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Thermo-physiological and Molecular Profiling of Two Indigenous Purebred Saudi Sheep under Acute Heat Stress Conditions

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ABSTRACT

In light of the escalating global concern regarding adaptation and resilience to elevated temperatures due to climate change, this experiment was designed to assess the thermo-physiological attributes of two native sheep breeds (Najdi and Naimi) and to delineate potential genetic factors conferring heat tolerance amidst acute exposure to elevated ambient temperatures. Meteorological and thermo-physiological parameters were scrutinized at distinct intervals (0 min, 30 min, 120 min, 24 hr, and 48 hr), alongside the analysis of heat-responsive gene expression at 0 min, 30 min, and 120 min, following the exposure of nine healthy male lambs from each breed (mean body weight: 25 kg; age: 4 months) to a bio-meteorologically-simulated environment, maintaining an average ambient temperature of 45 °C (approximately 93 units in the temperature-humidity index). In addition, blood samples were collected from each lamb, with total RNA isolated and purity assessed, followed by qRT-PCR analysis of 16 heat stress candidate genes using validated primers and standardized thermocycling protocols, including controls to ensure accuracy. Data were analyzed using statistical methods, including PROC GLM and PROC MEANS in SAS, one-way ANOVA, and pairwise differences with the LSD test for significance, while gene expression differences were calculated using the comparative Ct method and 2^ (−ΔΔCt) for relative quantification. The findings elucidate that the Najdi breed manifests heightened thermotolerance relative to the Naimi breed, as evidenced by diminished indicators of heat stress, encompassing skin temperature, respiratory rate, packed cell volume, adaptability coefficient, serum total protein, glucose levels, and triiodothyronine concentration. Moreover, analysis of gene expression patterns revealed widespread activation of heat stress-responsive genes in both breeds under thermal stress conditions; however, Najdi lambs consistently exhibited elevated expression levels of these genes compared to their Naimi counterparts. Notably, genes including HSP90AB1, HSPB6, HSF1, STIP1, HSP60, HSP90, and HSPB1 demonstrated particularly pronounced upregulation in Najdi lambs. In conclusion, the integrative thermo-physiological and molecular profiling highlights the superior thermotolerance and evolutionary adaptation of the Najdi breed to the hot climate of the KSA, in contrast to the Naimi breed.

Keywords: gene expression; heat-shock protein; lambs; Naimi; Najdi; RNA

INTRODUCTION

The Kingdom of Saudi Arabia (KSA) experiences persistent drought and high daytime temperatures in its tropical environment. Particularly, the central and northern areas of KSA report exceptionally high temperatures during the summer season, reaching up to 43 °C for maximum ambient temperature and 85.9 units for the temperature-humidity index (THI). In fact, recordings of ambient temperatures up to 54 °C are not uncommon (SNCM, 2024). Sheep, constituting 53% of KSA's livestock, is crucial in the economy, providing milk, meat, and other products. The predominant breeds, Najdi and Naimi sheep, are raised for meat and milk production, with distinct characteristics such as non-horned and hairy black wool for Najdi, and horned fat-tailed sheep with coarse wool for Naimi. However, sheep phenotype significantly influences heat tolerance traits. For instance, haired sheep exhibit better heat tolerance than wool sheep, and fat-tailed sheep are generally more heat-tolerant. Additionally, white-colored body wool sheep display increased heat-tolerant traits (McManus *et al.*, 2022). Despite these observable differences, there is a lack of comprehensive genetic and thermo-physiological studies comparing the heat tolerance and adaptation of these breeds to the prevailing hot climate conditions in KSA.

In order to mitigate the effects of heat stress, animals commonly employ various adaptive strategies that bolster their resilience at both physiological and molecular levels (Gaughan *et al.,* 2018). Physiologically, these strategies may manifest as heightened rectal temperature, increased respiration and pulse rates, and augmented sweating. At the molecular scale, a spectrum of thermally-responsive genes is triggered into action when endogenous adaptive processes fall short in reinstating homeostatic equilibrium, thereby safeguarding cellular integrity against the deleterious impacts of heightened temperatures (Mishra *et al.,* 2021). The intricate cascade of altered gene expression and protein activation triggered by the molecular response to heat stress has been a focal point of research. Traditionally, investigations have revolved around the regulation of heat shock transcription factors (HSFs) and heat shock proteins (HSPs) as primary components of gene expression during heat stress, where they play a pivotal role in cellular protection, contributing to the maintenance of cellular integrity and homeostasis under heat stress conditions (Kumar *et al.,* 2017; El-Zarei *et al.,* 2019; Rong *et al.,* 2019; Bai *et al.,* 2020; Mishra *et al.,* 2021). Nevertheless, as scientific interest in this field burgeons, researchers have broadened their inquiry to encompass other genes that potentially influence responses to heat stress in cattle and ruminants (El-Zarei *et al.,* 2019; Corazzin *et al.,* 2020; Grewal *et al.,* 2021). Transcriptomic analyses have revealed differentially expressed genes (DEGs) in sheep subjected to heat stress across various tissues (Li *et al.,* 2019; Lu *et al.,* 2019; Luna-Ramirez *et al.,* 2023;). Recent RNA-Seq studies have further identified DEGs in sheep that may be associated with breed-specific sensitivities to heat stress (Yang *et al.,* 2021; Haire *et al.,* 2022).

