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Identification of HSPA1A SNPs and heat stress biomarkers using infrared thermography and gene expression analysis in *Bos indicus*

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ABSTRACT

A study was conducted to evaluate seasonal thermal adaptability in 1,044 Deoni cattle representing Wanera (n = 356), Balankya (n = 560), and Shewara (n = 128) strains under semi-arid conditions of western India using infrared thermography, physiological measurements, and HSPA1A gene expression analysis. Body surface temperature was recorded from multiple anatomical regions during summer and winter, and the relationship with physiological variables and molecular markers was evaluated using a linear mixed-effects model with animal as a random effect. Surface temperature differed significantly at anatomical regions ($P < 0.001$) and varied with season and physiological status. Among the evaluated regions, the hump maintained a relatively stable mean surface temperature (approximately 34°C) across seasons and showed significant positive correlations with thorax, flank, and hip temperatures ($r = 0.71–0.89$, $P < 0.001$). The temperature–humidity index (THI) was moderately associated with hump temperature at morning but only weakly associated with the evening observations. No significant differences were observed in rectal temperature or respiration rate between the AA and BB genotypes ($P > 0.05$). Relative HSPA1A expression, quantified using the $2^{-\Delta\Delta Ct}$ method after normalization to GAPDH, was found to be inversely associated with body surface temperature during morning observations ($r = -0.71$, $P < 0.001$) but showed no significant association during evening measurements ($P > 0.05$). These findings suggested that infrared thermography, when combined with physiological measurements and HSPA1A expression analysis, can provide complementary information for assessing thermal status in Deoni cattle.

Keywords: Deoni cattle, HSPA1A gene, Infrared thermal imaging, Rectal temperature

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Infrared thermography (IRT) is a non-invasive diagnostic imaging method that captures the infrared energy emitted from an object's surface, enabling visualization and measurement of surface temperature patterns (Ring and Ammer, 2012, McCafferty, 2007). In livestock, these temperature changes often reflect underlying physiological processes and can serve as valuable indicators of health, welfare, and environmental stress (Schaefer *et al.* 2012, Stewart *et al.* 2008, Montanholi *et al.* 2008). Exposure of dairy animals to high ambient temperatures triggers the production of heat shock proteins (HSPs), a group of highly conserved molecular chaperones that protect cells from heat-induced damage and other physiological stresses (Collier *et al.* 2019, Lindquist and Craig, 1988). The expression of HSPs generally increases in proportion to the severity and duration of thermal and physiological stress (Kamboh *et al.* 2018, Kiang and Tsokos, 1998). The HSP superfamily is classified according to molecular weight into HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, HSP10, and the small heat shock protein (sHSP) family (Kamboh *et al.* 2018). Among these, HSP70 is

one of the most abundant and extensively studied stress-responsive proteins because of its central role in protein folding, cellular protection, and thermotolerance (Mayer and Bukau, 2005, Dangi *et al.* 2012). Thermal imaging has become an important diagnostic tool in both human and veterinary medicine, particularly for the early detection of inflammation, musculoskeletal disorders, and heat stress (Ring and Ammer, 2012). In livestock production, IRT enables rapid, contact-free assessment of animal health while minimizing handling stress and reducing the risk of injury to both animals and handlers (Schaefer *et al.* 2012, Stewart *et al.* 2008). Thermal imaging has emerged as an important component of precision livestock farming, supporting early disease detection, welfare monitoring, and evidence-based management decisions (Williams, 2019; Berckmans, 2017). The present study hypothesized that body surface temperature varies by region and season, correlates with heat-stress indicators, and is associated with HSPA1A expression rather than genotype alone. The identified thermal imaging sites and HSPA1A variants were evaluated as candidate biomarkers requiring validation in larger independent populations before routine application.

MATERIALS AND METHODS

A total of 15,500 infrared thermal images from 1,044 adult Deoni cattle (Wanera: 356, Shewara: 128, Balankya: 560) aged 2.5–12.5 years were analyzed. Data were collected during summer (15 April–30 June 2023) and winter (1 December 2023–31 January 2024), with measurements recorded twice daily (07:00–09:00 and 16:00–18:00 h). The Deoni breeding tract experiences temperatures of 9–44°C, annual rainfall of 750–890 mm, and lies at approximately 18°23′N, 77°07′E and 426.46 m above mean sea level. Measurements were recorded in the morning (07:00–09:00 h) and evening (16:00–18:00 h). Ambient temperature ranged from 20–35°C, relative humidity from 40–85%, wind speed from 0.2–3.0 m s^{-1}, and THI from 62–85, representing thermoneutral to moderate/severe heat-stress conditions.

