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Heat Stress, Antioxidant Supplementation and HSP70 mRNA Expression in Growing Pigs

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Heat stress poses significant challenges to livestock productivity, particularly in pigs, due to their limited thermoregulatory mechanisms. This study aimed to evaluate the seasonal effects of heat stress on growth performance, haematological and hormonal level, as well as the relative mRNA expression of HSP70 in growing pigs supplemented with melatonin and vitamin E. Results showed that haematological parameters, serum cortisol, lactate dehydrogenase, and average body weight were adversely affected during the summer season. While antioxidant supplementation did not

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significantly influence haematological profiles or LDH levels, it reduced cortisol levels during summer. Vitamin E supplementation resulted in a significant decrease in HSP70 mRNA expression during summer, indicating improved oxidative stability. Seasonal variations notably affect pigs' growth and health, with vitamin E supplementation reducing HSP70 expression during summer, highlighting the challenges of tropical climates.

Keywords: Heat stress; growing pigs; antioxidants supplementation; HSP70 expression.

1. INTRODUCTION

Under tropical climatic conditions, elevated temperature and humidity adversely affect the productive and reproductive performance of pigs. The limited thermoregulatory capacity of pigs, due to their underdeveloped sweat glands and thick subcutaneous fat layer, impairs effective heat dissipation. High ambient temperatures trigger excessive immune responses, often accompanied by increased white blood cell counts. Additionally, average daily gain, daily feed intake, and feed efficiency significantly decline during the summer season (Lewis & Berry, 2006). Increased leukocyte counts in pigs are commonly associated with stress-induced infections (McGlone & Pond, 2002). The higher humidity had a more detrimental impact than low humidity (Adenkola *et al.,* 2011). De *et al.* (2013) observed significantly higher ($p>0.05$) RBC, PCV, and haemoglobin concentrations in Andaman wild pigs compared to other local breeds, suggesting better adaptation of Andaman wild pigs to the hot and humid climate of the Andaman and Nicobar Islands. The lower serum T3 and T4 level was estimated in pigs during summer season than winter season (Chakraborty *et al.,* 2017a). In a related study, serum SOD levels were higher ($p<0.01$) in summer, however, reduced SOD activity in pigs supplemented with melatonin and vitamin E indicated their ameliorative effect during heat stress (Chakraborty *et al.,* 2017b). Furthermore, delayed onset of puberty ($p<0.01$) in pigs during summer was observed, although melatonin supplementation showed improvement in both seasons (Chakraborty *et al.,* 2017c). Elevated rectal temperature, respiration rate, and pulse rate ($p<0.01$) during summer confirmed the presence of heat stress in pigs (Chakraborty *et al.,* 2017d; Devi et al., 2021). Huynh *et al.* (2006) found that implementing cooling systems (water bath and sprinkling) improved daily weight gain in pigs exposed to heat stress. In contrast, a meta-analysis by Guo *et al.* (2018) concluded that although heat stress did not affect litter size or offspring birth weight, it reduced litter weight gain due to decreased sow milk production. Pigs exposed to heat stress showed a linear increase in serum cortisol levels (7.06 mg/dl) compared to those in thermally comfortable environments (4.82 mg/dl) (Fagundes *et al.,* 2008). Plasma cortisol levels were positively correlated with the temperature-humidity index (THI) (Borah *et al.,* 2023). The lower cortisol levels in pigs provided with a cooling system (Escribano *et al.,* 2023), reinforcing the importance of environmental control in mitigating thermal stress (Wirthgen et al., 2018). Heat stress further impaired pig performance, with significantly reduced body weight, average daily gain (ADG), and average daily feed intake (ADFI) during high-temperature periods (Oh *et al.,* 2017; Ortega et al., 2023). The higher LDH concentrations in finisher pigs and boars during summer, while in growing pigs and sows, LDH levels were higher during the spring season (Vashi *et al.,* 2018). The upregulation of HSP70 expression during summer has been attributed to elevated ambient temperatures, acting as a protective mechanism against cellular damage under heat stress (Vashi *et al.,* 2018; Parkunan *et al.,* 2015, 2016). This study explores how summer and winter affect blood profile, stress and growth hormones, LDH levels, and HSP70 gene expression in growing crossbred female pigs.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Eighteen healthy, weaned crossbred female pigs (Hampshire × Assam local) were selected for the study during both the summer and winter seasons. For each season, animals were divided into three groups: a control group (n=6), a melatonin-supplemented group [n=6; Meloset at 3 mg per animal], and a vitamin E-supplemented group [n=6; Evion at 100 mg per animal]. To ensure experimental integrity, separate groups of pigs matching in age and weight were chosen for each season; the pigs used in summer were not reused in winter.

