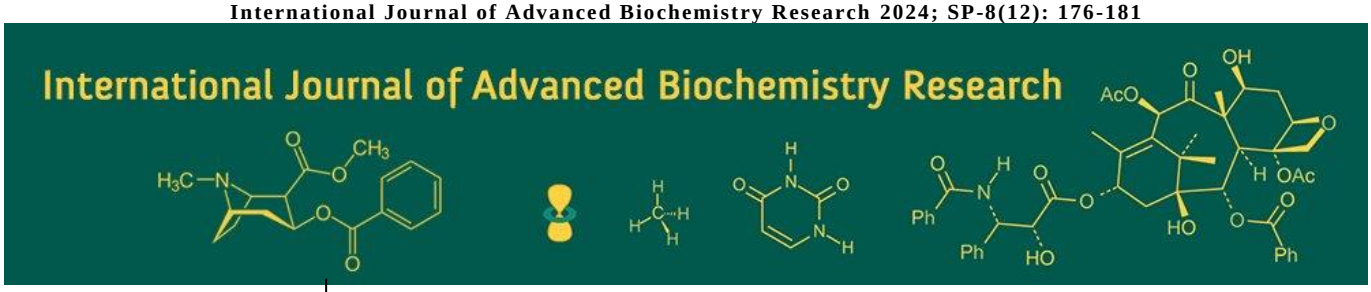

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Exploring the biochemical adaptations of crossbred calves to acute heat stress and its correlation with rectal temperature in controlled climatic conditions

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Abstract
Heat stress generally affects the overall production, productivity and well-being of livestock. Cattle are homeotherms, initiate thermo-regulatory mechanisms to maintain homeostasis. Rectal temperature is the ideal physiological biomarker of quantifying HS response in cattle. They also employ biochemical adaptations to counteract the oxidative stress due to temperature-humidity challenges. Antioxidant defense responses of cattle is their mechanism to neutralise the reactive oxygen species produced during heat stress. Enzymatic antioxidants such as glutathione peroxidase (GPx) scavenge the free radicals and prevent from cellular damage due to oxidative stress during heat stress. The present study was conducted to explore the biochemical adaptations exhibited by crossbred (Holstein Friesian × Sahiwal) calves to acute heat stress (Treatment) and compared it with thermo-neutral zone (Control) in an environmentally-controlled chamber. Rectal temperature was recorded on days zero, one, five and 10 in both the groups to correlate with the biochemical responses. Venous blood samples with anti-coagulant were collected on same days for spectrophotometric estimation of GPx activity and total antioxidant capacity by ferric reducing ability of plasma (FRAP) assay in both the groups. The study shown that the rectal temperature was significantly increased ($p<0.001$) in the heat-stressed calves. The biochemical antioxidant responses were significantly increasing ($p<0.001$) from day one to ten of heat stress and remained constant in thermo-neutral zone. Further, there was a strong positive correlation of rectal temperature with FRAP and GPx values found under heat stress. It was evident from the findings that crossbred calves were trying to maintain thermal homeostasis by adapting physiological and biochemical adaptations when they were exposed to controlled environmental challenges and elucidated the antioxidant defense mechanisms adapted by them to counteract the oxidative stress. The findings also thrown light on the normal cellular functioning of crossbred calves under thermo-neutral zone.

Keywords: Heat stress, thermo-neutral zone, crossbred calves, climatic chamber, rectal temperature, biochemical adaptations

1. Introduction
Climate change associated heat stress (HS) in livestock is one of the major challenges affecting their overall well-being, production and productivity. Cattle are homeotherms, maintains their thermal homeostasis by initiating various thermo-regulatory and adaptive mechanisms which promote their welfare and survival under stressful conditions (Indu and Pareek, 2015) [14]. All of the physiological and biochemical functions of cattle will be normal within their thermo-neutral zone (TNZ) or comfort zone. If the temperature exceeds the upper critical limit of TNZ, they experience HS (Bagath *et al*., 2019) [3]. Rectal temperature is considered as the ideal and gold standard bio-marker for quantifying HS response in cattle (Falkenberg *et al*., 2014). Heat stress in cattle often leads to the production of reactive oxygen species (ROS) such as superoxide, peroxide and hydroxyl radicals (Gupta *et al*., 2013[12]; Ganaie *et al*., 2013) [9]. The endogenous antioxidant mechanism is the first line of defense responses in cattle to overcome the oxidative stress due to thermal stress. The overproduction of ROS or decreased antioxidant defense mechanism will result in lipid peroxidation of plasma membrane and disrupt the normal cellular metabolism. Antioxidant enzymes were synthesised in the body and protect from ROS generated during HS. (Gupta *et al*., 2021) [13]. Their role is to scavenge intracellular and extracellular superoxides and inhibition of lipid peroxidation of plasma membrane (Zhang, *et al*., 2017) [23].