Infrared thermal imaging study: Infrared thermal images were captured using a FLIR E76 infrared camera (320 × 240 pixels) at approximately 1.5–2.0 m from the animal, with the camera positioned nearly perpendicular (90°) to the target body surface. Imaging was performed without disturbing the animals, and the tail was held aside for vulvar imaging. Images were analyzed using FLIR Thermal Studio, with bovine skin/hair emissivity set at 0.98 and reflected apparent temperature standardized to 20°C.

Physiological parameters: Respiration rate (RR) was determined by counting flank movements, while rectal temperature (RT) was measured using a digital thermometer. Measurements were recorded twice monthly during morning and evening periods from May to October, and mean values were used for analysis. Mean temperatures of the respective ROIs from infrared thermal images were also included in the analysis.

Temperature–Humidity Index (THI): Ambient temperature (°C) and relative humidity (%) were recorded at the time of infrared thermographic imaging using a portable digital thermo-hygrometer. The Temperature–Humidity Index (THI) was calculated using the equation proposed by Mader *et al.*:

$$\mathrm{THI} = (0.8 \times T_{bd}) + \left[\left(\frac{RH}{100} \right) \times (T_{db} - 14.4) \right] + 46.4$$

where:

THI = temperature–humidity index,

Tdb = dry-bulb ambient temperature (°C),

RH = relative humidity (%).

THI was used to classify heat-stress conditions: <72 indicated comfort, 72–78 mild heat stress, 79–88 moderate heat stress, and >88 severe heat stress. During each infrared thermography session, ambient temperature, relative humidity, wind speed, and THI were recorded using a digital thermo-hygrometer.

Molecular study of HSPA1A gene in Deoni cattle: HSPA1A (Heat Shock Protein 70) gene was selected as a candidate gene due to its key role in the cellular response to thermal stress. The bovine HSPA1A gene (Gene ID: 281825) is located on chromosome 23 (BTA23), with reference accession NC_037350.1 (ARS-UCD1.2). The promoter region was targeted for SNP analysis. Seasonal infrared thermal imaging was conducted during summer and winter to assess associations with HSPA1A expression and body surface temperature. Blood samples (4–5 mL) were collected from jugular vein of 60 Deoni cattle for genomic DNA analysis. DNA concentration and purity were assessed spectrophotometrically at 260 and 280 nm. DNA concentration (µg/mL) was calculated using the standard formula:

$$\text{DNA concentration (µg/mL)} = \text{A260} \times 50 \times \text{Dilution Factor}$$

where an absorbance of 1.0 at 260 nm corresponds to 50 µg/mL of double-stranded DNA. Polymerase chain reaction (PCR) conditions were optimized to obtain specific and reproducible amplification of the target DNA fragment.

PCR Optimization and amplification: Reaction conditions were refined by fine-tuning of annealing temperature and magnesium chloride () levels to secure the highest possible yield while preventing non-specific binding. Once finalized, this optimized framework was applied uniformly to all genetic templates (Table 1).

PCR assays were performed in 25 µL reactions containing Taq Gold Fast PCR Master Mix, nuclease-free water, forward and reverse primers, and genomic DNA. The reactions were amplified using optimized thermal-cycling conditions. The amplified fragment was located within the HSPA1A promoter region (5′ upstream regulatory region) and excluded 5′ UTR, exon, and intron sequences, enabling identification of polymorphisms potentially affecting HSPA1A transcription under heat stress.

Table 1. Primer sequences used for quantitative real-time PCR analysis

Gene	Primer	Primer sequence (5′–3′)	Primer length (bp)	Amplicon size (bp)	Reference
HSPA1A	F	GCCAGGAAACCAGAGACAGA	20	539	Ramesha *et al.* 2016
	R	CCTACGCAGGAGTAGGTGGT	20		
GAPDH	F	GGTGATGCTGGTGCTGAGTA	20	265	AF030943
	R	TCATAAGTCCCTCCACGATG	20		

Restriction Fragment Length Polymorphism (PCR-RFLP) analysis: The 539 bp HSPA1A PCR amplicons were digested with HindIII restriction endonuclease (recognition sequence: 5′-AAGCTT-3′) in a final reaction volume of 30 µL and incubated at 37°C for 14 h according to the manufacturer's instructions. PCR products containing the HindIII restriction site yielded the expected digestion fragments, whereas products lacking the restriction site remained undigested. The digested products and a 100-bp DNA ladder were separated on a 1.5% agarose gel at 100 V for 45 min. Representative genotype-specific PCR products were purified and subjected to Sanger sequencing (Eurofins Genomics India Pvt. Ltd., Bengaluru) to validate the identified SNPs.