Contemporary research approaches, such as functional genomics, offer a promising avenue to establish a link between genes and phenotypes (Brown-Brandl, 2018; Gaughan *et al.,* 2018). Gene expression arrays, particularly utilizing quantitative real-time PCR, have proven successful in identifying stress response genes, such as heat shock proteins, in various species. However, genes are increasingly recognized as a vital source of phenotypic diversity and genetic variation in the ovine genome (Kalds *et al.,* 2022). Besides, the suitability of genes for quantitative PCR (qPCR) normalization in sheep's heat tolerance remains unresolved. For example, recent developments, including the ovine 50K SNP Beadchip, provide an array of approximately 50,000 evenly spaced single-nucleotide polymorphisms (SNPs) (Woolley *et al.,* 2023). This array could allow for the analysis of genomic regions and genes that have undergone selection during the specialization of sheep to their unique environment.

Nevertheless, while acquiring heat tolerance is a priority for producers and owners (Fuller *et al.,* 2016; Rawash *et al.,* 2022), solely selecting for heat tolerance can be counterproductive, diverting energy from production to heat exchange balancing. Therefore, simultaneous selection for increased animal production and improved heat tolerance is a desirable goal at the genomic level. Understanding the genes associated with heat tolerance and productivity is crucial for achieving this balance. Despite the significance of sheep to the KSA's economy and the observable differences in heat tolerance traits among breeds, previous studies have lacked comprehensive thermo-physiological and genetic investigations into these traits in the local context under acute heat stress conditions (Abdoun *et al.,* 2012; Al-Haidary *et al.,* 2012, 2021; Fonsêca *et al.,* 2019; Samara *et al.,* 2023). Consequently, this experiment aims to bridge that gap by integrating thermo-physiological assessments with the identification of potential genes associated with acute heat tolerance in two indigenous sheep breeds, Najdi and Naimi, thereby providing in-depth understanding of how these sheep cope with sudden exposure to high ambient temperatures. The outcomes of this research are expected to contribute to the agricultural technology sector in KSA, supporting the nation's strategic goals for sustainable development and food security.

MATERIALS AND METHODS

Location and Ethical Approval

The experiment was carried out at the experimental animal station associated with the Department of Animal Production, College of Food and Agriculture Sciences, King Saud University (coordinates: 24°48′20.8″N, 46°31′14.2″E). All procedures involving animal use for scientific purposes described in this experiment adhered to the guidelines outlined in the Animal Welfare Practices Act (Process number: KSU-SE-20-18).

Animals, Management, and Experimental Design

Eighteen healthy male growing lambs from two indigenous sheep breeds (Najdi and Naimi), initially weighing 24.71±4.65 kg and aged 4 months, were utilized in a two-phase experiment (preliminary and experimental). The lambs were individually housed in spacious pens within climate-controlled chambers measuring 5×5×4 m, with 10 lambs per chamber. Water was provided *ad libitum*, while a predetermined quantity of feed was dispensed to each lamb daily at 8:30 A.M. The feed amount was gradually adjusted as the lambs grew to minimize refusal rates. Throughout the experiment, lambs were fed a commercial pelleted complete diet (ALWAFI-ARASCO, KSA), and contained, according to the manufacturer's specifications, 13% crude protein, 2% ether extract, 9% crude fiber, 8% ash, 1% calcium, 0.50% phosphorus, 0.70% sodium chloride, and provided 2.95 Mcal/kg of digestible energy on a dry matter basis.

As noted, the experiment comprised two distinct phases: the preliminary phase and the experimental phase. During the preliminary phase, which spanned three weeks and served as an acclimation period, the climatic chambers were outfitted with air conditioning units featuring efficient climate control systems. Within these chambers, individual animal pens were established. Additionally, the lambs underwent various preparations, including weighing, ear tagging, prophy-

lactic treatment for internal and external parasites, and placement under stable thermoneutral conditions (Ta= 25 °C). They were also familiarized with their feed and the measuring equipment. Notably, several baseline measurements were taken during the final week to establish control values. Subsequently, during the experimental phase lasting 54 hours, meteorological and thermo-physiological measurements were conducted. Additionally, potential heat-induced candidate genes were identified. These assessments were performed at specific intervals: 0 min, 30 min, 120 min, 24 hr, and 48 hr following the transfer of the lambs to another chamber with a hot climatic condition (Ta= 45 °C).

