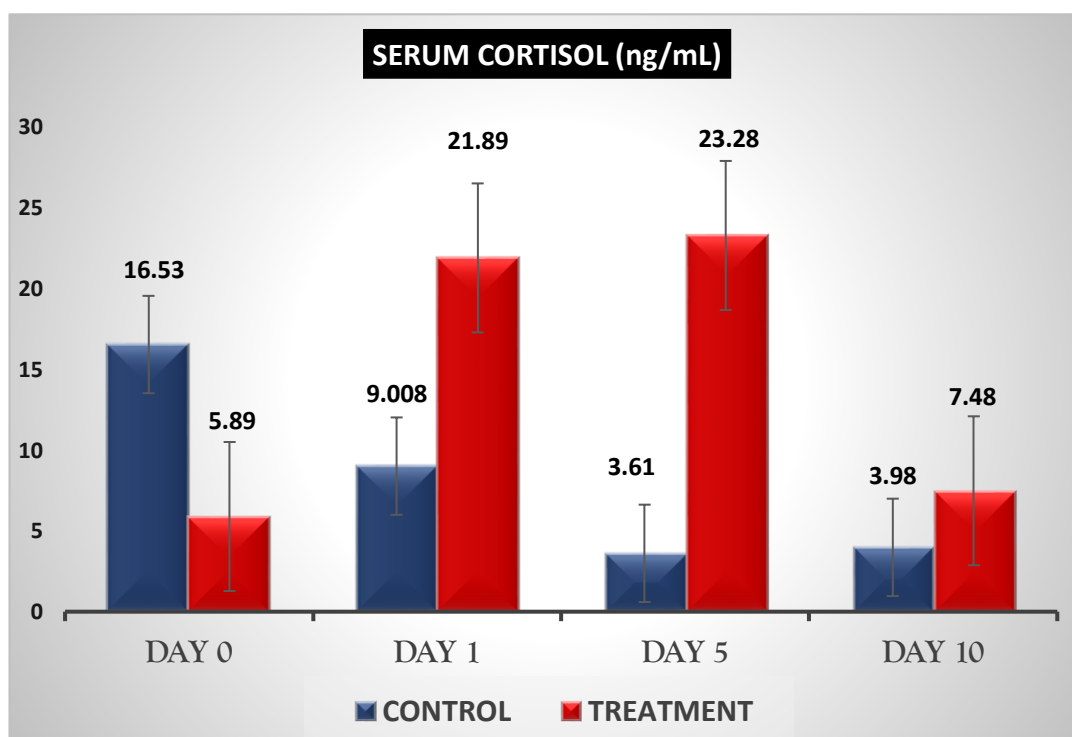


Fig. 4.16b. Day wise comparison of serum cortisol level (ng/mL) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

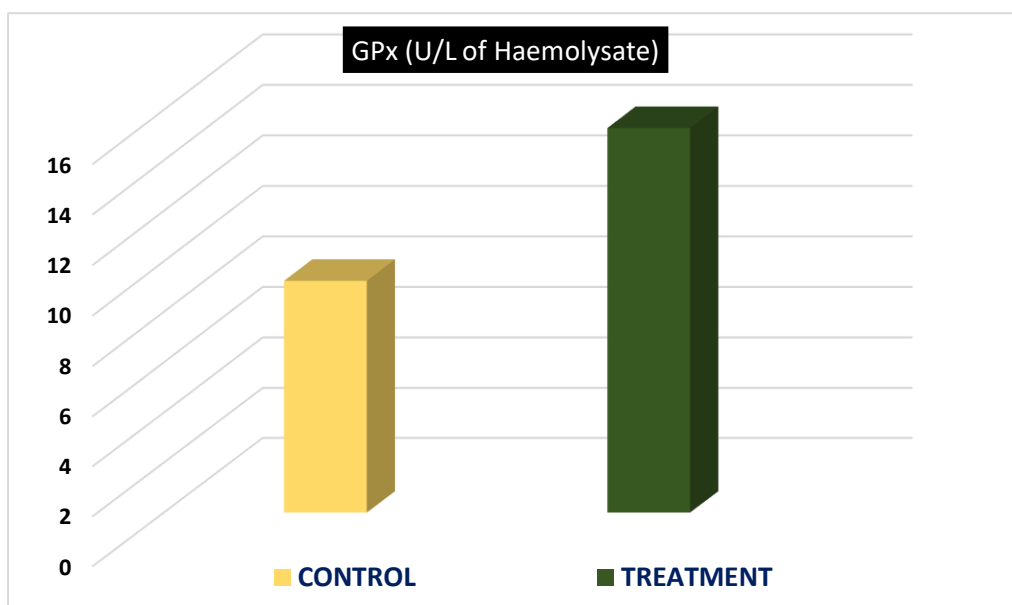


Fig. 4.17a. Glutathione peroxidase level (U/L of Haemolysate) of crossbred calves under thermo-neutral zone and acute heat stressed conditions.

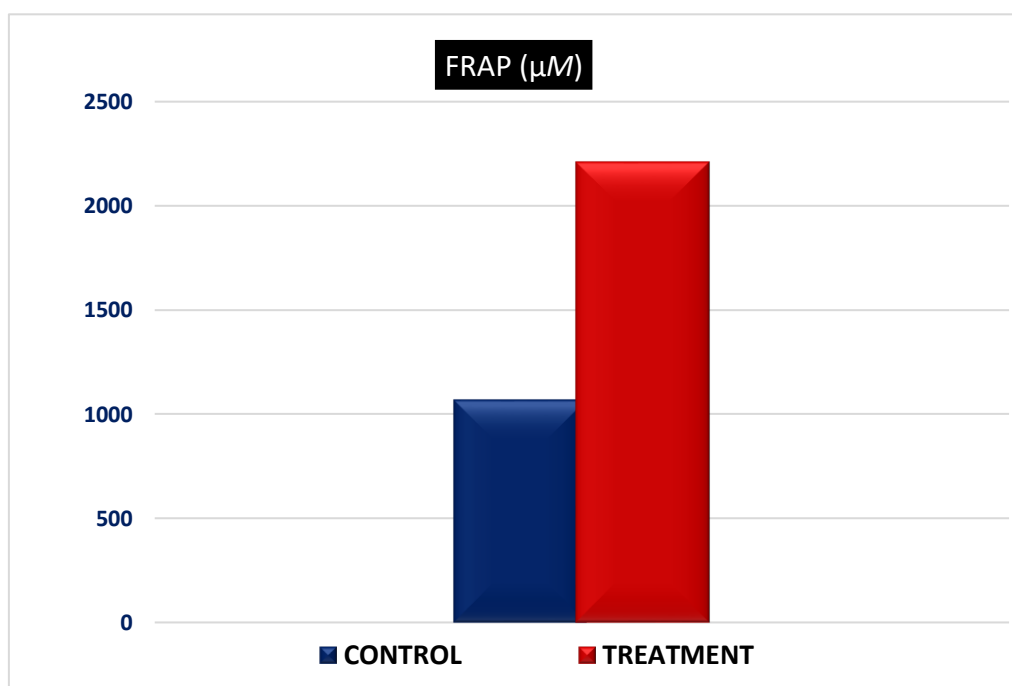


Fig. 4.17b. Ferric Reducing Ability of Plasma (μM) in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

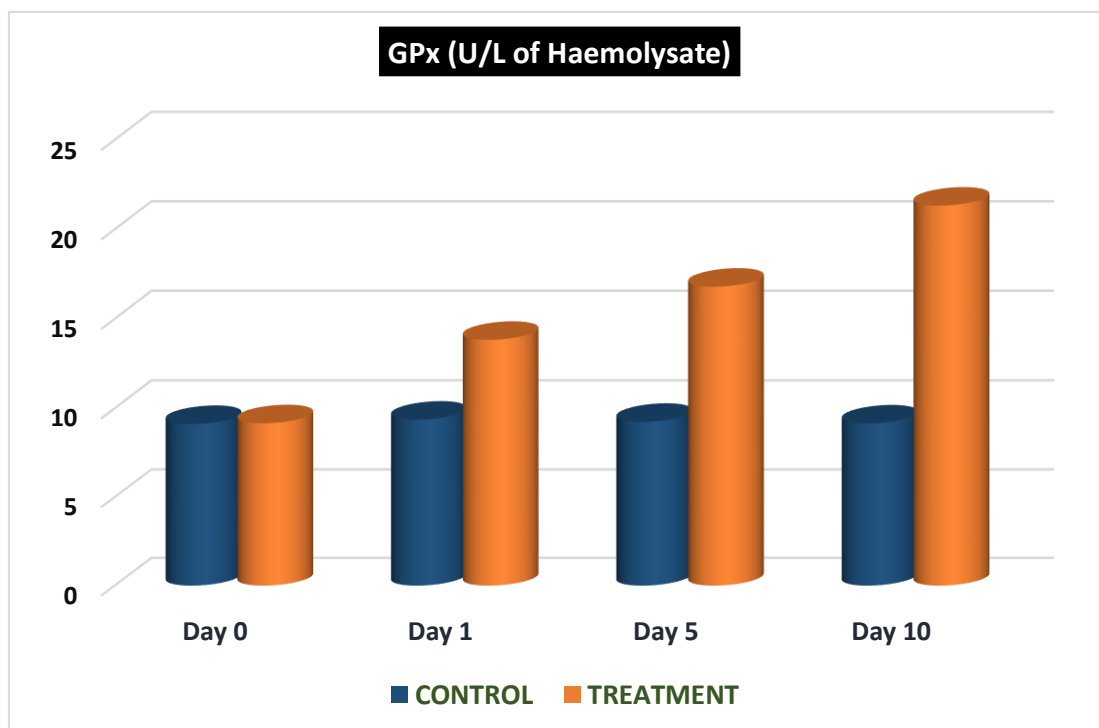


Fig. 4.18. Day wise comparison of glutathione peroxidase activity (U/L of Haemolysate) in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