Quantitative Real-Time PCR (qPCR) expression profiling: Total RNA was isolated from peripheral blood leukocytes of 30 Deoni cattle (15 heat-tolerant and 15 heat-susceptible) using TRIzol® Reagent. RNA quality was assessed by NanoDrop spectrophotometry and denaturing agarose gel electrophoresis. DNase-treated RNA (1 µg) was used for cDNA synthesis with oligo(dT)18 primers. qRT-PCR was performed on a LightCycler Nano System using Maxima SYBR Green/ROX Master Mix, with GAPDH as the reference gene. Each 25 µL reaction contained 12.5 µL master mix, 1.0 µL each of forward and reverse primers, 1.5 µL cDNA, and 9.0 µL nuclease-free water.

Thermocycling profile and data analysis: qRT-PCR was performed using 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, following initial denaturation at 94°C for 3 min and final extension at 72°C for 10 min. Melting-curve analysis confirmed amplification specificity. HSP70 expression was quantified from Cq values, with no amplification in control reactions. GAPDH showed stable expression and was used for normalization. The assay included 60 biological samples (30 heat-tolerant and 30 heat-susceptible cattle), each analyzed in technical triplicate.

Statistical analysis: Statistical analyses were conducted using SPSS version XX (IBM Corp., Armonk, NY, USA). Data were expressed as mean ± SE. Effects of season, strain, genotype, reproductive status, and observation time were evaluated using GLM and linear mixed-effects models, with animal identity as a random effect to account for repeated measurements. Significant differences were assessed using Tukey's HSD test.

RESULTS AND DISCUSSION

Infrared thermal imaging study: Mean infrared surface temperatures ranged from 30.32 ± 0.83°C (muzzle) to 35.41 ± 0.15°C (flank) across the 11 body regions (Fig. 1), and were consistent with reported thermal adaptation in indigenous cattle under harsh climates (Gaughan *et al.* 2010).

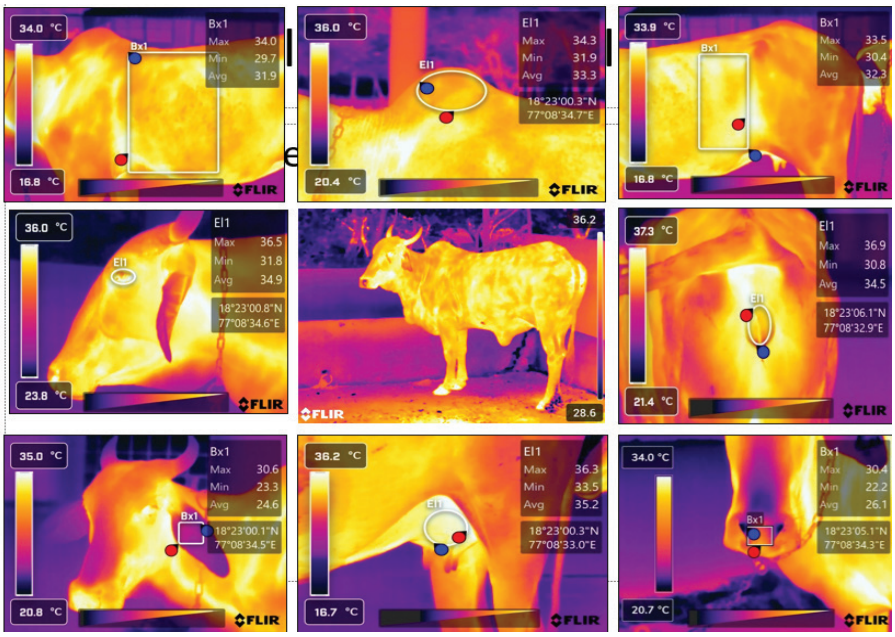


Fig. 1. Infrared thermal images of various body parts (thorax, hump, flank, eye, vulva, ear, udder, muzzle, neck, dewlap, hip) of Deoni cattle