Experimental Measurements

Meteorology. Two ThermoWorks high-precision data loggers (TW-USB-2-LCD+, Lindon, Utah, USA) were positioned at an approximate height of 2 meters above the lambs to continually monitor the ambient temperature (Ta) and relative humidity (RH) at 15 min intervals. Utilizing Box-Car-Pro 4 software (Onset Co, USA), the loggers were programmed and data retrieval was facilitated. Subsequently, the temperature humidity index (THI) was computed according to the methodology outlined by Kelly & Bond (1971) to assess potential environmental stress severity.

Thermo-physiology. During the experimental phase, an assessment was conducted to ascertain the thermo-physiological response and heat tolerance efficiency. Rectal temperature (Tr) was determined using a calibrated digital rectal thermometer with a precision of 0.10 °C, while skin temperature (Tsk) in shaved areas at the right shoulder and hip regions was measured utilizing a Traceable Mini IR™ infrared thermometer (Friendswood, TX, USA) (Samara *et al.,* 2016). Additionally, respiratory rate (RR) was assessed by visually counting 10 breaths and recording the time in seconds, subsequently expressed as breaths per minute. Heat tolerance coefficient (HTC) was evaluated for both breeds employing Rhoad's Iberia heat tolerance test (Rhoad, 1944), whereas the adaptability coefficient (AC) was calculated using Martins Júnior *et al.* formula (2007): [AC= (newly recorded Tr value / control Tr value) + (newly recorded RR value / control RR value)], where Tr represents rectal temperature in Celsius and RR denotes respiratory rate in breaths per minute. As a result, AC values approaching 2 indicate heightened heat adaptation.

Blood samples (~ 6 mL) were obtained via jugular venipuncture using EDTA-coated and plain vacutainer tubes. These samples were promptly refrigerated in an ice box and transported to the laboratory for analysis. Packed cell volume (PCV) was measured using the capillary tube method within approximately 1 hour of collection. Serum separation was accomplished by centrifugation of plain tubes at 1500 *g* for 10 minutes followed by storage at −20 °C. Subsequently, spectrophotometric analysis was conducted on the sera to determine total protein, albumin, glucose, and triiodothyronine (T3) levels (Samara *et al.,* 2016).

Molecular. Two peripheral whole blood samples of 10 mL were collected into heparinized tubes from each lamb in the last two weeks of the preliminary phase (Ta= 25 °C). During the experimental phase (Ta= 40 °C), similar samples were taken following thermo-physiological samples at 0 min, 30 min, and 120 min. Handling and storage of the samples followed the recommended protocols for maximum utilization of RNA isolation. The total RNA was isolated from the whole blood following instructions of RiboPure™-Blood Kit (Life technologies), incorporating on-column digestion of any DNA with RNase-free DNase I. The purity and quality of the RNA were determined using a Nano-Drop spectrophotometer (Ammar *et al.,* 2017).

For gene selection for qPCR, potential heat induced candidate genes were previously identified from goat and sheep available in the public domain in the NCBI gene bank EST database. For this experiment, a total of 16 potential candidate genes for heat stress were selected for quantitative real time PCR (qRT-PCR) analysis. Primers were designed using Batch Primer3 software and listed in Table 1. All selected gene primers were validated on both breeds at 25 ^{o}C and 40 ^{o}C for 30 min and 120 min of heat stressed conditions. All reactions were performed as triplicate on an ABI PRISM 7500 RT-PCR (Applied Biosystems, CA, USA) following the manufacturer's cycling parameters. Each reaction consisted of 100 ng RNA, 10 µL SYBR Green PCR master mix (Applied Biosystems), 1.5 µL (10 µM) of each forward and reverse primers and water up to 20 µL and 0.4 µL of RT mix (Ammar *et al.,* 2017). The thermocycling protocol consisted of 37 ^{o}C for 15 min and reverse transcriptase denaturation for 10 min at 96 ^{o}C, then 40 cycles each of denaturation at 95 ^{o}C for 0.15 min and annealing/extension at 60 ^{o}C for 1 min. A melting curve analysis protocol was performed to confirm the absence of multiple amplicons and primer dimers. A negative control (without a template) was included as well to ensure the absence of contamination. Expressions of the two internal housekeeping genes, GAPDH and Actin, were used as endogenous references. The arithmetic mean of their Ct-values was then utilized in the delta Ct-calculation to quantify the expression level.

Data Analysis

Meteorological and thermo-physiological measurement data were analyzed using a completely randomized design with the PROC GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The mathematical model was as follows: [$y_{ijk} = \mu + A_i + B_j + T_k + \varepsilon_{ijk}$], where y_{ijk} is the observation from the i^{th} animal of the j^{th} breed at the k^{th} time; μ is the overall mean; A_i is the random effect of the animal (i=A1, A2 … A9); B_j is the fixed effect of the breed (j=B1 and B2); T_k is the fixed effect of the time (k= 0 min, 30 min, 120 min, 24 hr, and 48 hr; and ε_{ijk} is the residual term, including the random error. Descriptive statistics for all parameters were obtained using the PROC MEANS procedure. Subsequently, the data were subjected to one-way analysis of variance (ANOVA) with a significance level set at α=0.05. Any