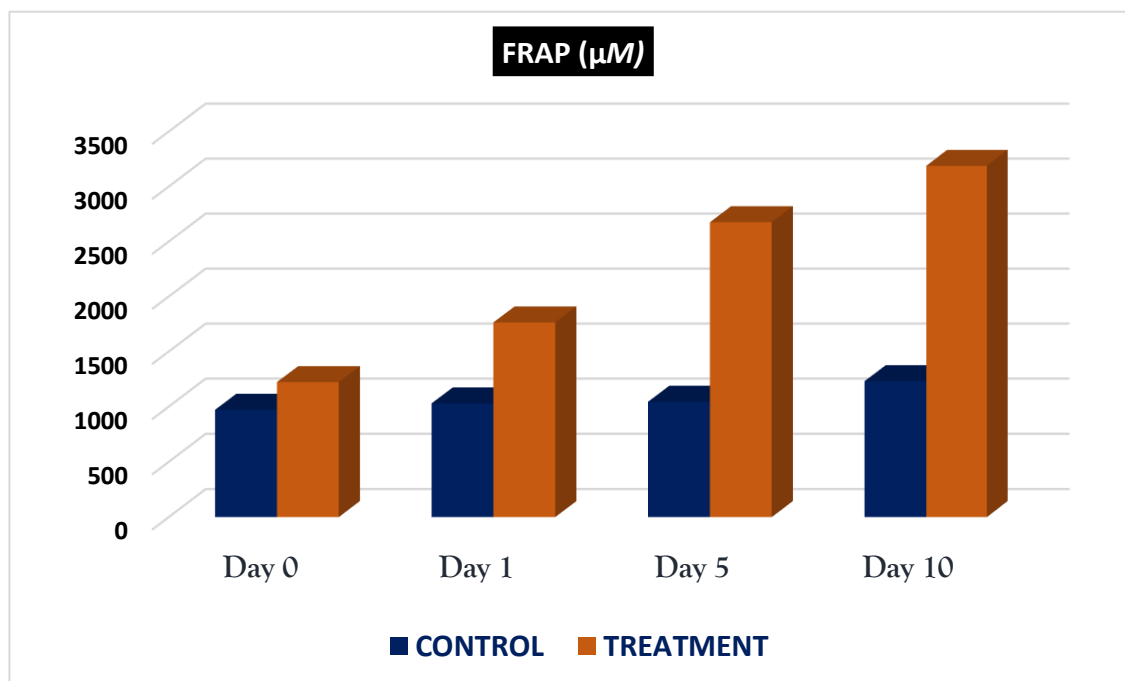


Fig. 4.19. Day wise comparison of ferric reducing ability of plasma (μM) in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

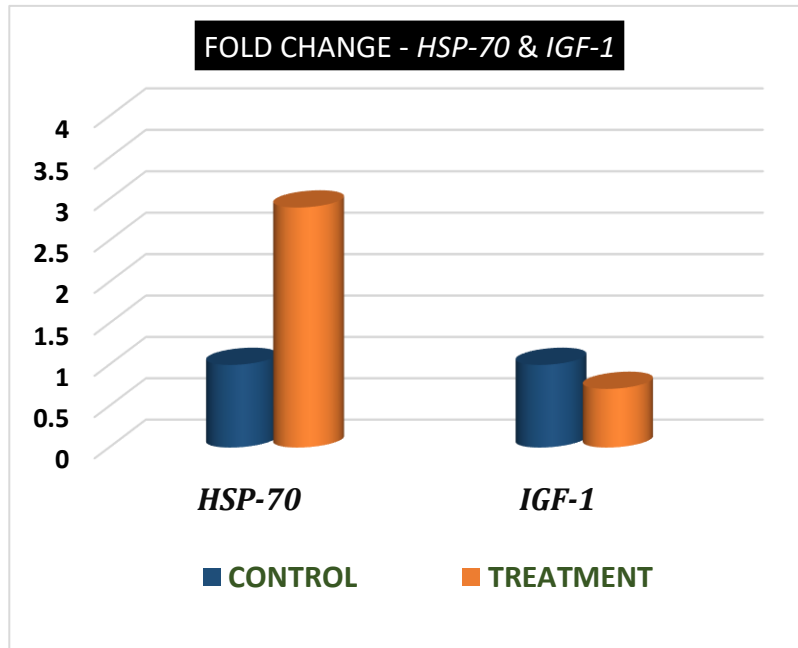


Fig. 4.20. Fold change in *HSP-70* and *IGF-1* in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

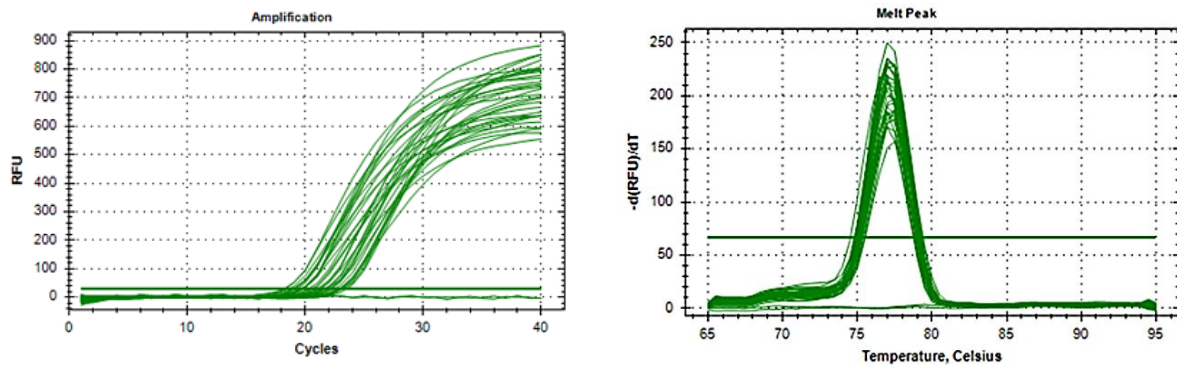


Fig. 4.21. Amplification plot and melt peak of *HSP-70* gene in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

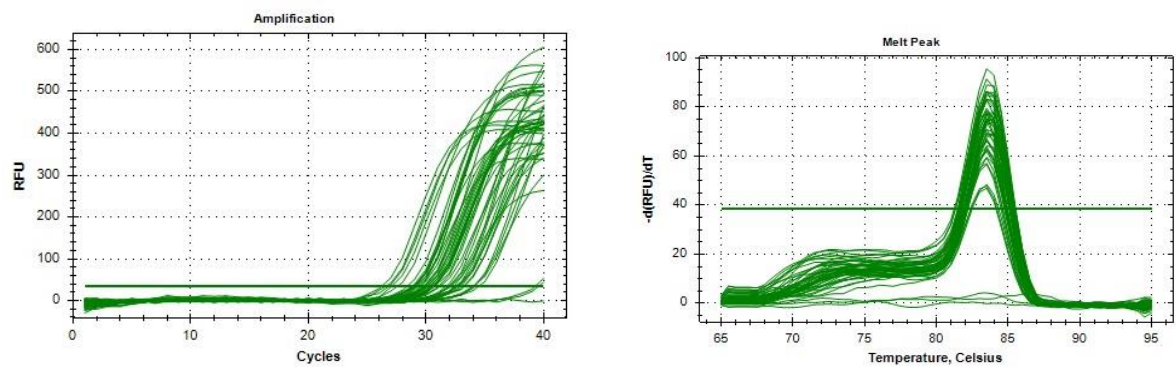


Fig. 4.22. Amplification plot and melt peak of *IGF-1* gene in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