Diurnal variations in surface temperature correlations: Pearson's correlation analysis was performed using data from 46 animals (n = 46) to evaluate the relationships between anatomical surface temperatures and environmental variables. The thorax, hump, flank and hip temperature observations in morning exhibited strong positive correlations with the mean body surface temperature (Fig. 2). Hump temperature did not differ significantly between seasons (*P*=0.275) or between morning and evening observations (*P=0.157*), and no significant season × time interaction was detected (*P=0.213*). These findings indicated that hump temperature remained relatively stable under different environmental conditions, supporting its suitability as a reference anatomical site for infrared thermographic assessment. Flank temperature showed the strongest association with mean body surface temperature (r = 0.87, 95% CI: 0.77–0.93, P < 0.0001), followed by hump temperature (r = 0.86, 95% CI: 0.75–0.92, P < 0.0001). THI and dry-bulb temperature showed moderate positive correlations with body surface temperature (r = 0.41, P = 0.0048 and r = 0.34, P = 0.0206, respectively). In the evening, these associations were weak and non-significant. In contrast, ear, eye, muzzle, and dewlap temperatures remained strongly correlated with mean body surface temperature (r ≈ 0.70, n = 46, P = 0.0001), indicating their potential as reliable indicators of thermal status (figure 3 & 4). A significant positive association was also observed between rectal temperature and selected surface regions.

Relationships between vital signs and thermal profiles: Core rectal temperature showed a significant positive association with specific surface temperatures, while muzzle, eye, hump, thorax, flank, and vulva temperatures were positively but non-significantly associated with RT. Respiration rate was positively associated with ear, dewlap, hump, flank, vulva, and udder temperatures, but negatively associated with the remaining regions.

Strain-wise comparison of infrared surface temperatures (°C) in Deoni cattle: Strain-wise comparison of Wanera (n = 16), Balankya (n = 15), and Shewara (n = 15) Deoni cattle revealed no significant differences in the infrared surface temperatures of muzzle, eye, ear, neck, dewlap, hump, thorax, flank, hip, vulva, or udder (P > 0.05). Although Balankya animals exhibited marginally higher mean

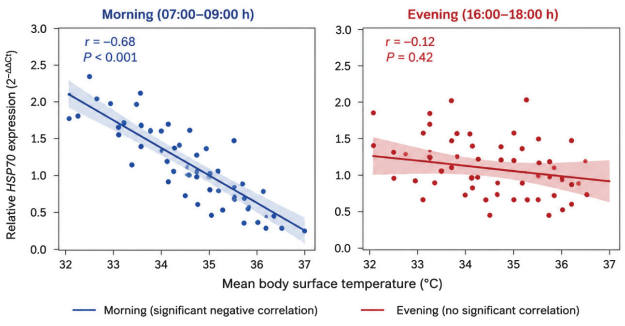


Fig. 2. Correlation of Infrared thermal images of various body parts and environmental factors of Deoni cattle

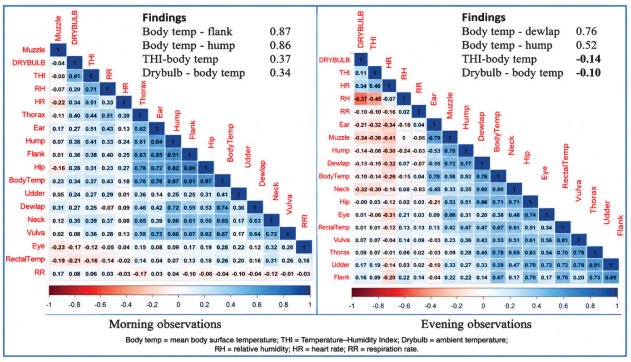


Fig. 3. Correlation of Infrared thermal images of various body parts and environmental factors of Deoni cattle.

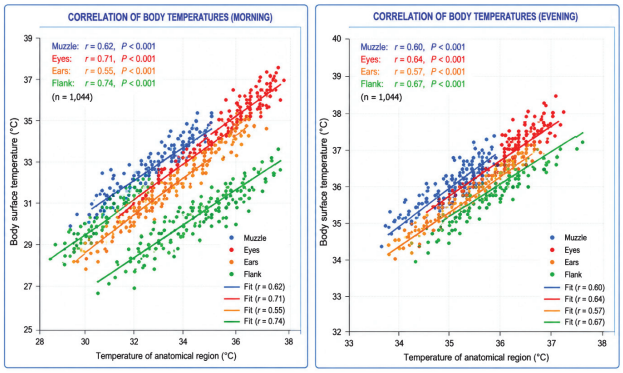


Fig. 4. Correlation of body temperatures morning and evening

temperatures for thorax (35.27 ± 0.17 °C), flank (35.45 ± 0.14 °C), and hip (34.58 ± 0.12 °C), these differences were not statistically significant. The overall similarity among strains suggested comparable thermoregulatory responses and supported pooling of the data for subsequent correlation and molecular analyses.