Measuring these bio-markers played a significant role in evaluating the impact of high temperature-humidity-index (THI) on oxidative stress levels of cows. Enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are the chief intracellular antioxidant defense system against superoxides and hydroperoxides significantly contribute to the overall antioxidant capacity of plasma. SOD enzymes are Mn, Zn and Cu dependent. GPx is a selenium dependent antioxidant enzyme converts H_2O_2 to water. The increased production of H_2O_2 due to increased activity of SOD during HS often results in a coordinated increase in GPx. Protein antioxidants such as albumin and thiols, vitamin antioxidants such as water-soluble vitamin C and lipid soluble vitamin E also increase the total antioxidant capacity. These antioxidants play a role in quenching the free radicals and counteract the oxidative stress in cattle. Another important oxidative stress marker in the plasma is the thio-barbituric acid reactive substances (TBARS). Higher enzyme activity results from the upregulation of mRNA expressions of antioxidant genes such as GPx, SOD and CAT. This enhances the ability of cells to detoxify ROS and maintain redox equilibrium or homeostasis (Akbarian *et al*., 2014) [1]. In view of this, the present study was conducted with the objective of exploring the biochemical adaptation mechanisms exhibited by crossbred (Holstein Friesian × Sahiwal) calves to acute heat stress and correlated with their rectal temperature in an environmentally-controlled chamber.

2. Materials and Methods

2.1 Ethical approval

The research was carried out at the Climate Controlled Research Complex (CCRC) of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS), Kerala Veterinary and Animal Sciences University following the approval of Committee for the Control and Supervision of Experimentation on Animals (CCSEA), New Delhi.

2.2 Experimental layout

Six healthy eight to twelve months aged crossbred (Holstein Friesian × Sahiwal) female calves were selected randomly from University Livestock Farm and Fodder Research Development Scheme (ULF & FRDS), Mannuthy and the same were distributed in two experimental groups: thermo-neutral zone (TNZ) or control group; acute heat stress or treatment group. The experimental animals were given an acclimatisation period of 10 days each initially in the animal holding facility and in the climatic chamber for adaptation of the animals to novel conditions of the climate-controlled chamber. It was followed by experimental period of 10 days each housed under TNZ and in climatic chamber for acute HS study. In TNZ, the comfort chamber was maintained at a temperature of 27ºC and relative humidity of 45-55 per cent. For acute HS study, maximum temperature of 40ºC and relative humidity of 55-65 per cent was simulated in the climatic chamber for three hours a day for 10 days. The animals were re-shifted to the respective sheds of the cattle farm for rehabilitation and reuse after the experiment, as per the recommendations of CCSEA.

2.3 Recording of rectal temperature

The rectal temperature of the calves was recorded using a clinical thermometer by inserting 6–7 cm inside the rectum inclined towards the wall of the rectum, at three-hour interval from 8.00 a.m. to 6 p.m. on days zero, one, five and ten of the experiment in both TNZ and acute heat stressed conditions.

2.4 Collection of Blood samples

Venous blood samples were collected in lithium heparinised vacutainers in both the experimental groups on days zero, one, five and ten of the experiment and aliquoted into two. One aliquot was used for the preparation of haemolysate for the estimation of GPx activity using a commercial kit. Another aliquot was immediately centrifuged at 2700×g at 4 ºC for 15 minutes to separate plasma and were stored at –80 °C until complete preparation for analysis of total antioxidant capacity (TAC) by the FRAP assay. This assay was instantaneously done on the same day after the separation of plasma for higher accuracy of antioxidant capacity.

2.5 Estimation of Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase activity was determined by Perkin Elmer Lambda UV – VIS spectrophotometer using GPx activity assay kit (Origin Diagnostics and Laboratory, Kerala).

Principle

Glutathione peroxidase 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 GPx, so the portion of GSH reduction by non-enzymatic reaction should be subtracted. GSH can react with di-nitrobenzoic acid to produce 5-thio-dirutrobenzoic acid anion, which showed a stable yellow colour.

Table 1: Kit components of Glutathione peroxidase activity kit.