At the start of the experiment, the initial body weights were as follows: during summer, the control, melatonin, and vitamin E groups weighed 10.25 ± 0.02 kg, 10.32 ± 0.07 kg, and 10.32 ± 0.05 kg, respectively; during winter, their weights

were 10.34 ± 0.06 kg, 10.25 ± 0.09 kg, and 10.15 ± 0.17 kg, respectively. All pigs were sourced from the All-India Co-ordinated Research Project (AICRP) on Pig at the College of Veterinary Science, Khanapara, Guwahati, Assam (coordinates: 26°07'16"N, 91°49'15"E).

The animals were fed according to the standard farm practices and observed over a three-month period in both seasons. Monitoring continued until furrowing to determine litter size and ensure comprehensive data collection.

2.2 Temperature-Humidity Index (THI)

Temperature-Humidity Index was calculated from the data of ambient temperature and relative humidity by using the formula of Mader *et al.,* (2006). The dry bulb temperature and relative humidity were recorded daily during the experimental period from the Automatic Weather Station (AWS). THI was calculated for the entire period using the following formula:

THI = (0.8 x Tdb) + [(RH/100) x (Tdb – 14.4)] + 46.4

The THI recorded above 82 (stress) during the summer season while THI recorded below 72 (comfort) during the winter season (Table 1).

2.3 Blood Collection

About 5 mL of blood was collected at around 9:30 am in the morning from vena cava following all the ascetic protocol as well as the ethical committee consideration. Around 1.5 mL of blood was transfer to EDTA coated vial for haematological profile and total RNA isolation. The other part of the blood was allowed to coagulate, and serum was separated and stored at -20°C for estimation of Cortisol, growth hormone and Lactate dehydrogenase (LDH). About 500 µL of blood immediately transfered to a Tube (RNAprotect® Animal Blood Tubes, Cat. No. 76554) for Total RNA isolation. Haematological profile carried out using MS4 Automated Haematological Cell Counter.

2.4 Body Weight Measurement

The body weight of the animals was measured using a digital balance from the day of experiment i.e. ‘0’ day and repeated after every 15 days interval for 105 days post weaning.

2.5 Hormones and LDH Estimation

The level of cortisol was estimated by Radioimmunoassay (RIA) technique using RIA kits (Immunotech, France). The tracer I-125 was used in the estimation technique which involved competition between free and isotope tagged hormone for binding to the limited antibody sites and subsequently quantification was made through calibration curve (6 well Automatic gamma counter procured from Stratec W. Germany). The intra and inter assay co-efficient of variation were found to be 5.8 per cent and 9.2 per cent. Growth hormone (LDN Immunoassays and services) and Melatonin (Genway, Biotech Inc.) were estimated by ELISA technique using Elisa kits as per the manufacturer protocol. The Lactate Dehydrogenase (LDH) was estimated using LDH assay kit (Cayman Chemical Company, USA) as per manufactures protocol. The estimation used 96 wells plates namely Dynamica, Halo MPR 96 visible Microplate Readers (Australia).

2.6 Estimation of Vitamin E

Vitamin E was estimated by Ferric Chloride Dipyridyl Method (Emmerie-Engel Reaction). The absorbency was determined at 520mµ. The difference between the absorbencies of the control sample and the test sample was converted to mg of tocopherol.