Visualization and sequencing of the HSPA1A gene: Following electrophoresis, a single 539-bp band was observed under UV illumination, confirming successful HSPA1A amplification (Figure 5). The amplicons were subjected to HindIII-based PCR-RFLP and Sanger sequencing. Representative products were purified and commercially sequenced by Eurofins Genomics India Pvt. Ltd. The chromatograms were edited and aligned using BioEdit, generating a high-quality 359-bp consensus sequence for SNP identification and analysis.

Mutation and SNP analysis: Sequence analysis identified an A→G transition at position 284 in the HSPA1A promoter. As a regulatory-region variant, it did not alter the protein sequence but may affect promoter activity or transcription-factor binding and thereby influence HSPA1A expression under heat stress. Chromatogram analysis confirmed the identified promoter SNP. Genotyping by PCR-RFLP classified the animals into two genotypic groups, designated as AA and BB, based on the observed restriction fragment patterns. No significant differences were observed between the two genotypes with respect to rectal temperature or respiration rate, indicating that the

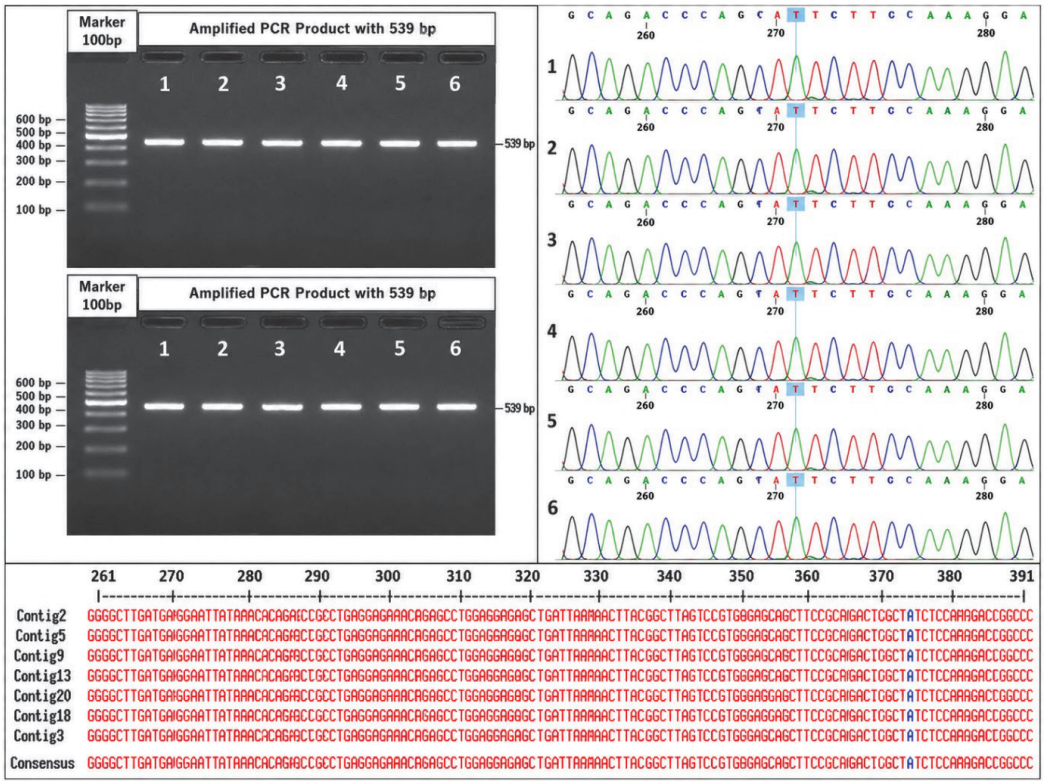


Fig. 5. PCR amplification of the HSPA1A promoter region and representative sequencing chromatogram showing the A>G polymorphism

identified promoter polymorphisms were not associated with the physiological indicators of heat stress evaluated in the present study. An additional SNP was identified at position 397, involving a thymine-to-cytosine (T→C) substitution within the promoter region. Similar promoter-region polymorphisms in the HSPA1A gene have been reported in Karan Fries and Sahiwal cattle by Ramesha *et al.* (2016). Likewise, Deb *et al.* (2013) demonstrated that promoter polymorphisms in HSPA1A were associated with increased gene expression and enhanced heat tolerance in *Bos indicus* cattle compared with crossbred animals. Sodhi *et al.* (2013) also reported considerable genetic polymorphism within the HSPA1A promoter region in Frieswal and Sahiwal cattle, highlighting its potential role in regulating the cellular response to thermal stress.