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

Added 0.405 mL diluent to get a standard concentration of 80 µmol/mL.		
Diluent	20mL	4°C

Preparation of Haemolysate for GPx assay

Haemolysate samples were prepared and used for the assay. Blood sample was collected using lithium heparin coated vacutainer. RBC was spin down by centrifugation at 3000 rpm for 10 min at 4 ℃. Plasma was removed from the cells by drawing it off from the top. Buffy coat (the white interface between the pelleted RBCs and the plasma) was removed and discarded. RBC pellet was washed with saline at 4 ℃ and centrifuged at 3000 rpm for 10 min at 4℃. The clear saline from top was discarded and repeated once. To an aliquot of RBC, an equal volume of deionised water was added and vortexed well for complete lysis of the cells. 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. Hemolysate sample aliquots were stored at < -65 ºC freezer until analysis. Frozen samples were thawn out and vortexed well before analysis. Thawed out red blood cell lysate was diluted in deionised water to 6-7 g/L of hemoglobin. Mixed well and left the diluted samples on ice before analysis.

Procedure of GPx Assay

Preheated the spectrophotometer for 30 min and adjusted the wavelength to 412 nm and set zero with distilled water. Diluted the standard solution (80 µL mol/mL) to 0.08 µmol/mL with diluent. Performed the subsequent assay in a 1.5 mL microcentrifuge tube. Added the reagents in the order as mentioned in the Table 2. Then, 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 and continued the assay as given in the Table 3. 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.

$\Delta A_T = A_C - A_T$

$\Delta A_S = A_S - A_B$

Table 2: Assay Procedure 1

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

Table 3: 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

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.

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

Hence, GPx (U/mL) = 200 x ΔAT ÷ ΔAs

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

Actual value = GPx (U/mL) × 10

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

2.6 Estimation of Total Antioxidant Capacity

The ferric reducing ability of plasma (FRAP) assay by Benzie and Strain (1996) [4] was used as a direct method for estimating the total antioxidant capacity of plasma samples. At low pH, ferric 2,4,6-tripyridyl-s-triazine [Fe (III)-TPTZ] complex gets reduced to ferrous 2,4,6-tripyridyl-s-triazine [Fe (II)-TPTZ] complex, which gives an intense blue colour, can be monitored spectrophotometrically by measuring the change in absorption at 593 nm. Working FRAP reagent was prepared by using 300 mM acetate buffer, pH 3.6 (3.1 g sodium acetate trihydrate, plus 16 mL glacial acid); 10 m*M* TPTZ [2,4,6-tris (2-pyridyl)-s-triazine], in 40 m*M* HCl; and 20 m*M* $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1. Plasma sample (100 µL) was mixed with 3 mL of FRAP reagent and kept for 10 minutes. Then the sample was centrifuged at 10,000 rpm for 5 minutes and supernatant solution was taken. The absorbance of the supernatant solution was monitored at wavelength 593 nm with Perkin Elmer's LAMBDA UV-Vis spectrophotometer in the Central Instrumentation Laboratory (CIL). Aqueous solution of Ascorbic acid (1000 µ*M*) was used as standard. The change in absorbance was translated into FRAP value (µ*M*) by relating ΔA_{593} of test sample with that of standard solution of known FRAP value using formula.

Calculation of FRAP value

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

2.7 Statistical analysis

The results of the experiment were expressed as Mean ± Standard Error. The statistical analysis of the data was done by linear mixed model analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) version 24.0 software.

3. Results and Discussion

3.1 Rectal temperature

The mean rectal temperature of crossbred calves was significantly ($p<0.01$) increased in the different days of heat exposure (Table 4) when compared to calves under TNZ which was in accordance with Garner *et al*. (2017) [10] who evidenced that mean rectal temperatures was significantly higher in cows under HS (40 ºC) than under TNZ of 25 ºC. 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 tenth day of heat exposure. Also, the mean RT values of each days increased significantly in the treatment group. The significantly increased rectal temperature of calves with increase in temperature and humidity of the chamber 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) [21] also found a similar finding that rectal temperature increased with increase in THI suggesting that rectal temperature was an early warning signal where the balance between heat gain and heat loss in the animal might be disturbed.

Table 4: Day wise recordings of rectal temperature in 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)	38.15^{aA}±0.06	37.84^{bA}±0.06	37.92^{bA}±0.06	37.75^{bA}±0.06
TREATMENT (HS)	38.59^{aB}±0.06	38.99^{bB}±0.06	38.92^{bB}±0.06	39.25^{cB}±0.06
p-value	<0.04	p<0.001	p<0.001	p<0.001

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

3.2 Glutathione peroxidase activity

The GPx activity was significantly higher (p<0.001) in the acute heat stressed group when compared to the control group. The GPx values remained constant in different days of the TNZ and was gradually increasing from day one of heat exposure to day ten. The highest GPx value of 21.33±2.13 U/L of Haemolysate was recorded on the 10^{th} day of heat exposure and the lowest value of 9.17±0.26 U/L of Haemolysate on day zero or before exposure. There were also high significant differences (p<0.01) found in mean GPx values of days one, five and ten between the control and treatment groups.