2.7 Total RNA Isolation

RNA was isolated from blood using RNAprotect Animal Blood Tubes (Qiagen), which stabilized RNA at room temperature before storage at -20°C. Blood (500 µL) was added to the RNAprotect Animal Blood Tube, mixed gently, incubated for at least 2 hours at 15-25°C, and stored until further processing. For mRNA preparation, the tubes were centrifuged (5000×g, 3 min), and 100 µL of blood was transferred to a new tube. After washing with RNase-free water and vortexing, the pellet was resuspended in buffer RSB. The sample was then mixed with buffer RBT and proteinase K, incubated at 55°C, and processed through a QIAshredder spin column. Ethanol (96-100%) was added to the supernatant, followed by purification using a RNeasy Min-Elute spin column. DNase I treatment was performed to remove genomic DNA, with sequential washes using buffers RW1, RPE, and 80% ethanol. The purified RNA was eluted in buffer REB, incubated at 65°C for 5 min, and immediately chilled on ice. The purity of the isolated RNA was assessed using a spectrophotometer at 260/280 nm, with samples a ratio between 1.8 and 2.1 selected for cDNA preparation.

Table 1. Relative humidity, temperature and temperature humidity index (THI) during summer and winter season

Season	RH (%)	Ambient temperature (ºC)	THI
Summer	81.97±1.12	29.28±0.30	82.01±0.50
Winter	68.75±5.98	17.91±0.14	63.16±0.30

2.8 cDNA Preparation and Reverse Transcription Cycling Program

cDNA was synthesized using the Verso cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's protocol. The reaction was set up in a final volume of 20 µL, consisting of 4 µL of 5× cDNA synthesis buffer (1× final concentration), 2 µL of dNTP mix (500 µM each), 1 µL of RNA primer, 1 µL of RT enhancer, 1 µL of Verso Enzyme Mix, and 1-5 µL of RNA template (1 ng). Nuclease-free water was added to adjust the total volume to 20 µL. The cDNA synthesis was performed at 42°C for 30 minutes (1 cycle), followed by enzyme inactivation at 95°C for 2 minutes (1 cycle).

2.9 Polymerase Chain Reaction (PCR) and Semi-Quantitative PCR (qPCR)

Polymerase Chain Reaction (PCR) for HSP 70 and GAPDH genes was carried out to check the specificity of the primers used and to confirm the size of the amplicons. The PCR reaction mixture included 10X Taq DNA polymerase buffer with 15 mM Mg+2 (2.5 µL), forward primer (10 pmol) (1.0 µL), reverse primer (10 pmol) (1.0 µL), dNTPs mix (10 mM each) (2.5 µL), cDNA (1.0 µL), Taq polymerase (0.2 µL), and nuclease-free water (16.8 µL), making a total volume of 25 µL. The thermal cycling conditions included an initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The primers used for qPCR included HSP 70 forward primer (GCCCTGAATCCGCAGAATA, HM025989.2, 152 bp), HSP 70 reverse primer (TCCCCACGGTAGGAAACG), GAPDH forward primer (GAAGGTCGGAGTGAACGGAT, NM_002046.7, 149 bp), and GAPDH reverse primer (CATGGGTAGAATCATACTGGAACA). Confirmation of specific products for HSP 70 and GAPDH genes was performed using Gel Electrophoresis, where the gel was run until the marker dyes migrated the desired distance. The bands were visualized under UV light in a gel documentation system (Gel Doc XR + BioRad, USA). Semi-Quantitative PCR (qPCR) for HSP 70 was performed using SYBR Green Chemistry on the Applied Biosystems Step One Plus platform, with GAPDH as the internal control. All samples were prepared in triplicate, and reactions were performed in a total volume of 20 µL, comprising SYBR Green Master Mix (2x) (10 µL), forward primer (1 µL), reverse primer (1 µL), cDNA (2 µL), and nuclease-free water (6 µL). The Non-Template Control (NTC) included all the components except cDNA, with nuclease-free water adjusted. The thermal cycling conditions involved an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. For melt curve analysis, the temperature was held at 95°C for 1 minute, followed by ramping down to 60°C while fluorescence data was collected continuously over the 60–95°C range.