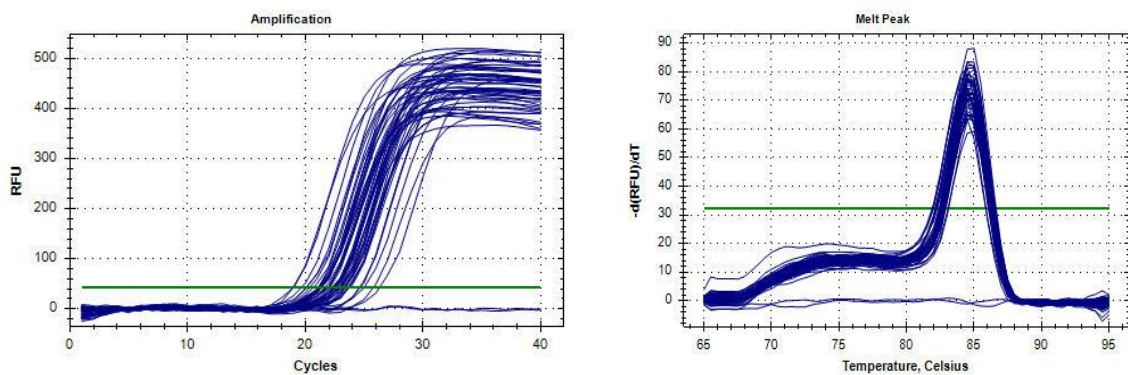


Fig. 4.23. Amplification plot and melt peak of *GAPDH* gene (reference gene) in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

Discussion

5. DISCUSSION

Climate change associated heat stress (HS) causes tremendous impacts on overall health, well-being and production of cattle. Understanding various adaptive responses of cattle to environmental challenges has become vital to eliminate multiple adverse effects and to adopt optimal mitigation strategies that ultimately favour the welfare and production of cattle. Hence the present study was conducted exclusively with the objective of comparing the physiological, behavioural, metabolic, endocrine, antioxidant and molecular responses of crossbred calves to acute HS with that of thermo-neutral zone (TNZ) under controlled climatic conditions. In this context, various responses of the current study are discussed.

5.1 PHYSIOLOGICAL RESPONSES

5.1.1 Rectal Temperature (RT)

In the present study, mean rectal temperature of crossbred calves was significantly ($p<0.01$) increased in the acute heat stressed group (38.99 ± 0.03 °C) compared to those of TNZ (37.87 ± 0.03 °C). This result was in accordance with Garner *et al.* (2017) who evidenced that mean rectal temperatures was significantly higher in cows under HS (40°C) than under TNZ of 25°C. The significantly increased rectal temperature with an increase in THI was an indication that the thermoregulatory responses of the heat-stressed calves were not sufficient to fully counteract the external and internal heat load. Woo *et al.* (2024) also found a similar finding that rectal temperature increased with increase in THI suggesting that rectal temperature was a warning signal where the balance between heat gain and heat loss in the animal might be disturbed. In heat stressed group of the current study, maximum temperature of 39.81 ± 0.05 °C was noticed at 2 p.m. where the chamber THI was 92.48 to 95.04 which was in consistent with the findings of Woo *et al.* (2024). They found that RT was above 39°C when the THI was 85 to 99. In the present study, highest rectal temperature was observed on day 10 of heat stress treatment which was in concurrence with study by Wankar *et al.* (2020). Hou *et al.* (2021) also found a similar significant elevation of RT in heat stressed cows than cows under TNZ.

5.1.2 Respiratory Rate (RR)

In the present study, RR was significantly increased in the acute heat stressed crossbred calves (37.014 ± 0.37 breaths/min; $p < 0.01$) when compared to calves under TNZ (21.48 ± 0.37 breaths/min). This finding was in accordance with Shaji *et al.* (2016) who reported that respiratory rate acted as an early warning signal of heat stress in livestock. This marked increase in the RR was an indication that the animals were trying to maintain homeostasis by dissipating heat load from their body. Similar results were also observed by Bernabucci *et al.* (2003) and Hou *et al.* (2021), where they found that RR was normal in TNZ and significantly increased in heat stressed cows. In heat stressed group of the current study, highest RR of 53.5 ± 0.6 breaths/min was noticed at 2 p.m. when the chamber THI was 92.48 to 95.04 which agreed with the findings of Woo *et al.* (2024) and Kendall *et al.* (2006). The increased RR of crossbred calves during HS condition was an attempt to upsurge the adaptive respiratory evaporative cooling mechanisms for effective heat dissipation from the body to maintain homeostasis. The present findings completely agreed with the previous work done by Wankar *et al.* (2020) who opined that cattle increase their RR to reduce core temperature by allowing heat dissipation by the process of convection. The increased RR also acted as a crucial adaptation in maintaining acid base balance of the animal's body as previously observed by Garcia *et al.* (2015). Heat stress often resulted in elevated metabolic rates which could increase oxygen demand and hence cattle might increase their RR to meet the increased oxygen requirement and to expel more carbon dioxide out from the body. In the present study, there was a strong positive correlation (significant at 0.01 level) between rectal temperature and respiratory rate as evidenced by Wankar *et al.* (2014).

5.1.3 Heart Rate (HR)

The mean HR was significantly increased in calves under HS (83.50 ± 1.74 beats/min) when compared to calves under thermo-neutral zone (64.04 ± 1.74 beats/min) in the present study which was in accordance with Hou *et al.* (2021). They evidenced a significantly increased heart rate of 5–7 beats per minute (bpm) in the heat-stressed group when compared to thermo-neutral control group in their

study on responses of dairy cows to short-term and long-term heat stress under controlled climatic conditions. The metabolic rate might increase during HS, leading to greater oxygen demand by the tissues. An increased heart rate in heat stressed cows ensured that oxygenated blood is delivered more rapidly to meet the oxygen demand for vital cellular functions and preventing hypoxia. In the present study, maximum HR was observed at 2 p.m. in crossbred calves in the climate-controlled chamber where the THI was 92.83 to 95.04. This result was consistent with the results reported by Woo *et al.* (2024). They found that HR was more than 75 bpm when the THI was 85 to 99. Yadav *et al.* (2021) found an increase in HR in crossbred cattle exposed to HS at 40°C than at 25°C which was in accordance with our findings. They reported that higher HR at 40°C might be due to higher secretion of catecholamines and higher expression of adrenergic receptors on cardiomyocytes. They also opined that peripheral vasodilation during HS might lead to drop in blood pressure, hence increased HR acted as compensatory adaptive mechanism to maintain adequate blood pressure and increased the cardiac output for sufficient blood flow to the vital organs during HS. The results of the present study were also consistent with the findings of Wankar *et al.* (2020). The increased HR might be involved in redistribution of fluids within the body, ensuring that blood volume remained adequate to support both thermoregulation and physiological functions. In the present study, there was a strong positive correlation (significant at 0.01 level) between rectal temperature and heart rate as previously reported by Kim *et al.* (2018) and Woo *et al.* (2024).

5.2 BEHAVIOURAL RESPONSES

5.2.1 Standing Time (ST)

The standing time was significantly increased in calves under HS (255.83 ± 5.25 min/10 hrs) when compared to calves under TNZ (165.25 ± 3.28 min/10 h) in the present study which is consistent with the findings of Allen *et al.* (2015). They reported that the duration of standing time was significantly increased when the body temperature of cows exceeded 38.6°C during exposure to HS. They also suggested that standing time was one of the important eye-tracking indicators of HS in cattle. The increased standing time during HS might increase the airflow

around the body for convective heat loss. Standing also allowed cattle for better lung expansion and increased respiratory rate which could enhance respiratory evaporative cooling mechanisms. In the present study, the standing time was accelerating from day zero to 10 in the treatment group whereas it was decreasing in the control group. This finding was similar to the results previously reported by Kim *et al.* (2021). They opined that standing time was increased with increasing THI. Kim *et al.* (2018) studied the behavioural responses of beef calves in a climate-controlled chamber and found a significantly increased standing time at THI of 74.22 to 87.72 (345 to 392 min/day) in comparison to THI of 70.01(236 min/day) which further supports the present findings. An increase in standing time in heat-stressed calves might render more evaporative and convective heat loss resulting in more heat elimination to the surroundings and minimised heat gain *via* conduction and radiation from hot ground (Kamal *et al.* 2016).