Table 5: Day wise comparison of glutathione peroxidase activity (U/L of Haemolysate) in 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)	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	0.917^{ns}	<0.01 (p=0.004)	<0.001	<0.01 (p=0.002)

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 GPx value of calves in the present study was significantly higher (p<0.001) on different days of 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) [19], Lallawmkimi *et al*. (2013) [16], Chaudhary *et al*. (2015) [7] and Rathwa *et al*. (2017) [17] who reported a significantly increased GPx activity under HS when the animals were exposed to higher THI. Zeng *et al*. (2013) [22] 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. Guo *et al*. (2018) [11] found a contradictory finding of significantly decreased plasma GPx activity (p<0.05) during heat stress when compared to the comfortable autumn period in dairy cows. Chetia *et al*. (2017) [8] 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.

3.3 Total Antioxidant Capacity

The total antioxidant capacity of plasma samples measured as FRAP values were increased gradually from day one of heat exposure to day ten. The highest mean FRAP value of was recorded on day ten of heat exposure indicating that the calves undergone maximum oxidative stress on day ten when compared to other days of HS and the lowest value was recorded on day zero or before HS exposure. Hence the present findings of increased antioxidant capacity in plasma of the heat-stressed calves as the treatment days advanced indicates the gradual increase in oxidative stress throughout the acute HS period under climate-controlled conditions. This result of higher FRAP value under HS is in accordance with the previous reports of Almoosavi *et al*. (2020) [2], Chaiyabutr *et al*. (2011) [6], Chaudhary *et al*. (2015) [7] and Tejaswi *et al*. (2020) [20] further validating the present findings. The constant FRAP value on different days in calves kept in control group indicates the absence of oxidative stress in crossbred (Holstein Friesian × Sahiwal) calves under comfort zone or TNZ.

Table 6: Ferric reducing ability of plasma (μ*M*) in crossbred (Holstein Friesian × Sahiwal) calves exposed to thermo-neutral and acute heat stressed conditions (Mean± 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	1030.86^{aA} ±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**	0.123^{ns}	<0.001	<0.001	<0.001

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

During HS, significantly increased TAC value as the days advanced was attributed to the production of antioxidant enzymes such as SOD, catalase and GPx, and non-enzymatic antioxidant such as vitamin E that might have played a defensive role in quenching the free radicals to counteract the oxidative stress in heat-stressed calves

(Chaiyabutr *et al*., 2011) [6]. The upregulation of antioxidant genes such as GPx, SOD 1, SOD 2 and CAT leads to higher enzyme activity and might have increased the overall antioxidant capacity (Rimoldi *et al*., 2015) [18]. Another probable explanation for this could be due to upregulation of heat shock protein-70 (*HSP-70*) gene expression during HS which maintains the cellular integrity by co-working with antioxidants (Khan *et al*., 2020) [15].

Correlation of rectal temperature with biochemical adaptive responses in crossbred calves exposed to acute 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 (Table 7) shown a strong positive correlation of RT with GPx and FRAP values (significant at 0.01 level) found under acute heat stress in the crossbred calves in climate-controlled conditions. This finding of positive correlation is in consistent with the findings of Zeng *et al*. (2013) [22]. The increased rectal temperature along with GPx and FRAP values could be considered as an adaptive physiological and antioxidant defense mechanisms to mitigate the damage caused by ROS in crossbred calves for maintaining thermal equilibrium.

Table 7: Pearson's correlation coefficient of rectal temperature with biochemical adaptive responses in crossbred calves under acute 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)

4. Conclusion

It was evident from the current findings that the correlation studies of rectal temperature with biochemical adaptations of crossbred calves (Holstein Friesian × Sahiwal) thrown light on the physiological and biochemical adaptation mechanisms exhibited by them when exposed to a maximum temperature of 40 ºC and relative humidity of 55-65 per cent, to counteract the oxidative stress due to temperature-humidity challenges. This study also highlighted the normal physiological and cellular functioning of crossbred (Holstein Friesian × Sahiwal) calves under thermo-neutral zone. Hence in general, it is suggested to maintain crossbred cattle under their TNZ or comfort zone for their normal physio-biochemical functions, welfare and productivity.

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6. Conflict of Interest

The authors declared no conflict of interest.

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