2.10 Statistical Analysis

The statistical analysis was conducted following the methodology outlined by Snedecor and Cochran (1994), utilizing SAS Enterprise Guide (version 9.3). Graphs were generated using PRISM8 software.

3. RESULTS AND DISCUSSION

The seasonal variation significantly ($p<0.05$) influences the haematological parameters (table 2). The haemoglobin level (g/dl) were lower ($p<0.05$) in all groups during the summer season compared to winter values. Packed cell volume (PCV) also showed a similar trend, being lower ($p<0.05$) during summer compared to the winter season. The total erythrocytic count (TEC) was reduced in summer as compared to winter season, conversely, total leukocytic count (TLC) was higher ($p<0.05$) during the summer season as compared to the winter season. The supplementation of melatonin and vitamin E did not significantly ($p<0.05$) influence the haematological profile during the summer or winter season in pigs.

Table 2. Seasonal variation of Haematological parameters in control and supplemented (melatonin and vitamin E) groups

Groups/ Parameters	Season	
	Summer	Winter
Hemoglobin (g/dl)		
Control	10.91±0.14[b]	14.45±0.10[a]
Melatonin	11.13±0.14[b]	14.11±0.07[a]
Vitamin E	11.00±0.13[b]	14.33±0.08[a]
Packed Cell Volume (PCV) (%)		
Control	30.81±0.15[b]	43.42±0.25[a]
Melatonin	32.00±0.17[b]	43.12±0.26[a]
Vitamin E	32.38±0.16[b]	43.01±0.23[a]
Total Erythrocytic Count (million/cmm)		
Control	6.18±0.03[b]	7.08±0.07[a]
Melatonin	6.02±0.04[b]	7.20±0.07[a]
Vitamin E	5.98±0.06[b]	7.13±0.07[a]
Total Leukocytic Count (thousand/cmm)		
Control	22.66±0.07[a]	18.36±0.07[b]
Melatonin	22.35±0.06[a]	18.59±0.09[b]
Vitamin E	22.63±0.05[a]	18.40±0.07[b]

Different superscripts differ significantly (p>0.05) in row-wise

Table 3. The serum concentration of Cortisol (ng/ml), Growth hormone (pg/ml), LDH (UL-1), Melatonin (pg/ml) and Vitamin E (mg/l) in control and supplemented (melatonin and vitamin E) groups during summer and winter seasons

Groups/ Parameters	Season	
	Summer	Winter
Cortisol level (ng/ml)		
Control	7.08±0.06[a]	3.49±0.05[b]
Melatonin	6.70±0.03[a]	3.39±0.04[b]
Vitamin E	6.79±0.03[a]	3.43±0.04[b]
Growth Hormone (pg/ml)		
Control	1.87±0.10[a]	2.11±0.12[a]
Melatonin	1.91±0.10[a]	2.18±0.13[a]
Vitamin E	1.92±0.10[a]	2.16±0.13[a]
LDH (U/L)		
Control	940.16±2.73[a]	799.00±4.55[b]
Melatonin	941.18±2.08[a]	800.78±4.59[b]
Vitamin E	948.56±1.81[a]	794.41±6.00[b]
Melatonin (pg/ml)		
Control	126.02±0.49[b]	141.6±00.31[a]
Melatonin	167.09±3.05[a]	170.92±2.75[a]
Vitamin E	126.20±0.53[b]	141.72±0.39[a]
Vitamin E (mg/l)		
Control	1.74±0.04	1.80±0.05
Melatonin	1.76±0.05	1.82±0.05
Vitamin E	4.17±0.09	4.27±0.10

Different superscripts differ significantly (p>0.05) in row-wise

The serum cortisol level (ng/mL) was significantly higher (p>0.01) during the summer season as compared to the winter season in all groups, however, supplemented groups (melatonin and vitamin E) showed lower cortisol level during summer season as compared to control group (Table 3). The concentration of growth hormone (pg/mL) did not differ significantly (p>0.01) between the season in all the groups; however, higher values were observed during the winter

season as compared to the summer season (Table 3).