A total of 60 Deoni cattle were genotyped for the HSPA1A promoter polymorphism by PCR-RFLP, and all samples were successfully assigned to a genotype, resulting in a 100% genotyping success rate. Two homozygous genotypes, AA (n = 42; 70.0%) and BB (n = 18; 30.0%), were identified, whereas the heterozygous genotype (AB) was not detected. The allele frequencies were 0.70 for allele A and 0.30 for allele B, yielding a minor allele frequency (MAF) of 0.30. Hardy–Weinberg equilibrium analysis revealed a significant deviation from equilibrium ($\chi^2 = 36.73$, df = 1, $P < 0.0001$), primarily due to the absence of heterozygous individuals. The amplified fragment was mapped to the *Bos taurus* reference genome (ARS-UCD1.2), and the identified SNP is located within the promoter region upstream of the HSPA1A coding sequence. An A>G SNP was identified in the promoter region of the HSPA1A gene. The HSPA1A promoter polymorphism was successfully genotyped in 60 Deoni cattle using PCR-RFLP. Bidirectional Sanger sequencing confirmed the A>G promoter polymorphism, with 100% concordance with PCR-RFLP genotyping. Genotype and allele frequencies were calculated from observed counts, with MAF defined as the less frequent allele. HWE was assessed using the χ^2 goodness-of-fit test.

Genotypic association with physiological traits: Targeted amplification and restriction profiling of the HSP70 locus in Deoni cattle identified two homozygous genotypes (AA and BB). The association between these genotypes and physiological traits was evaluated using rectal temperature and respiration rate. Statistical analysis revealed no significant differences between the AA and BB genotypes for either trait (P= 0.152), indicating that the identified HSP70 polymorphism was not associated with physiological responses under the prevailing field management conditions. These findings were consistent with the observations of Deb *et al.* (2013).

Polymorphic architecture and sequence conservation: Applying the PCR-RFLP technique successfully confirmed the polymorphic nature of the HSPA1A gene within the Deoni population. Sequence characterization of the polymorphic region highlighted an A → G single nucleotide polymorphism (SNP). Despite the molecular variation, no differences were observed in respiration rate

or rectal temperature, while the HSPA1A sequence showed high evolutionary conservation across domestic livestock species. The identified SNPs were queried against the NCBI dbSNP and Ensembl variation databases, and no matching records were found. Therefore, these variants are considered putatively novel. However, additional validation in larger populations is required before formal designation as novel variants. An A>G SNP was identified in the promoter region of the HSPA1A gene. Two SNPs were identified within the promoter region of the HSPA1A gene. SNP1 involved an A/G substitution, while SNP2 involved a T/C substitution, with the respective flanking sequences retained as follows:

SNP1: 5′-AGCTTGACCTGA/GTGGCCTGATCGACT-3′, located in the promoter region of HSPA1A.

SNP2: 5′-GGTCCAGTTAT/CGGATCGAGCTTCCA-3′, located in the promoter region of HSPA1A.