5.2.2 Lying Time (LT)

In the present study, calves under thermo-neutral zone spent more time lying down (434.58 ± 3.26 min/10 h) when compared to calves under HS with higher core temperature (339.50 ± 6.59 min/10 h) as reported by Goble (2012). According to Kim *et al.* (2018), as the severity of HS increased, cattle might have felt discomfort and preferred to stand for more heat dissipation *via* evaporation and convection and this might be the reason behind the gradual reduction in lying down duration with an increase in THI. In their study, they found that the lying time was significantly decreased at THI of 74.22 to 87.72 ($p < 0.05$) compared to that at THI of 70.01. This further validated our present findings of decreased lying time in heat-stressed calves with increasing THI. The decreased lying time also could help the calves to maintain an upright posture for effective respiratory heat dissipation. Increased lying time in calves under TNZ in the current study was an indicator of their welfare and comfort as reported by Solano *et al.* (2016) and Tullo *et al.* (2019).

5.2.3 Water Intake Frequency (WIF)

In the present study, water intake frequency was significantly increased ($p < 0.001$) in the animals in acute heat-stressed conditions when compared to

thermo-neutral zone which was in accordance with the findings of Kim *et al.* (2018). They reported that beef calves exposed to high THI in a climate-controlled chamber ($\text{THI} > 82$) had a significantly increased water intake compared to the calves exposed to comparatively lower THI. Yadav *et al.* (2021) found a significant increase in water intake in CB cattle subjected to heat stress at 40°C in contrast to TNZ at 25°C further validating the present findings. Their study suggested that heat exposure at 40°C and 35°C would have caused excessive sweating which might have triggered the hypothalamic thirst centre which resulted in increased water intake. Another reason might be that heat exposure at 40°C could have directly activated the preoptic area and anterior hypothalamus and resulted in increased water intake. Similar findings were also observed by Hamzaoui *et al.* (2013), Yadav *et al.* (2016) and Woo *et al.* (2024). The increased frequency of water intake was found as a protective behaviour of heat-stressed calves to prevent dehydration, compensation for electrolyte loss and maintain blood volume for effective circulation (West, 2003).

5.2.4 Rumination Time

In the present study, rumination time was significantly decreased in the treatment group (72.91 ± 1.002 min/3h) than the control group (99.36 ± 0.95 min/3h) which was consistent with the findings of Kim *et al.* (2018) and Park *et al.* (2019). Similarly, Moretti *et al.* (2017) found a strong correlation between THI and rumination time in Holstein dairy cows and they suggested that rumination could be a valuable parameter for evaluating the impact of heat stress on cattle. According to Grant and Dann (2014), cattle preferred to ruminate while lying down rather than while standing which supported the present results. The results of the current study were also in accordance with the study by Soriani *et al.* (2013) and Antanaitis *et al.* (2024). They suggested that decreased rumination time in cows was attributed to high THI (> 78) and dehydration during HS. The decreased rumination might also be due to depressed blood flow to rumen epithelium which affected the rumino-reticular motility in heat-stressed calves. Decreased rumination in heat-stressed cattle might allow them to conserve energy which would redirect the energy towards thermoregulatory mechanisms.

5.2.5 Feed Intake

In the current study, the mean feed intake of acute heat-stressed calves (16.81 ± 0.052 kg/day) was significantly lower ($p < 0.001$) than the calves under thermo-neutral zone (15.21 ± 0.114 kg/day) as previously reported by Rhoads *et al.* (2013). Similar to our results, Nonaka *et al.* (2008) found a significant reduction in feed intake in Holstein Friesian heifers exposed to heat load at 33°C than at TNZ of 28 or 20°C . This marked decrease in feed intake could be due to inhibition of the appetite centre located in hypothalamic arcuate and paraventricular nuclei which might relay a negative signal to the lateral hypothalamic area. Min *et al.* (2015) also reported decreased feed intake in HF cows under high THI at 81.7 (17.89 kg/day) than under low THI at 53.4 (24.45 kg/day), which further validates the present findings. A study by De Andrade Ferrazza *et al.* (2017) also supported the results of the current study. The decreased feed intake in heat-stressed calves acted as an adaptive mechanism to reduce the internal metabolic heat production. Because the heat increment of feeding was an important source of heat production in ruminants and might exacerbate the impacts of heat stress (Kadzere *et al.*, 2002).

5.2.6 Feeding Time

In the present study, the feeding time was significantly increased in the heat-stressed group ($p < 0.001$) than the thermo-neutral group which agreed with the results previously reported by Tripon *et al.* (2014) and Eslamizad *et al.* (2015). The results were contradictory to the findings of Cook *et al.* (2007). They found that there was no consistent reduction in total feeding time in dairy cows with increasing THI. The increased feeding time in heat-stressed cattle might have acted as an adaptive mechanism that helped them adjust their feeding behaviour to cope with HS. Another reason for increased feeding time was that HS could cause discomfort and lethargy in heat-stressed calves leading to a slower eating rate. Hence heat-stressed calves might have taken longer time to consume the same amount of feed and thereby increasing the total time spent for feeding.

5.3 METABOLIC RESPONSES

5.3.1 Blood Gases

5.3.1.1 Partial Pressure of Oxygen (pO_2)

The mean value of pO_2 in the venous blood of crossbred calves when exposed to high THI in a climate- controlled chamber (38.95 ± 0.51 mm Hg) was significantly higher ($p < 0.05$) than the control group (37.58 ± 0.51 mm Hg). This finding is in accordance with Garcia *et al.* (2015). They investigated the metabolic responses of dairy cattle to varying THI (low, moderate and severe) and found that severe heat-stressed cows had a significantly higher pO_2 values. There was a strong positive correlation found between respiratory rate and partial pressure of oxygen (pO_2) in the current study under heat stress (significant at 0.01 level) similar to the findings of Pandey *et al.* (2017). The significantly increased blood pO_2 values during HS was attributed to increased alveolar ventilation. Heat-stressed cattle increase their RR to facilitate evaporative cooling and that increased ventilation might improve the oxygen exchange in the lungs, leading to a higher pO_2 level in the blood. Similarly, Pandey *et al.* (2017) and Yamin *et al.* (2022) also observed a significantly increased pO_2 values during HS and opined that pO_2 in the blood would increase with an increase in THI further validating the present findings. The increased pO_2 in heat stress was also attributed to increased cardiac output which might improve oxygen delivery to tissues and resulted in higher pO_2 levels in the blood.

5.3.1.2 Partial Pressure of Carbon dioxide (pCO_2)

In the current study, the observed mean value of pCO_2 in the treatment group (40.29 ± 0.569 mm Hg) was significantly lower ($p < 0.05$) than the control group (42.71 ± 0.569 mm Hg). This result was in accordance with the findings of Al-Qaisi *et al.* (2020). Hamzaoui *et al.* (2013) also reported a significantly lower value of pCO_2 at high THI of 77 – 85 in milking goats kept in the climate controlled-chamber compared to those in TNZ which was further consistent with the current findings. The significantly lower blood pCO_2 value during HS was attributed to hyperventilation of animals and the diminished ability of haemoglobin to bind with

CO₂. Joy *et al.* (2020) also found a significantly lower ($p < 0.01$) blood pCO₂ value under HS at 40°C where there was a strong negative correlation between RR and partial pressure of carbon dioxide (pCO₂) which is in consistent with the present findings. Similar results were also reported by Pandey *et al.* (2017) and Yamin *et al.* (2022). The reduced metabolic activity during HS also might have contributed to decreased pCO₂ levels in the blood.

5.3.2 Blood Electrolytes

5.3.2.1 Bicarbonate Ion (HCO₃⁻) Concentration

In the present study, bicarbonate level in the heat-stressed calves were significantly lowered when compared to thermo-neutral controls. This finding is in accordance with Hamzaoui *et al.* (2013). They found that bicarbonate (HCO₃⁻) level was significantly lowered ($p < 0.001$) in the heat-stressed goats (37°C and 40 per cent RH) when compared with the thermo-neutral goats (15 to 20°C and 45 per cent RH) under controlled climatic conditions. They opined that increased respiratory rate and panting during HS contributed to significant loss of CO₂, diminishing the carbonic acid content of the blood. This consequently resulted in the transfer of HCO₃⁻ from the blood to urine *via* the renal pathway to maintain a constant blood pH. According to Sivakumar *et al.* (2010), higher THI (40°C temperature and 30 per cent RH) tremendously decreased blood HCO₃⁻ levels with a strong negative correlation between RR and HCO₃⁻ level under acute heat-stressed conditions. This was attributed to hyperventilation which increased the elimination of CO₂ dissociated from carbonic acid, which was formed by the combination of hydrogen ion and bicarbonate ion. Garcia *et al.* (2015) revealed that cows in comfortable THI showed higher blood bicarbonate values than heat-stressed group supporting the present findings. Since HS could lead to shifts in the acid-base balance, increased loss of bicarbonate through respiratory and renal mechanisms might be a way to compensate for changes in blood pH caused by several stress-induced factors, thereby maintaining overall homeostasis.