The concentration of LDH (U/L) was significantly ($p>0.01$) higher during the summer season as compared to the winter season, however, the concentration did not differ significantly between the supplemented (melatonin and vitamin E) groups as compared to control in both the season (Table 3).

The serum melatonin was significantly ($p<0.05$) higher during winter season than summer season in control and vitamin E supplemented group, the melatonin supplemented group showed non-significantly higher values in winter than summer (Table 3). The serum vitamin E (mg/L) level was four times higher ($p>0.01$) in vitamin E supplemented group than other groups; the concentration was slightly higher during the winter than the summer season (Table 3).

The average body weight of the animals showed significant ($p>0.01$) difference between summer and winter season, the average body weight is better during winter season as compared to summer season (Fig. 1). The supplementation of melatonin and vitamin E did not improve the average body weight in both seasons. The litter size did not differ significantly between the groups as the experimental animals were observed till furrowing (Table 4).

The relative mRNA expression of HSP70 was decreased significantly ($p>0.01$) in vitamin E supplemented group during summer as compared to winter season (Fig. 2C). The expression level of HSP70 did not change significantly ($p>0.05$) in melatonin supplemented and control groups (Fig. 2B, 2A) during summer and winter season.

Heat stress poses a major challenge to livestock productivity in tropical climates, adversely impacting animal performance. In pigs, the lack of functional sweat glands and the presence of a thick subcutaneous fat layer limit thermoregulation, making them particularly vulnerable to heat stress. This study observed significantly lower ($p<0.05$) Hb, PCV, and TEC values during the summer season compared to winter, accompanied by a significant increase ($p<0.05$) in leukocyte counts. Despite antioxidant supplementation (melatonin and vitamin E), no improvement was noted in the haematological profiles. This aligns with previous findings, which reported decreased Hb, PCV, MCV, MCH, and MCHC levels during summer in growing pigs (Mayengbam & Tolenkhomba, 2015). Heat stress has been shown to reduce feed intake by 37%, affect blood profiles, and impair oxygen transport (Collin *et al.,* 2001).

Table 4. Litter size in control and supplemented (melatonin and vitamin E) groups during summer and winter seasons

Groups	Summer	Winter
Control	4.50±0.22	5.00±0.45
Melatonin	5.50±0.43	5.83±0.31
Vitamin E	5.00±0.26	5.17±0.31