Table 1. List of primers used for quantitative PCR

Gene name	Primer-sequence 5′-3′	Product size (bp)	Tm	Gene description
HSP70-F	TACGTGGCCTTCACCGATAC	171	61.50	Heat shock protein 70
HSP70-R	GTCGTTGATGACGCGGAAAG		61.10	U09861
HSP90-F	GACTCCCAGGCATACTGCTC	169	62.90	Heat shock protein 90 Family alpha class A member 1
HSP90-R	GGCGCTGATATCTCCATGAT		58.40	
IL-2-F	CCTGAGCAGGATGGAGAATTACA	141	61.30	Interleukin 2
IL-2-R	TCCAGAACATGCCGCAGAG		62.60	
LEP-F	ACCAACAGATCCTCGCCAGT	148	62.00	Leptine
LEP-R	AGGCTCTCCAAGCTCTCCAG		61.00	
HSP90AB1-F	TCTGGTAGACACGGGCATTG	146	62.00	Heat shock protein 90 Family alpha class B member 1
HSP90AB1-R	AGAAGCCCACACCAAACTGC		62.05	
HSPB6-F	GCCTACCGCCCAGGTATCTA	157	61.00	Heat shock protein Family B (Small) member 6
HSPB6-R	CGCAATGTATCCGTGCTCAT		62.00	
HSF1-F	AAGCCAGAGAGGGACGACAC	153	62.00	Heat shock Transcription factor 1
HSF1-R	CAGCAGCTTGGTAACGCTGT		61.45	
ST1P1-F	GTGCTGGCAACATTGACGAT	156	62.60	Stress induced Phosphoprotein 1
ST1P1-R	TCAGGTCAACGGTTTTGCAG		62.00	
HSP60-F	GGAAAAGGTGACAAGGCTCA	214	60.60	Heat shock protein Family D (HSP60) member 1
HSP60-R	CAGCTCGTGTGGCATTAAGA		59.00	
HSPB1-F	ATTTCCCGTTGCTTCACTCG	153	62.13	Heat shock protein Family B (small) member 1
HSPB1-R	GGTGACGGGAATGGTGATCT		62.45	NM_001025569
CAMKK1-F	AGAGAGTGGCAGCCATTGAT	173	60.50	Calcium/Calmodulin dependent protein kinase kinase 1
CAMKK1-R	CAGGGAGAACTTCCTTGTCG		61.50	
CEBPB-F	ATCGACTTCAGCCCCTACCT	164	63.20	CCAAT Enhancer binding protein beta
CEBPB-R	CCGTAGTCGTCGGAGAAGAG		62.60	
MAPK13-F	CAAGCAGGACGTCAACAAGA	168	59.90	Mitogen-activated protein kinase 13
MAPK13-R	GCAAAGATCTCGGACTGGAA		59.90	
ENPP1-F	AAAGTTGCAAAGGTCGCTGT	167	58.80	Ectonucleotide pyrophosphatase / phosphodiesterase 1
ENPP1-R	TCGGGACAACCTTTTCTCAC		60.70	
BDKRB1-F	GCACCCTACCACTTCTTTGC	162	61.10	Bradykinin receptor B1
BDKRB1-R	CTGGCCCCAAAAGACATAAA		58.00	
UCP-F	TGGACGACAGGGAAGCCAG	181	66.30	Uncoupling protein
UCP-R	ATTCGCAGATTCCTCTCATC		56.30	Uncoupling protein
β-ACTIN-F	GTCCGTGACATCAAGGAGAAG		60.80	Indigenous control
β-ACTIN-R	AGGAAGGAAGGCTGGAAGAG		62.80	Indigenous control
GAPDH-F	CAGGGCTGCTTTTAATTCTGGC		60.90	Indigenous control
GAPDH-R	AGGATCTCGCTCCTGGAAGATG		64.00	Indigenous control

Note: Tm= melting temperature.

means showing significant differences in ANOVA were further analyzed using the pairwise differences (PDIFF) option and compared using the least significant difference (LSD) test. The threshold for statistical significance was set at $p<0.05$, and the means along with their pooled standard error of the mean (SEM) were presented unless otherwise indicated.

For molecular measurements, real-time PCR software was used to calculate the Cycle threshold (Ct) values automatically. The obtained data were then exported to MS Excel for further analysis. The comparative Ct method was employed to estimate expression differences, which normalizes the expression of the target gene to an internal control gene and compares the expression level of the target gene between different experimental groups. In fact, Ct values of samples collected from control and stressed lambs were first normalized with an internal standard. Subsequently, the ΔΔCt was calculated using the following formula: $\Delta\Delta Ct = [\Delta\Delta Ct= \Delta Ct_{target}(mCt_{target}-mCt_{endogenous\ control}) -\Delta Ct_{control}(mCt_{control}-mCt_{endogenous\ control})]$. Relative estimation of target genes was obtained according to Livak & Schmittgen (2001) using the fold difference calculated by the $2^{\wedge (-\Delta\Delta Ct)}$ method. The expression levels of the target gene between different experimental groups were compared quantitatively.