5.3.2.2 Sodium Ion (Na^+) Concentration

The mean value of Na^+ ion concentration in the heat-stressed calves of the present study was significantly lower ($133.87 \pm 0.48 \text{ mEq/L}$; $p < 0.01$) than that of calves under TNZ ($138.79 \pm 0.48 \text{ mEq/L}$) which is in accordance with the results of Chetia *et al.* (2016). They found that blood Na^+ level was decreased at higher THI during summer and significantly increased ($p < 0.01$) at lower THI during winter. Similarly, Yamin *et al.* (2022) also reported a significantly decreased Na^+ at higher THI of 89.65. They suggested that hyponatraemia might be due to sodium loss *via* sweating during HS. Wankar *et al.* (2020) reported that serum Na^+ levels did not vary significantly at 25°C supporting the results of the present study. But Wankar *et al.* (2014), in their study on thermoregulatory responses during hyperthermia in an environmentally controlled chamber found a significantly increased value of Na^+ at 40°C than at 30°C which is in contrast to the present findings. Increased water intake during HS also might dilute the blood Na^+ concentration. Another reason might be HS could affect the hormones involved in electrolyte balance such as aldosterone and antidiuretic hormone (ADH) affecting blood sodium concentration.

5.3.2.3 Potassium Ion (K^+) Concentration

In the present study, the blood K^+ concentrations did not differ significantly between crossbred calves under TNZ ($3.97 \pm 0.05 \text{ mEq/L}$) and under heat stress ($4.07 \pm 0.05 \text{ mEq/L}$). This result is in consistent with the earlier reports by Hamzaoui *et al.* (2013) and Antanaitis *et al.* (2024) who found that blood electrolytes did not differ significantly in varying THI. They also opined that potassium is less concentrated in sweat compared to sodium, hence the relative loss of blood K^+ was lower even during HS. Wankar *et al.* (2014) also found no significant variation in K^+ values at different temperature exposures which further validating the present results. They suggested that optimum concentrations of potassium levels during HS might be due to low levels of aldosterone with increased renal conservation. Another reason for no significant variation in blood K^+ between TNZ and HS could be potassium ion is essential for normal cellular functions and maintenance of electro-chemical gradients across the cell membranes. Hence the animal's body might conserve it more strictly. But Yamin *et al.* (2022) found a significantly

increased K^+ value in summer stress at a THI of 89.65 which is contradictory to the present findings. They suggested that hyperkalaemia was due to defects in the functioning of the $Na^+-K^+-ATPase$ pump during HS.

5.3.3 Blood Glucose

The blood glucose level of crossbred calves was significantly decreased in the acute heat stress treatment compared to the TNZ which is in accordance with the results previously reported by Kim *et al.* (2018) and Woo *et al.* (2024). Woo *et al.* (2024) reported that blood glucose level was significantly decreased in the severe THI group (59.15 mg/dL) when compared to the comfort THI group (69.59 mg/dL). Kim *et al.* (2018) inferred that blood glucose level decreased with increasing THI. The significant decline in blood glucose level in response to heat stress in cattle might be attributed to glucose utilisation due to declined feed intake (Baumgard and Rhoads, 2013). Salama *et al.* (2021) found a contradictory result of no significant difference in blood glucose values of thermo-neutral (58.6 mg/dL) and heat-stressed (58.1mg/dL) goats under controlled climatic conditions. Similarly, Garner *et al.* (2017) also found no significant difference in blood glucose values between heat-stressed and thermos-neutral Holstein Friesian cows contradictory to the present findings. Effective heat dissipation in cattle involves peripheral vasodilation, which redirects the blood flow to the skin's surface. This redistribution might compromise blood flow to the vital organ liver, potentially affecting glucose metabolism and production, which could contribute to decreased blood glucose levels. Decline in feed intake during HS might affect the availability of substrates for glucose production, thereby could lower the blood glucose levels (Hamzaoui *et al.*, 2013). The energy demand for thermoregulatory mechanisms might also be a contributing factor for reduced blood glucose levels during HS. Reactive Oxygen Species (ROS) production during HS might alter metabolic functions and impair insulin signalling phosphoinositide-3-kinase–Akt (PI3K–Akt) pathway which could disrupt glucose utilisation and decrease the blood glucose levels (Baumgard and Rhoads, 2013).

5.3.4 Blood pH

The blood pH in the present study was significantly decreased ($p < 0.01$) in the calves exposed to higher THI when compared to the calves under TNZ. This result is in accordance with the findings of West *et al.* (1999), Berian *et al.* (2019), Meneses *et al.* (2021), Yamin *et al.* (2022) and Zeng *et al.* (2023). The major cause of significant reduction in blood pH during HS is mainly due to the respiratory evaporative cooling mechanism. Increased respiratory rate and panting lead to loss of CO₂ which diminishes the carbonic acid content of blood. This ultimately resulted in respiratory alkalosis and therefore, the heat-stressed calves would undergo renal compensation mechanism to excrete HCO₃⁻ *via* urine to reduce the blood pH. The prolonged exposure to HS may lead to a condition called ‘metabolic acidosis’. Hence the present study suggested bicarbonate supplementation for crossbred calves to prevent such conditions.

5.4 ENDOCRINE RESPONSE

5.4.1 Serum Cortisol

In the present study, the serum cortisol level of crossbred calves under acute heat stressed condition was significantly higher (14.64 ± 0.78 ng/mL; $p < 0.001$) when compared to thermo-neutral condition (8.28 ± 0.38 ng/mL) which is consistent with the results reported by Woo *et al.* (2024). They found a significantly increased ($p < 0.05$) serum cortisol values with an increase in THI. In their study, serum cortisol value was lowest at comfort THI (11.96 ng/mL) and highest in severe stress group (17.17 ng/mL) which further validating the present findings. Similar results were also shown by Kim *et al.* (2018) who found a higher serum cortisol level in calves exposed to THI of 87.72 (17.1 ng/mL) than those exposed to THI of 76.51 (4.6 ng/mL). Hence rise in serum cortisol concentrations was temperature-dependent in accordance with the findings of Yadav *et al.* (2015). The increase in cortisol level in calves during acute stress might have acted as a stimulus for the immune system (Ju *et al.*, 2014). In the current study, the mean serum cortisol level in calves was significantly higher ($p < 0.01$) on day one of sudden heat exposure when compared to day zero. It was further increased on day five of heat stress and

there was a significant dip ($p < 0.01$) on 10th day of HS when compared to all other days of treatment. This finding is in accordance with Yadav *et al.* (2021) who evidenced a significantly higher serum cortisol level in crossbred cattle on day one of heat exposure at 40°C than under TNZ at 25°C followed by a gradual dip from 6th day up to 21st day with a significant dip on 16th day of heat exposure at 40°C. The initial peak in serum cortisol level might be attributed to sudden activation of the hypothalamo-pituitary-adrenal (HPA) axis and gradual reduction from the 5th day to significant reduction on the 10th day might be due to the cortisol acclimation pathway in heat-stressed crossbred calves. Similarly, Bharati *et al.* (2017) found that serum cortisol concentration was significantly elevated ($p < 0.05$) during short-term heat stress acclimation and it was gradually decreased during long-term heat stress acclimation (LTHSA). They opined that the decline in cortisol levels during LHTSA was attributed to negative glucocorticoid feedback to HPA axis as an adaptation to prolonged HS in cattle. Garner *et al.* (2017) found no significant difference in mean cortisol value between thermoneutral (3.7 ng/mL) and temperature challenged (3.2 ng/mL) cows kept in a climate-controlled chamber for four days which was contradictory to the present findings.

5.5 ANTIOXIDANT STATUS

5.5.1 Glutathione Peroxidase Activity (GPx)

The mean GPx value in the present study was significantly higher ($p < 0.001$) in the acute heat stressed group at high THI when compared to the control group at low THI which is in accordance with the findings of Sakatani *et al.* (2012), Lallawmkimi *et al.* (2013), Chaudhary *et al.* (2015), Chetia *et al.* (2017) and Rathwa *et al.* (2017). Zeng *et al.* (2013) studied the effects of HS on the health status of dairy cows through blood oxygen availability and found that GPx concentration was significantly greater in the mild-HS group (55.9 U/mL) when compared to the no-HS group (40.9 U/mL) further supporting the present findings. There was strong positive correlation of RT with GPx values (significant at 0.01 level) under heat stressed conditions in the present study which is in consistent with the findings of Zeng *et al.* (2013). Guo *et al.* (2018) found a significantly decreased plasma GPx activity ($p < 0.05$) during heat stress when compared to the comfortable autumn

period in dairy cows which is contradictory to the current findings. Chetia *et al.* (2017) opined that increased 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 could result in a coordinated increase in activity of GPx. This could be helpful in preventing the accumulation of harmful reactive oxygen species (ROS) and might protect the cells from oxidative damage during HS. The increase in GPx activity during HS might have helped to restore and maintain redox homeostasis for maintaining cellular function and preventing oxidative stress.