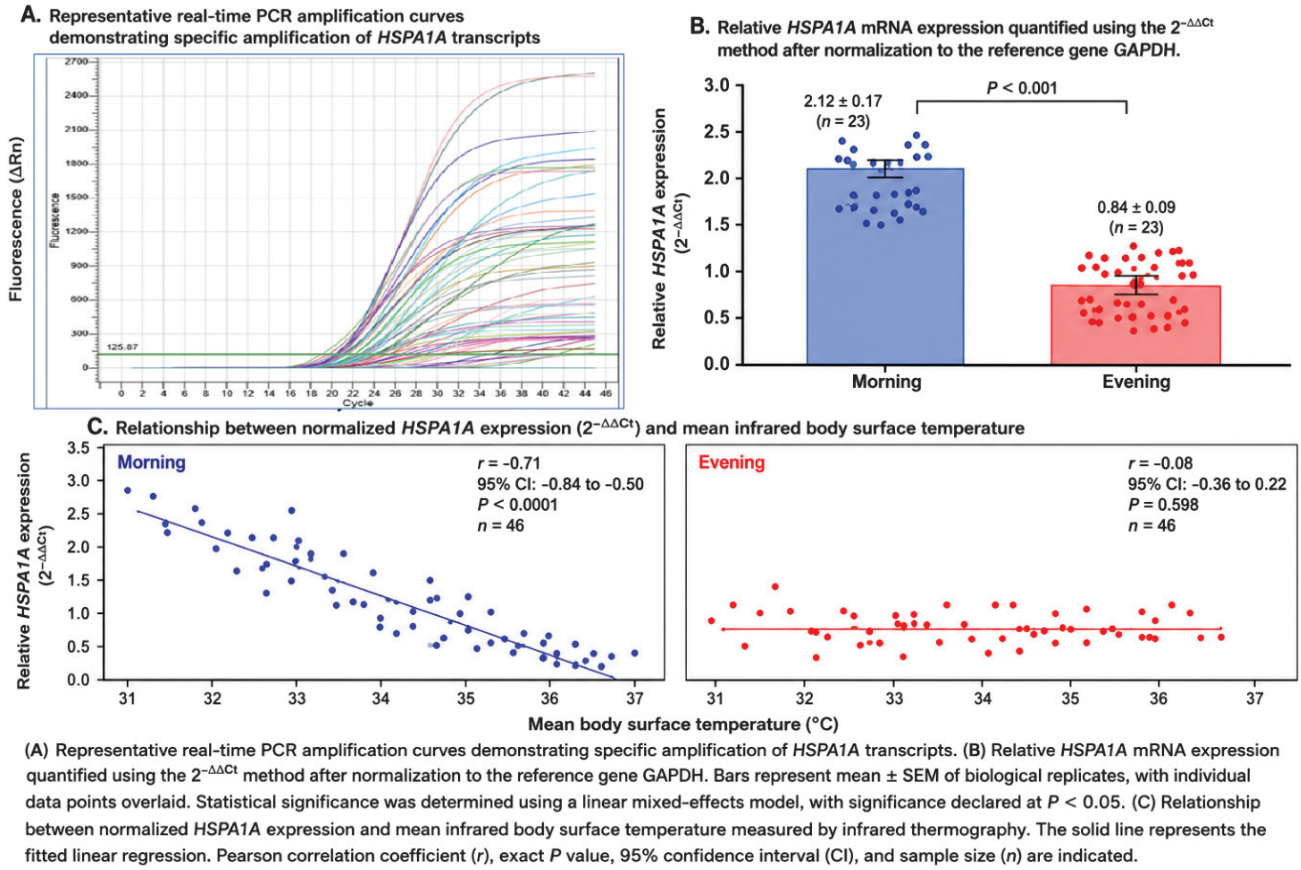
Evolutionary homology and sequence alignment: Comparative sequence analysis revealed that the Deoni *HSPA1A* sequence shared its highest identity with *Bos gaurus* × *Bos taurus* at 99.09%, followed by *Bos taurus* at 98.77%. Lower identity percentages were observed with more distant ruminants, including *Bos mutus* (96.05%), *Bubalus bubalis* (95.46%), *Ovis aries* (91.24%), *Capra hircus* (91.18%), and *Cervus elaphus* (89.80%). The elevated sequence identity seen among members of the *Bos* genus highlighted their tight phylogenetic relationship. Conversely, the reduced homology with sheep, goats, and red deer reflected a wider evolutionary distance within the suborder Ruminantia. Such strong evolutionary preservation across diverse species underscored the critical biological role of *HSPA1A* plays in cellular defense mechanisms and thermal tolerance, aligning with the previous reports by Gupta *et al.* (2013) and Deb *et al.* (2013).

Genotypic effects and real-time expression trends: Statistical evaluations linking *HSPA1A* polymorphisms to vital signs showed no significant differences () between the identified variants. Specifically, cows carrying the AA genotype showed a rectal temperature of 100.52 ± 0.023°F and a respiration rate of 19.07 ± 0.117 breaths per minute, while those with the BB genotype exhibited a rectal temperature of 100.65 ± 0.019°F and a respiration rate of 19.17 ± 0.108 breaths per minute. Quantitative real-time PCR (qPCR) tracking revealed an inverse relationship between *HSPA1A* gene expression levels and the animals' infrared surface temperatures. The negative correlation was more pronounced during morning observations, whereas the evening data showed little or no association. These findings suggested that, although genetic variation exists within the HSPA1A locus, its direct effects on basic physiological traits are limited under standard environmental conditions. This pattern matched with the historical literature showing that genetic variations within heat-shock or stress-response pathways did not always result in clear, outward phenotypic or physiological changes (Basiricò *et al.* 2011).