RESULTS

Meteorological Data

Analysis of the meteorological data observed during the present investigation revealed that the environmental conditions within the climatic chamber to which the lambs were relocated, exhibited ($p<0.05$) higher values of Ta and THI compared to chambers with neutral conditions (Figure 1). Conversely, RH ($p<0.05$) displayed an inverse relationship. These findings underscore the variation in environmental conditions experienced by the lambs upon relocation, aligning with the primary objective of our experiment.

Assessment of Thermo-physiological Changes

Exposure of both breeds to hot conditions for 30 min resulted in pronounced ($p<0.05$) elevations almost linearly in the overall means of all thermo-physiological variables over their control levels, which did not return to their normal levels in both breeds even after 48 hr of the first exposure (Table 2 and Figure 2). However, during this period, lambs showed ($p<0.05$) some breed differences in overcoming such sudden environmental change. As a matter of fact, Naimi lambs showed ($p<0.05$) an elevated response in Tsk, RR, and AC as well as a lower response in both PCV, total protein, glucose, and T_3 compared to their Najdi counterparts, while both had the same response in their Tr, HTC, and Albumin level (Table 2 and Figure 2).

Assessment of Molecular Changes

We conducted real-time PCR analysis on genes that exhibited changes in response to acute heat stress or played a crucial role in regulating important biological processes. Results generally revealed that breed and duration of heat exposure (MAPK13, IL2, HSP90AB1, HSPB6, HSF1, STIP1, HSP60, HSP70, HSP90AA, HSPB1, CAMKK1, CEBPB, ENPP1, BDKRB, and LEP) had influenced the expression of heat shock protein genes, and variations were observed among both factors (Table 3).

Additionally, the obtained findings indicated that the heat shock response genes HSP90AB1 and HSP 60 increased expression in both breeds after 120 min of heat exposure, but Najdi lambs exhibited higher expression levels than the Naimi lambs (Table 3). Similar pattern was noted for the small heat shock proteins HSPB1 and HSPB6, which have higher level of expression in Najdi lambs compared to Naimi lambs. The transcription factor HSF1, which regulates the heat shock response, showed more expression in Najdi lambs than in Naimi lambs at both 30 min and 120 min of heat exposure (Table 3). In contrast, HSP90 expression was higher in Naimi lambs than in Najdi lambs after 120 min of heat exposure (Table 3). Moreover, HSP70 expression was also different between the two breeds, with Najdi exhibiting a greater level of expression compared to Naimi after 120 minutes of heat exposure. These results indicate that both HSP70 and HSP90 participate in the heat stress adaptation of both breeds, but with different levels of expression (Table 3). The expression of STIP1,

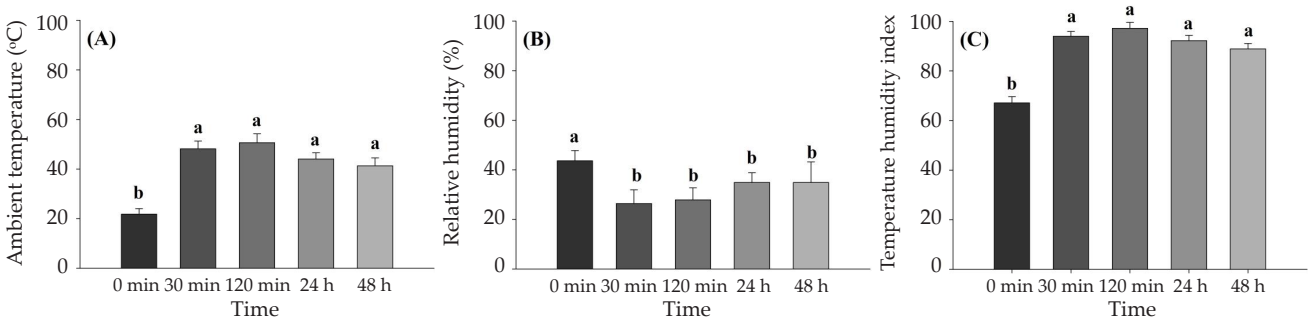

Figure 1. Meteorological data (ambient temperature (Ta), relative humidity (RH), and temperature humidity index (THI)) collected throughout the current experiment (Means±SE). Means bearing different superscripts are significantly different at $p<0.05$.