5.5.2 Total Antioxidant Capacity (TAC)

In the present study, the overall mean TAC value measured as ferric reducing ability of plasma (FRAP) was significantly higher ($p < 0.001$) in the acute heat stressed group when compared to the thermo-neutral group. The present finding is consistent with Almoosavi *et al.* (2020) who reported that FRAP value was highly significant ($p < 0.001$) in the heat-stressed cows when compared to the cows under cooled conditions. Similar findings of increased FRAP value during HS was also reported by Chaiyabutr *et al.* (2011), Tejaswi *et al.* (2020) and Cecchini and Fazio (2020) further validating the current results. Turk *et al.* (2015) found that TAC value was significantly decreased at high THI during summer ($p < 0.001$) than winter which is contradictory to the present results. Higher FRAP value during HS was attributed to the production of antioxidant enzymes such as SOD, catalase and GPx, and non-enzymatic antioxidant such as vitamin E that might play a role in quenching the free radicals. Another reason might be that the enzymatic antioxidants could neutralise the ROS and increased the overall antioxidant capacity of plasma. The increased FRAP value might also be due to increased expression of heat shock protein-70 (*HSP-70*) during HS which works with antioxidants in maintaining cellular integrity. Hence increased FRAP value could be considered as an indicator of adaptive antioxidant defense mechanism in cattle to HS.

5.6 MOLECULAR RESPONSES

5.6.1 Heat Shock Protein 70 (*HSP-70*) gene expression

In the present study, relative quantification of *HSP-70* gene expression in peripheral blood leukocytes showed a significant ($p < 0.05$) 2.98 fold upregulation in crossbred calves under acute HS exposure to 40°C when compared to thermo-neutral condition of 27°C which is in consistent with the findings previously reported by Muhammed (2014), Dangi *et al.* (2012), Kishore *et al.* (2014), Manjari *et al.* (2015), Bhanuprakash *et al.* (2016) and Kim *et al.* (2020). Muhammed (2014) conducted thermotolerance and gene expression studies in different cattle breeds of Kerala and found that crossbred cattle showed significantly increased fold ($p \leq 0.05$) in *HSP-70* expression to increased heat exposures. Kishore *et al.* (2014) reported that *HSP-70* was the most responsive gene and a potential bio-marker for quantifying the heat stress response compared to other HSP chaperones. They also opined that the peripheral blood mononuclear cells (PBMC) system was a potential model for elucidating the differential heat shock responses in cattle. Similar findings of higher expression of *HSP-70* mRNA in PBMCs under HS were also reported by Bhanuprakash *et al.* (2016) and Manjari *et al.* (2015). They also inferred that *HSP-70* expression was positively correlated with changes in physiological variables such as respiratory rate (RR), pulse rate (PR) and rectal temperature (RT) which is precisely supporting the present findings. Guerriero and Raynes (1990) also found that *HSP-70* transcripts were expressed in cattle lymphocytes after several minutes of heat stress exposure supporting the present study. Kim *et al.* (2020) conducted an *in vivo* experiment on *HSP* gene expression in PBMCs to heat stress in beef calves in a climate-controlled chamber and found that the *HSP-70* gene expression was significantly increased ($p < 0.05$) after sudden exposure to HS (severe THI of 88 to 90) further validating the present results. They opined that *HSP-70* could serve an important role in cellular protection by inhibiting caspase-3 activity in response to acute HS. The upregulation of *HSP-70* gene might act as first line of molecular defense mechanism during HS, assisting in the repair of heat-damaged cellular structures and restoring normal cellular functions. These proteins acted as “molecular-chaperones” which facilitate the assembly and translocation of newly synthesized proteins within the cell and the repair and refolding of damaged proteins during HS (Sejian *et al.*, 2019).

5.6.2 Insulin like Growth factor 1 (*IGF-1*) gene expression

In the present study, relative quantification of *IGF-1* gene expression in peripheral blood leukocytes showed a non-significant ($p>0.05$) 0.71fold downregulation in crossbred calves exposed to acute HS when compared to thermo-neutral condition which is in accordance with the results reported by Pragna *et al.* (2018) and Shashank *et al.* (2023). Pragna *et al.* (2018) found a significantly lower expression of *IGF-1* mRNA at high THI during hotter periods of summer stress. Fabris *et al.* (2020) evidenced that Holstein cows exposed to HS had downregulation of *IGF-1* ($p\leq 0.10$) when compared to cows provided with cooling further validating the present findings. Kim (2023) found that extreme heat stress of 41°C upregulated ($p < 0.01$) the expression of *IGF-1* in bovine myocytes when compared to the control group (38°C) which is contradictory to the present findings. The downregulation of *IGF-1* in the present study might be involved in energy conservation for thermoregulation. The downregulation of *IGF-1* and upregulation of *HSP-70* expressions during HS could also be considered as a broader shift in gene expression towards heat stress adaptation. During HS, there might be decline in growth hormone (GH) levels which could downregulate *IGF-1* expression (Pragna *et al.*, 2018).

The present study elucidated various adaptive and thermo-regulatory responses of crossbred calves to acute HS. Further long-term studies under controlled climatic conditions will be useful to understand the long-term responses of crossbred calves to HS to identify novel biomarkers. Cross-comparative studies can be done to identify thermos-tolerant breeds and to design effective housing systems for the well-being of livestock to maintain them in TNZ. Advanced proteomics and transcriptomics studies will explore further thermo-tolerant genes of crossbred calves.

Summary

6. SUMMARY

Climate change associated heat stress (HS) is the major global challenge affecting the livestock. The first step towards effective HS mitigation is to accurately monitor and quantify adaptive responses of cattle to HS. Hence the present study was conducted exclusively to compare the physiological, behavioural, metabolic, endocrine, antioxidant and molecular responses of crossbred calves under thermo-neutral zone and acute heat stressed conditions using a climate – controlled chamber.

The study was conducted at Climate Controlled Research Complex (CCRC) of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS) of Kerala Veterinary and Animal Sciences University, Mannuthy.

Six apparently healthy crossbred (CB) female calves of eight to twelve months of age were selected randomly from University Livestock Farm and Fodder Research Development Scheme (ULF and FRDS), Mannuthy for the study. The research consisted of two experimental groups: thermo-neutral zone (TNZ) or control group and acute heat stress or treatment group. Same six animals were used for experimentation in both the groups.

The animals were given an acclimatisation period of 10 days each in the animal holding facility near the climatic chamber and in a comfort chamber for 3 hours a day maintained at a temperature of 27°C.

Temperature Humidity Index (THI) for the experiment in climate – controlled chamber was fixed based on the formula by Mader *et al.* (2006). After acclimatisation period, animals were housed under TNZ in comfort chamber for a period of 10 days from 6 a.m. to 6 p.m. maintained at a temperature of 27°C and relative humidity of 45-55 per cent. Later they were transferred to climatic chamber for acute HS study. Maximum temperature of 40°C and relative humidity of 55-65 per cent was simulated for three hours a day for 10 days.

Physiological variables such as rectal temperature (RT), respiratory rate (RR) and heart rate (HR) were measured on all alternate days at three-hour interval in both the groups.

Venous blood samples with anticoagulant were collected on days zero, one, five and 10 of the experiment in both the experimental groups and aliquoted into two. One aliquot was immediately used for blood gases and electrolytes analysis using EPOC blood gas analyser. Another aliquot was used for separation of plasma for instantaneous estimation of total antioxidant capacity by measuring ferric reducing ability of plasma (FRAP) and for the preparation of haemolysate for glutathione peroxidase (GPx) estimation. Blood without anticoagulant were also collected from animals in both the groups for estimation of serum cortisol.

On final day of the experiment in both the groups, whole blood samples were drawn into 15 mL centrifuge tubes containing EDTA for *HSP-70* and *IGF-1* gene expression studies in the peripheral blood leukocytes.

Behavioural parameters such as standing time, lying time, water intake frequency, rumination time and feeding time were studied by recorded CCTV surveillance. Daily feed intake was also recorded in both the groups.

All the physiological variables were significantly increased ($p < 0.001$) in the treatment group when compared to calves under TNZ. A strong positive correlation of RT with RR and HR was found when the calves were kept under HS.