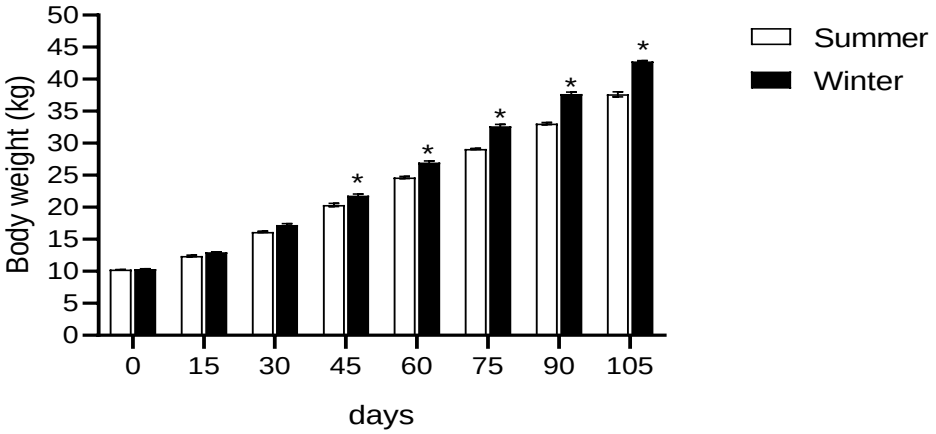

Fig. 1. Body weight (kg) of the animals during summer and winter

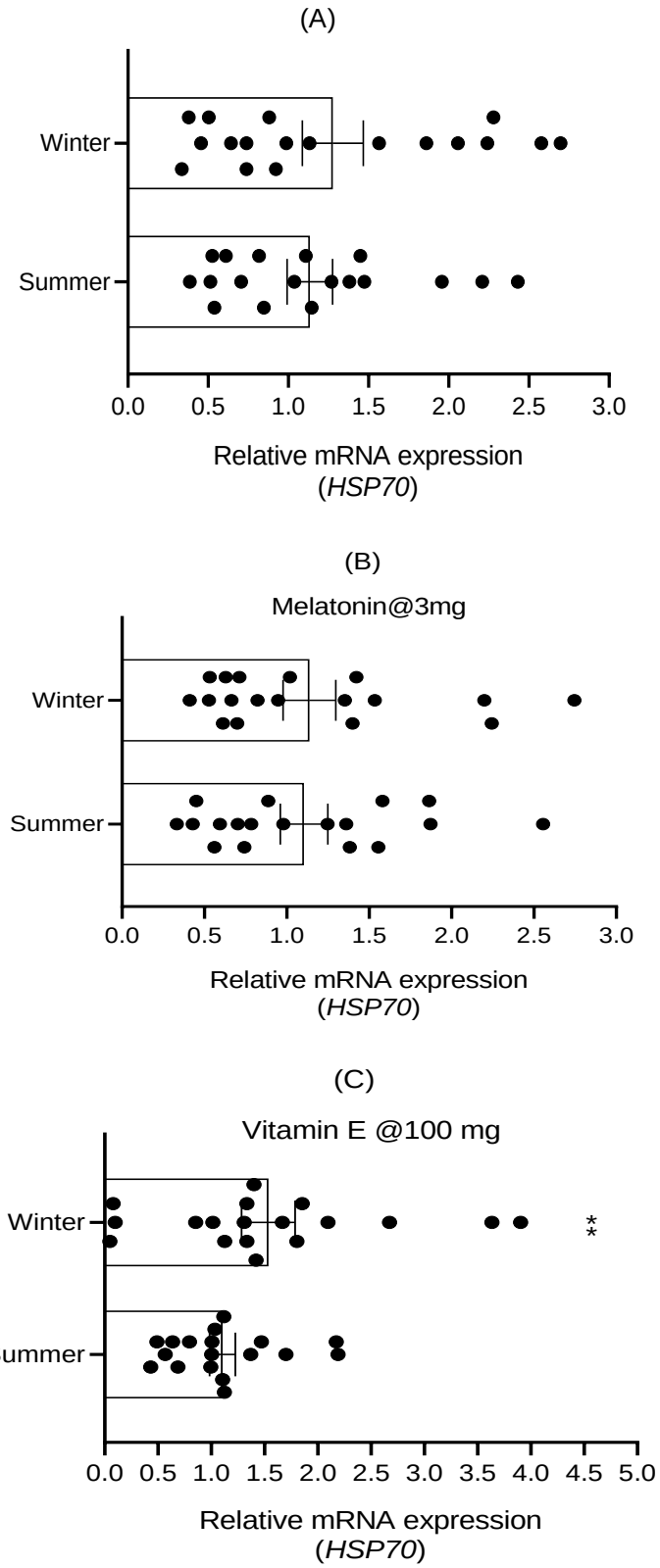

Fig. 2. The relative m RNA expression of HSP70 in control (A), Melatonin (B) and Vitamin E (C) supplemented groups

Elevated leukocyte counts during summer, unaffected by antioxidant supplementation, may reflect environmental stressors triggering immune responses (Adenkola *et al.,* 2011; Shengjun *et al.,* 2010). Seasonal variations in leukocyte levels are known to compromise immune function under prolonged heat exposure (Nascimento *et al.,* 2019). Dietary vitamin E is reported to protect RBCs from oxidative damage by increasing glutathione peroxidase activity (Liu *et al.,* 2016) and reducing inflammatory markers like C-reactive protein and haptoglobin (Lauridsen *et al.,* 2021). However, in this study, vitamin E supplementation showed no significant improvement in haematological parameters, consistent with similar findings in pigs under heat stress (Silva-Guillen *et al.,* 2024). While melatonin has been documented to improve blood cell production and immune function by reducing stress-induced inflammation (Bouroutzika *et al.,* 2022), this study found no significant improvements with melatonin supplementation during the summer season.