Table 2. Overall combined changes of thermo-physiological variables in two indigenous sheep breeds (Najdi and Naimi) as a function of acute heat exposure

Variables	Time					SEM
	Control	30 min	120 min	24 hr	48 hr	
Rectal temperature (°C)	39.42 ^{d}	40.18 ^{b}	40.77 ^{a}	39.87 ^{c}	39.72 ^{c}	0.07
Skin temperature (°C)	33.06 ^{d}	36.33 ^{b}	36.55 ^{b}	37.23 ^{a}	35.46 ^{c}	0.28
Respiratory rate (Breath/min)	31.01 ^{c}	138.52 ^{b}	197.30 ^{a}	134.51 ^{b}	130.14 ^{b}	5.78
Heat tolerance coefficient	100.00 ^{a}	92.45 ^{d}	86.50 ^{e}	95.50 ^{c}	97.00 ^{b}	0.65
Adaptability coefficient	2.00 ^{c}	5.59 ^{b}	7.66 ^{a}	5.52 ^{b}	5.37 ^{b}	0.20
Packed cell volume (%)	30.70 ^{a}	29.60 ^{b}	28.80 ^{c}	28.80 ^{c}	29.00 ^{b}	0.52
Total protein (g/dL)	4.43	4.36	4.36	4.26	4.50	0.18
Albumin (g/L)	3.35	3.38	3.50	3.38	3.50	0.10
Glucose (mg/dL)	53.90 ^{c}	61.23 ^{b}	76.50 ^{a}	55.35 ^{c}	65.30 ^{b}	3.48
T_3 (ng/mL)	2.71	2.58	2.56	2.12	2.34	0.18

Note: $^{a-d}$Means bearing different superscripts are significantly different at $p<0.05$. Control= 0 min.

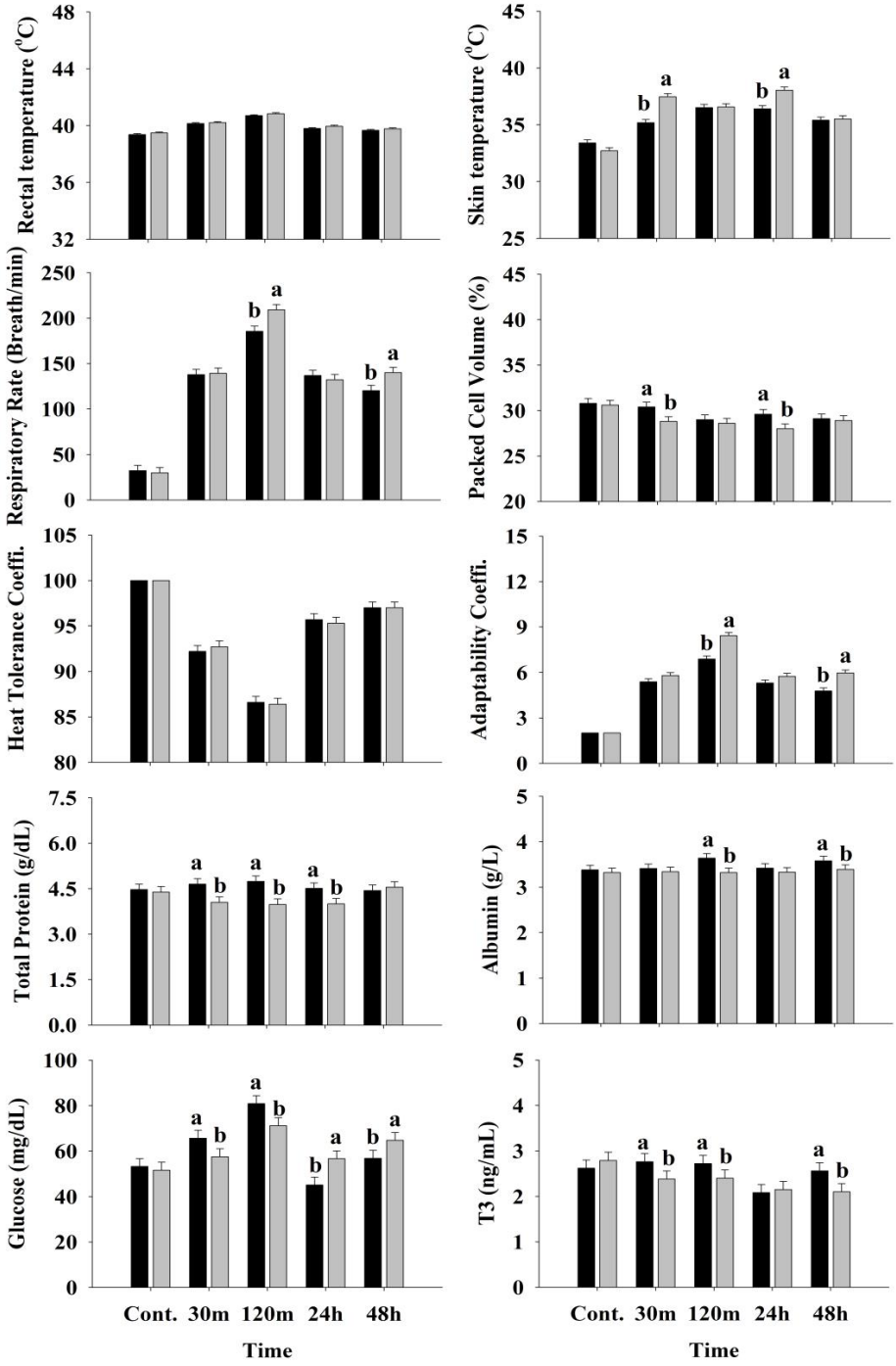

Figure 2. Changes of thermo-physiological variables in both Najdi (■) and Naimi (■) sheep as a function of acute heat exposure (Means±SE). Means bearing different superscripts are significantly different at p<0.05 in each collection time between both breeds. Cont.= 0 min.