In acute HS, the physiological parameters were increased with increasing chamber THI at different time periods of the day. The maximum values of RT, RR and HR were noticed at 2 p.m. when the chamber THI was 95.04. Under TNZ, there was no significant difference between different time periods indicating normal physiological functions of crossbred calves under TNZ.

The pO_2 level in the acute heat stressed calves was significantly higher ($p < 0.05$) and pCO_2 level was significantly lower ($p < 0.01$) in the treatment group than the control group. There was strong positive correlation between RR and pO_2 level and strong negative correlation between RR and pCO_2 level in acute heat stressed conditions.

The bicarbonate level and blood pH were significantly lower ($p < 0.01$) in the treatment group than the control group. There was strong negative correlation found between RR and bicarbonate level of crossbred calves under acute HS.

The blood sodium level in the treatment group was also significantly lower ($p<0.01$) than the control group. But no significant difference was found in blood potassium level between the groups.

Blood glucose level was significantly decreased ($p<0.01$) in the acute heat stress group than the control group.

Serum cortisol level was significantly higher ($p<0.01$) in the treatment group than the control group. But cortisol level had gradual dip on 10th day of heat exposure. Similarly, cortisol levels had declined as the days advanced in TNZ.

Both the GPx and FRAP values were significantly higher ($p<0.01$) in the treatment group when compared to the control group. These levels were gradually increasing from day one of heat exposure to day ten whereas it remained constant in thermo-neutral conditions.

Behavioural parameters such as standing time, water intake frequency, and feeding time were significantly increased ($p<0.01$) in the treatment group when compared to the control group. Lying time, rumination time and feed intake were significantly increased ($p<0.01$) in the calves under TNZ when compared to the acute heat stressed conditions.

Relative quantification of *HSP-70* gene expression in peripheral blood leukocytes showed a significant ($p<0.05$) 2.98 fold upregulation in crossbred calves under acute heat stress when compared to thermo-neutral condition. But *IGF-1* gene expression shown a non-significant downregulation under heat stress.

The present study elucidated various adaptive and thermo-regulatory responses of crossbred calves to acute HS. It was evident that crossbred calves were trying to maintain thermal homeostasis when they were exposed to maximum THI of 92.48 to 95.04. Since there was a significant reduction in bicarbonate level and blood pH during acute HS, bicarbonate supplementation for crossbred calves is suggested to prevent from metabolic acidosis. This study also highlighted the importance of TNZ for normal physiological functioning and welfare of crossbred calves.

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Abstract

**IMPACT OF ACUTE HEAT STRESS ON CROSSBRED
CALVES UNDER CONTROLLED CLIMATIC CONDITIONS**

**NAVIN B.
(22-MVM-57)**

ABSTRACT OF THESIS

Submitted in fulfilment of the requirement for the degree of

**MASTER OF VETERINARY SCIENCE
(Veterinary Physiology)
2024**

**Faculty of Veterinary & Animal Sciences
Kerala Veterinary and Animal Sciences University**



**DEPARTMENT OF VETERINARY PHYSIOLOGY
COLLEGE OF VETERINARY & ANIMAL SCIENCES
MANNUTHY, THRISSUR 680 651
KERALA, INDIA**

2024

ABSTRACT

Climate change associated heat stress is one of the major global challenges affecting the overall health and production of livestock. All animals possess a thermo-neutral zone (TNZ), within which their physiological functions will be normal. If the temperature exceeds the upper critical level of TNZ, the animal is said to be in heat stress (HS). Hence the present study was conducted exclusively to compare the physiological, behavioural, metabolic, endocrine, antioxidant and molecular responses of crossbred calves under TNZ and acute heat stressed conditions in the Climate Controlled Research Complex (CCRC) of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS) following the approval of CCSEA. Six healthy female crossbred calves of eight to twelve months of age were selected from ULF & FRDS and the same animals were used for experimentation in both TNZ and HS groups. The animals were given an acclimatisation period of 10 days each in a shed near the chamber and in comfort chamber, followed by experimental period housed under TNZ maintained at of 27°C and in climatic chamber for acute HS study where maximum temperature of 40°C and relative humidity of 55-65 per cent was simulated for three hours a day for 10 days each in both groups. Venous blood samples were collected for analysis of blood gases and electrolytes, antioxidant activity and gene expression studies.

Physiological variables such as rectal temperature (RT), respiratory rate (RR) and heart rate (HR) were significantly increased in the calves under HS when compared to calves under TNZ. There was strong positive correlation of RT with RR and HR found. The pO₂ level in the acute heat stressed calves was significantly higher ($p < 0.05$) than the calves under TNZ. But pCO₂ and bicarbonate levels were significantly lower in the treatment group ($p < 0.01$). There was strong positive correlation found between RR and pO₂ level and strong negative correlation between RR and pCO₂ and bicarbonate levels in HS. Blood sodium and blood glucose levels were significantly lower ($p < 0.01$) in the treatment group and no significant difference was found in blood potassium level between the groups. Serum cortisol level was significantly higher ($p < 0.01$) in the treatment group and it showed cortisol acclimation pathway on 10th day. Antioxidant parameters such as

GPx activity and FRAP values were significantly higher ($p < 0.01$) in the HS group. Behavioural parameters recorded by CCTV surveillance such as standing time, water intake frequency, and feeding time were significantly increased ($p < 0.01$) in the treatment group. Lying time, rumination time and feed intake were significantly increased ($p < 0.01$) in the calves under TNZ. Relative quantification of *HSP-70* gene expression using *GAPDH* as reference gene in peripheral blood leukocytes showed a significant ($p < 0.05$) upregulation and *IGF-1* gene showed a non-significant downregulation in crossbred calves under acute HS when compared to TNZ. Hence the present study elucidated various thermo-regulatory responses of crossbred calves to acute HS and highlighted the importance of TNZ for their normal physiological functioning and welfare. Significant decline in blood bicarbonate and pH levels in the present study suggested bicarbonate supplementation in crossbred calves during HS. It was evident that crossbred calves were trying to maintain thermal homeostasis when they were exposed to maximum THI of 92.48 to 95.04.

Synopsis

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY
Faculty of Veterinary and Animal Sciences
PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS
DEGREE

1. Title of thesis

Impact of acute heat stress on crossbred calves under controlled climatic conditions

2. a. Title of the departmental/KVASU research project of which this forms part

Nil

b. Code No. if any, and order by which the departmental/KVASU research project is approved

Not applicable

3. a. Name of student

Dr. B. Navin

b. Admission No.

22-MVM-57

c. Name of the discipline

Veterinary Physiology

4. a. Name of major advisor

Dr. K. Karthiayini

b. Designation

Professor,
Department of Veterinary Physiology,
College of Veterinary and Animal
Sciences, Mannuthy - 680651.

5. Objectives of the study

1. Assess and compare the behavioural, physiological and biochemical responses of crossbred (CB) calves under thermo-neutral and heat-stress (HS) conditions

2. Analysis of insulin like growth factor-1(IGF1) and heat shock protein- 70 (HSP70) expressions to evaluate growth and resistance to heat stress

6. Practical / Scientific utility

Heat stress is one of the major climate change impacts on livestock. Ambient temperature higher than an animal's thermo-neutral zone, can adversely affect live weight gain, milk yield and fertility. The first step towards effective heat stress mitigation is to accurately monitor and quantify animal responses associated with the impact of thermal stressors. The adaptive capacity of cattle to HS varies between genetic groups. Holstein Friesian cattle evolved in the temperate climatic zone with high production capacity and increased metabolic rates are less heat tolerant. The temperature humidity index (THI) in Thrissur district during summer (March to May) may reach upto 95.52 which is far above the thermo-neutral zone (THI < 72) for CB cattle (Prasad, 2014). This high temperature along with high humidity impair the evaporative cooling mechanism and increase the thermal load on animals.

The impact of thermal stress in CB calves and cows, during the summer season of Kerala has been studied and documented. However, these studies reveal the impact of multiple stressors than the lone effect of thermal stress and none of the studies were carried out in controlled climatic conditions to understand the exclusive effect of

temperature and humidity on CB cattle of Kerala. Climate chambers are the specialised enclosed spaces used to evaluate the impact of specific environmental conditions on animals. Hence, the present study is undertaken to assess the physiological, biochemical and behavioural responses of CB calves under HS in climatic chamber and compare it with that of the control (animals in thermo-neutral zone).

7. Important publications on which the study is based

According to Bouraoui *et al.* (2012) in lactating cows under Mediterranean climatic conditions, with the value of THI varying from 68 to 78, the average cortisol concentration had increased from 21.75 to 23.5 nMol/L.

Anderson *et al.* (2013) reported that ambient temperature and THI were lowest at 5 A.M. to 6 A.M. and highest at 3 P.M.