Lactate dehydrogenase (LDH), an enzyme involved in energy metabolism, was significantly elevated ($p>0.01$) during summer, indicating tissue damage or metabolic shifts under heat stress. Similar observations have been reported, with elevated LDH levels in pigs during summer linked to stress and reduced carcass quality (Ijiri *et al.,* 2022; Okab *et al.,* 2008). Increased LDH levels due to tissue leakage reflect adaptive metabolic shifts and tissue damage during heat stress (Vashi *et al.,* 2018). Although melatonin and vitamin E supplementation did not mitigate LDH levels, their potential benefits warrant further investigation.

Cortisol levels, a biomarker of stress, were higher ($p>0.05$) during summer. Elevated cortisol contributes to metabolic disruptions, oxidative stress, and immune suppression (Knezevic *et al.,* 2023). However, antioxidant supplementation resulted in lower cortisol levels during summer, suggesting their mitigating effects. Seasonal variations in serum melatonin, with higher levels observed in winter, may be attributed to shorter day lengths and endogenous pineal gland activity (Andersson, 2001).

Growth performance metrics, including average body weight and growth hormone levels, were higher during winter due to favorable environmental conditions. Despite antioxidant supplementation, no improvement in growth hormone levels or body weight was observed during summer. Pathak *et al.,* (2018) reported similar seasonal effects, with reduced growth under heat stress conditions where THI exceeded 72. Cortisol-mediated suppression of growth hormone secretion during heat stress may contribute to impaired feed intake and overall performance (Sillence & Etherton, 1987). Genetic components, such as the growth hormone receptor (GHR) gene, may regulate GH responses to heat stress (Kim *et al.,* 2018).

The expression pattern of HSP70 mRNA in pigs is breed-specific and influenced by thermal tolerance, with higher expression reported in exotic and crossbred pigs during summer under tropical conditions (Vashi *et al.,* 2018). The present study observed a decrease ($p<0.05$) in HSP70 mRNA expression in vitamin E-supplemented pigs during summer compared to winter, potentially attributed to reduced ROS and stabilized antioxidant status (Bora *et al.,* 2024; Jang *et al.,* 2014). Vitamin E and selenium supplementation may restrict HSP expression through enhanced superoxide dismutase activity (Kumbhar *et al.,* 2018; Dokladny *et al.,* 2006). While melatonin has been shown to increase HSP70 expression in goats and porcine oocytes (Hasin *et al.,* 2017; Yang *et al.,* 2021), this study found no seasonal variation in HSP70 expression in melatonin-supplemented pigs. Heat stress impaired the jejunal integrity in pigs by reducing villus height, increasing HSP70, and disrupting tight junction proteins, while altering selenoprotein and inflammatory gene expression (He *et al*., 2022). The supplementation with organic selenium reversed these effects by restoring mucosal architecture, reducing oxidative stress and enhancing antioxidant selenoprotein levels (He *et al*., 2022). Zhang et al. (2015) reported that oregano essential oil supplementation significantly reduced serum cortisol and norepinephrine levels in transported pigs, indicating lowered stress response, enhancing antioxidant defenses while reducing oxidative stress markers. Zhang *et al*. (2015) also found that these effects were comparable to vitamin E, suggesting oregano essential oil as viable strategy to mitigate transportation-induced physiological stress.

4. CONCLUSION

Heat stress significantly impacted haematological parameters, cortisol levels, lactate dehydrogenase, and body weight in pigs, with adverse effects more pronounced in summer. While melatonin and vitamin E supplementation

reduced summer cortisol levels, however, did not improve haematological profiles or LDH levels. Vitamin E supplementation notably decreased HSP70 mRNA expression during summer, highlighting its oxidative stability benefits. These findings underscore the need for improved nutritional and management strategies to counteract heat stress in pigs.

ETHICAL APPROVAL

The experiment was approved by the Institutional Animal Ethics Committee constituted as per the article number 13 of the CPCSEA rules laid down by the Government of India and conducted following the code of ethics for animal experimentation.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The authors hereby declare that NO generation of AI technologies such as large language models and text-to image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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