Intersection of thermal profiles, genotypes, and transcriptional activity: Statistical analysis confirmed that the identified HSPA1A genetic variants did not significantly influence outward vital signs (). Specifically, Deoni cattle with the AA genotype presented a rectal temperature of 100.52 ± 0.023°F and a respiration rate of 19.07 ± 0.117 breaths per minute. Similarly, those with the BB genotype displayed a rectal temperature of 100.65 ± 0.019°F alongside a respiration rate of 19.17 ± 0.108 breaths per minute. These minimal variations suggested that HSPA1A polymorphism has limited effects on primary homeostatic traits under normal conditions. The negative association between HSP70 expression and body surface temperature should be interpreted cautiously, as HSP70 expression is influenced by circadian rhythms and delayed responses to heat exposure. Thus, the observed relationship likely reflects the complex temporal dynamics of the heat-stress response rather than a direct protective effect.

Thermoregulation and surface heat dissipation: The notable variations in surface heat observed across distinct body regions point to specialized regional heat-shedding capacities. This supports historical data emphasizing how peripheral zones such as the muzzle, ears, and dewlap serve vital roles in bovine vascular thermoregulation (McManus *et al.* 2009; Collier *et al.* 2008). Furthermore, the strong positive correlation linking respiration rates to the surface temperatures of the ear, dewlap, hump, flank, vulva, and udder reflected an active physiological reliance on evaporative cooling pathways to mitigate thermal stress (West, 2003).

HSPA1A transcriptional dynamics: Quantitative real-time PCR (qPCR) assays yielded an average cycle threshold () value of 25.91 ± 0.72 for the *HSPA1A* gene. Correlation analyses revealed an inverse relationship between *HSPA1A* transcript abundance and the animals' infrared surface temperatures. This negative correlation was highly pronounced during early morning observations, whereas evening data revealed virtually no statistical relationship (Figure 6). This inverse morning pattern suggested an active, protective cellular defence system. Because *HSPA1A* proteins operate as molecular chaperones that preserve structural protein integrity and assist in cellular adaptation (Lindquist and Craig, 1988; Kregel, 2002), their elevated expression at lower morning temperature may be indicative of a preparatory or highly sensitive cellular response before peak thermal load occurs later in the day. Standard curve analysis demonstrated efficient amplification of both HSP70 and GAPDH primer pairs, with amplification efficiencies within the acceptable range (90–110%) and correlation coefficients (R^2) greater than 0.99. Melt-curve analysis revealed a single specific amplification peak for each gene, confirming amplification specificity. Technical triplicates showed high reproducibility, with Ct standard deviations below 0.30 for all analysed samples. GAPDH showed minimal variation in Ct values across experimental groups, indicating stable expression under the experimental heat-stress conditions. The stability analysis supported its suitability as the endogenous reference gene for normalization of HSP70 expression. Use of a single



(A) Representative real-time PCR amplification curves demonstrating specific amplification of *HSPA1A* transcripts. (B) Relative *HSPA1A* mRNA expression quantified using the $2^{-\Delta\Delta Ct}$ method after normalization to the reference gene GAPDH. Bars represent mean ± SEM of biological replicates, with individual data points overlaid. Statistical significance was determined using a linear mixed-effects model, with significance declared at $P < 0.05$. (C) Relationship between normalized *HSPA1A* expression and mean infrared body surface temperature measured by infrared thermography. The solid line represents the fitted linear regression. Pearson correlation coefficient (*r*), exact *P* value, 95% confidence interval (CI), and sample size (*n*) are indicated.

Fig. 6. Real time qPCR of HSPA1A gene expression in Deoni cattle

reference gene is acknowledged as a limitation, and future studies will employ multiple validated reference genes in accordance with MIQE guidelines.

This study demonstrated that infrared thermography is a useful, non-invasive technique for assessing body surface temperature in Deoni cattle under field conditions. Among the anatomical regions evaluated, the hip, flank, thorax, and hump consistently exhibited strong associations with physiological indicators of thermal status. However, although the hump showed relatively stable surface temperatures across the study conditions, further studies are required to establish its repeatability, predictive accuracy, and suitability as a reliable thermal reference site. At the molecular level, HSPA1A gene expression was inversely associated with body surface temperature, indicating activation of the cellular heat-stress response under elevated thermal conditions. Although no statistically significant differences in physiological traits were observed among the identified HSPA1A genotypes, these findings should not be interpreted as evidence of the absence of biological effects. The identified thermal imaging sites and HSPA1Avariants can be regarded as candidate biomarkers for heat-stress assessment rather than validated diagnostic indicators, although, their diagnostic value and biological significance require confirmation in larger, independent populations encompassing diverse physiological states, environmental conditions, and management systems. The findings support the potential integration of infrared thermography with physiological measurements and molecular analyses as complementary approaches for heat-stress monitoring in cattle. Further validation is required before these biomarkers can be recommended for routine precision livestock management or breeding programmes aimed at improving thermotolerance.

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