a co-chaperone that interacts with HSP70 and HSP90, was marginally greater in Najdi lambs after 120 minutes of heat exposure compared to Naimi lambs; however, it was lower after 30 minutes (Table 3). Furthermore, the expression of BDKRB1, a receptor that mediates the inflammatory response to heat stress, was greater in Najdi lambs than in Naimi lambs following 30 min and 120 min of heat exposure (Table 3). The expression of ENPP1, which is involved in RNA metabolism and stability, was down-regulated in both breeds after heat exposure (Table 3). The expression of CAMKK1, a kinase that regulates cellular energy metabolism, was slightly higher in Naimi lambs than in Najdi lambs after 120 min of heat exposure (Table 3).

DISCUSSION

Heat stress is a serious challenge for sheep and other animals, affecting their health, productivity, and reproduction. Farmers and researchers have been investigating strategies to alleviate the adverse consequences of heat stress and prevent economic

Table 3. Relative expression patterns of heat tolerance genes in two local sheep breeds, Najdi and Naimi at 30 and 120 min heat exposure[1]

Gene[2]	Najdi breed		Naimi breed	
	Fold difference at 30 min	Fold difference at 120 min	Fold difference at 30 min	Fold difference at 120 min
HSP70	1.32±0.15	5.33±0.50	1.33±0.12	2.56±0.38
HSP90	1.62±0.12	4.63±0.58	1.51±0.15	11.06±0.45
HSP90AB1	6.68±0.35	14.12±0.42	1.95±0.18	9.31±0.52
HSF1	4.16±0.20	10.41±0.56	1.74±0.16	3.38±0.57
HSP60	9.64±0.28	15.77±0.68	2.29±0.20	11.87±0.58
HSPB6	4.69±0.18	7.56±0.52	1.40±0.14	2.82±0.68
HSPB1	9.18±0.22	10.12±0.48	7.26±0.30	6.06±0.35
CAMKK1	0.76±0.10	1.90±0.45	1.13±0.14	3.58±0.68
CEBPB	1.68±0.16	7.21±0.72	1.55±0.16	1.17±0.53
ENPP1	0.84±0.08	0.87±0.70	0.92±0.12	1.20±0.86
BDKRB	7.21±0.30	7.67±0.85	4.22±0.25	3.78±0.72
LEP	9.38±0.32	11.01±0.55	1.60±0.15	4.85±0.65
MAPK13	6.72±0.25	9.18±0.57	7.94±0.30	3.94±0.65
IL2	9.98±0.40	16.00±0.55	3.63±0.22	4.69±0.58
ST1P1	1.41±0.14	8.39±0.58	3.01±0.20	7.56±0.58
UCP	1.31±0.12	2.96±0.76	3.3±0.20	5.89±0.73

Note: [1]Relative expression levels were determined using the comparative Ct (ΔΔCt) method, normalizing the target gene's expression to an internal control gene. The expression levels of the target gene between different experimental groups were compared quantitatively. [2]HSP70= Heat Shock Protein 70, HSP90= Heat Shock Protein 90, HSP90AB1= Heat Shock Protein 90 Family Alpha Class B Member 1, HSF1= Heat Shock Transcription Factor 1, HSP60= Heat Shock Protein Family D (HSP60) Member 1, HSPB6= Heat Shock Protein Family B (Small) Member 6, HSPB1= Heat Shock Protein Family B (Small) Member 1, CAMKK1= Calcium/Calmodulin Dependent Protein Kinase Kinase 1, CEBPB= CCAAT Enhancer Binding Protein Beta, ENPP1= Ectonucleotide Pyrophosphatase/Phosphodiesterase 1, BDKRB= Bradykinin Receptor B1, LEP= Leptin, MAPK13= Mitogen-Activated Protein Kinase 13, IL2= Interleukin 2, ST1P1= Stress Induced Phosphoprotein 1, and UCP= Uncoupling Protein. Refer to text for more details.