Mishra and Palai (2014) reported that heat shock genes could serve as markers in marker-assisted selection for thermotolerance.

Glutathione peroxidase (GSH-Px) was one of the reliable stress indicators in animals and it helped the animals to maintain homeostasis during HS conditions (Harikumar, 2017).

High heat load in animal surroundings might have induced changes in behaviour such as changes in frequency of lying down, time spent resting and standing, reproductive behaviour, feed and water intake, aggression, fearfulness and increased or altered vocalisation (Polsky and Keyserlingk, 2017).

Angel *et al.* (2018) reported that insulin-like growth factor 1 (IGF-1) was the ideal biomarker for assessing the heat stress impact on growth performance of ruminants

and involved in regulation of carbohydrate and lipid metabolism.

The THI was the most frequently used indicator to assess the heat stress of dairy cows as it is simple and reliable (Wang *et al.*, 2018).

Heat shock protein genes (*HSPs*), were the foremost genes to respond to HS in animals and regulate cell viability by maintaining protein homeostasis through their molecular chaperone function (Pires *et al.*, 2019).

Jisha (2020) reported that the rectal temperature and the respiratory rate of the CB cattle were within the normal range at a THI of 77.48 ± 0.51 .

Cattle adopt physiological responses as a coping strategy for HS conditions, particularly changes in respiratory rate, heart rate, body temperature and endocrine status. The respiration rate, panting response and rectal temperature were the main indicators of HS in cattle (Rashamol *et al.*, 2020).

Yamin *et al.* (2022) observed that levels of PO₂ increased and PCO₂ decreased when native and CB goats were exposed to heat stress (THI = 89.65). They also reported a significantly lower Na⁺ and higher K⁺ concentrations at this THI.

8. Outline of the technical programme

The study will be conducted at Climate Controlled Research Complex of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS) in the College of Veterinary and Animal Sciences, Mannuthy, Kerala Veterinary and Animal Sciences University. Six apparently healthy CB female calves of 8 to 12 months of age with identical body weight will be selected randomly from University Livestock Farm and Fodder Research Development Scheme (ULF and

FRDS), Mannuthy. All the animals will be maintained as per standard feeding and management practices followed at the farm.

An acclimatisation and gentling period of seven days will be given to all the animals by keeping them in a shed near the climatic chamber. These animals will be then housed under thermo-neutral zone, in a comfort chamber maintained at a temperature of 27°C.

The animals will be housed in the comfort chamber from 6 a.m. to 6 p.m. for a period of seven days for acclimatisation followed by ten days in thermo-neutral zone. Then the animals will be transferred to the climatic chamber for HS study. Maximum temperature will be simulated for a period of three hours a day for ten days.

In the climatic chamber HS will be applied as follows:

Every day at 6 a.m. the animals will be taken to the climatic chamber maintained at 25°C. The temperature in the climatic chamber will be increased @ 3°C/h from 6 a.m. to 11 a.m. and the animals will be subjected to a maximum temperature of 40°C from 11 a.m. to 1 p.m. The temperature will be again reduced @ 3°C/h from 1 p.m. to 6 p.m. The relative humidity within the chamber will be maintained at 55 to 65 per cent throughout the study period. The animals will be taken out at 6 p.m. and kept in open sheds near the climatic chamber.

During the study period, if any animal exhibits signs of very high thermal stress, it will be taken immediately out of the chamber.

Time	Temperature (°C)	Relative Humidity (%)	THI
6 a.m.	25	55 to 65	72.23 to 73.29
7 a.m.	28	55 to 65	76.28 to 77.64
8 a.m.	31	55 to 65	80.33 to 81.99
9 a.m.	34	55 to 65	84.38 to 86.34
10 a.m.	37	55 to 65	88.43 to 90.69
11 a.m.	40	55 to 65	92.48 to 95.04
2 p.m.	37	55 to 65	88.43 to 90.69
3 p.m.	34	55 to 65	84.38 to 86.34
4 p.m.	31	55 to 65	80.33 to 81.99
5 p.m.	28	55 to 65	76.28 to 77.64
6 p.m.	25	55 to 65	72.23 to 73.29

In all the animals under study, physiological variables such as rectal temperature, respiratory rate, heart rate/pulse rate and rumination rate will be recorded at three-hour interval from 8 a.m.

to 6 p.m. on alternate days till the completion of study. Venous blood samples with and without anticoagulant will be collected at 2 p.m. on day zero (just before the start of the experiment), one, five and ten of the experiment and plasma and serum will be separated.

Blood with anticoagulant will be aliquoted into two. One aliquot will be immediately used for the estimation of electrolyte concentration and blood gas analysis using blood gas analyser. The second aliquot will be stored at -20°C for Glutathione peroxidase estimation using appropriate commercial kit to assess the oxidative stress status. Serum samples will be subjected for quantitative analysis of cortisol by radio immuno assay. The behavioural responses of the animals will be monitored during the entire period of study using a closed-circuit television camera. On day 10, the blood samples with anticoagulant will be collected from all animals before and after treatment to assess the differential expression profile of *HSP70* and *IGF1* in peripheral mononuclear cells by quantitative real-time PCR technique using appropriate reference genes. Statistical analysis of data will be done with the help of SPSS version 24.0.

9. Main items of observations to be made

1. Physiological parameters
 - a. Rectal temperature (°C)
 - b. Respiratory rate (breaths/min)
 - c. Heart rate/ pulse rate (beats/min)
2. Behavioural parameters
 - a. Standing time (min)
 - b. Lying time (min)
 - c. Feeding time (min)
 - d. Rumination time (min)
 - e. Water intake frequency (number/day)
3. Electrolyte concentration
 - a. Na^+ (mEq/L)

- b. K^+ (mEq/L)
 - c. HCO_3^- (mEq/L)
4. Partial pressure of blood gases
 - a. PO_2 (mm Hg)
 - b. PCO_2 (mm Hg)
5. Glutathione peroxidase (U/L of haemolysate)
6. Cortisol (ng/mL)
7. Cycle threshold values of
 - a. *IGF1*
 - b. *HSP70*
8. Feed intake (kg)

10. Facilities

a. Existing

The facilities existing in the ULF and FRDS, Mannuthy, Department of Veterinary Physiology, Central Instruments Laboratory (CIL) and CAADECCS of Kerala Veterinary and Animal Sciences University will be utilised.

b. Additional

Chemicals and reagents

11. Duration of study

Four semesters

12. Financial estimate

- | | |
|---|-------------|
| a. Disposables, instruments, chemicals and glasswares | : Rs. 20000 |
| b. Contingencies | : Rs. 5000 |
| Total | : Rs. 25000 |

Sd/-

Signature of student

Project coordination group

Animal Welfare, Wildlife and Climatology

Sd/-

Signature of major advisor

Place: Mannuthy

Date : 25.11.2023

Name, address and signature of other members of the advisory committee

1. Dr. V. Ramnath, Sd/-
Professor and Head,
Department of Veterinary
Physiology, College of Veterinary
and Animal Sciences, Mannuthy.
2. Dr. V. Beena, Sd/-
Professor,
Department of Veterinary
Physiology, College of Veterinary
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3. Dr. Aziz Zarina, Sd/-
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Department of Veterinary
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4. Dr. S. Harikumar, Sd/-
Associate Professor and Head,
Instructional Livestock Farm
Complex, Pookode, Wayanad -
673576.

13. References

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CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to Animals Act (Amendment, 1998).

Sd/-

Dr. K. Karthiayini
Major advisor

Place : Mannuthy

Date : 25.11.2023

14. Time frame of work

Semester I

1. Collection of literature
2. Planning of programme of research work
3. Preparation of synopsis

Semester II

1. Collection of literature
2. Procurement of essential chemicals and reagents
3. Standardisation of procedure

Semester III

1. Conducting the experiment
2. Collection of data

Semester IV

1. Completion of research work
2. Statistical analysis of collected data and interpretation of results
3. Preparation and submission of thesis

Curriculum Vitae

CURRICULUM VITAE

1. Name of the candidate : Dr. B. NAVIN
2. Date of birth : 20.11.1998
3. Place of birth : Coimbatore, TN
4. Marital status : Unmarried
5. Permanent address : 3/2, Teacher's colony, Anthiyur, Erode Dt.
6. Major field of specialization : Veterinary Physiology
7. Area of specialization : Stress Physiology
8. Educational status : B.V.Sc. & A.H, PGDAW
9. Professional experience : Nil
10. Publications made : 1 (International Journal of Veterinary Sciences and Animal Husbandry)
11. Book chapters : 1
12. International Conference/Seminar : 1 (Awarded 1st Place in Oral presentation)
13. Membership in professional bodies
 - a) Tamilnadu State Veterinary Council (Reg. No. 7365)