losses. One of the possible approaches is to study the expression of genes stimulated by heat stress to understand how they help animals to cope with heat stress. In order to optimize the usefulness of selective breeding for low-heritability traits in sheep, the identification of the pivotal genes that regulate thermoregulation becomes critical. The intention of this experiment is to recommend one out of two important indigenous sheep purebreds (Najdi and Naimi) with increased or at least maintained heat tolerance at the organ and molecular level after acute exposure to high ambient temperatures. This experiment represents the inaugural investigation into the assessment of certain breeds under bioclimatic conditions akin to those found in semi-arid environments. Abrupt exposure to elevated temperatures markedly impacted the thermoregulatory mechanisms of Najdi and Naimi lambs. As homeothermic organisms, these animals rely on maintaining a stable internal body temperature within their thermoneutral zone (da Silva & Maia, 2013). Given their status as small ruminants, subjecting sheep to temperatures above their thermoneutral zone is anticipated to necessitate a reduction in water expenditure and thermogenic processes while triggering the activation of water conservation and thermolytic mechanisms (Al-Haidary *et al.*, 2012; Renaudeau *et al.*, 2012). Consequently, discernible alterations in their thermo-physiological and genetic responses are expected to manifest.

During the acute heat exposure, the computed average THI values indicated that the animals experienced heat stress. According to Silanikove (2000), THI values below or equal to 70 signify a comfortable condition, while those falling between 71 and 78 are deemed stressful, and values exceeding 78 induce heightened discomfort, potentially leading to the inability of the animal to regulate its body temperature. Our findings corroborate this, revealing alterations in all measured parameters within 30 minutes of exposure (Figure 1). This suggests that the prevailing conditions of our experiment were sufficiently intense to perturb the thermoregulatory system of the lambs, albeit without evident signs of thermoregulatory failure, despite THI readings surpassing 78 during this phase. However, even after 48 hours following the initial exposure, the overall means of all variables did not revert to their baseline levels in either breed. Nonetheless, lambs exhibited some disparities in coping with the abrupt environmental shift during this period, with Najdi lambs demonstrating a quicker recovery compared to Naimi lambs. This discrepancy is likely attributable to the genetic lineage and productive traits inherent to these breeds, where the indigenous Najdi breed is renowned for its adaptation to hot climates, boasting moderate live weight and daily gain compared to other breeds, whereas the Naimi breed is more geared towards enhanced production (Al-Haidary *et al.*, 2012).

Comparing the two breeds, it was obvious that Naimi lambs exhibited herein higher values in T_r, T_{sk}, RR, and SR than Najdi lambs, thereby suggesting that the Naimi breed experienced greater difficulty in dissipating heat from the body through evaporation compared to Najdi breed. This was reflected on the observed lower HTC and high AC in Naimi compared to Najdi lambs (Table 2 and Figure 2). These observations could

be attributed to a higher metabolic heat production in the Naimi compared to the Najdi breed. The intensity of heat stress in ruminants is assessed as well using RR as a quantitative measure (Silanikove, 2000). During the heat stress period, the RR ranged from 130.1 to 194.49 breaths per min during the current experimental conditions. Andersson & Jónasson (2006) reported that sheep exhibit open mouth polypnea when their T_r exceeds 41 °C, a finding that was observed herein. A strong link between RR and T_r was documented by Starling *et al.* (2002) in their studies on sheep in Brazil. Additionally, they reported a positive association between RR and T_r, suggesting that the respiratory system plays a crucial role in thermolysis and the maintenance of homeothermy in animals, hence preventing an elevation in body temperature. The presence of positive correlations indicates that the animals are able to absorb heat from their environment. This increase in skin temperature triggers homeothermic mechanisms, leading to an elevation in RR. Polypnea, a crucial heat regulatory process in this particular species, is likely responsible for this observed increase in RR (Andersson & Jónasson, 2006).

The aforementioned results are supported by the observation that circulatory T3, was lower in the Naimi breed compared to Najdi lambs throughout the experiment. Heat stress has been found to decrease the production of thyroid hormones, which has been suggested as a potential physiological indicator of adaptation (da Silva & Maia, 2013). The reduction in the production of thyroid hormones in animals experiencing heat stress is attributed to a decrease in the need for internal heat production (Pugh *et al.,* 2020). Nevertheless, a decline in metabolism has the potential to diminish the expression of production features, including growth and fattening (West, 2003). Therefore, it can be inferred that the Naimi breed tried to lower its internal heat production (i.e. , metabolic rate) compared to the Najdi breed under heat stress conditions. Sivakumar *et al.* (2010) observed a reduction in plasma concentrations of T3 and T4, as well as elevated levels of cortisol and prolactin in goats subjected to heat stress. The elevation of cortisol levels facilitates the process of protein breakdown into amino acids, hence providing support for gluconeogenesis during the heat stress condition (Sejian *et al.,* 2010). Marai *et al.* (2007) assumed that the increase in serum glucose concentration during hot summer conditions may be attributed to the activation of cortisol secretion caused by stress, which subsequently stimulates gluconeogenesis and inhibits cellular glucose uptake and utilization. Similarly, the observed overall mean of blood glucose level in both breeds showed a gradually increasing trend with the duration progress of the long-term heat stress. Moreover, the blood glucose level in the Najdi breed was increased within a span of 120 minutes during the short-term heat stress, suggesting a rapid adaptive response in the Najdi breed. The findings presented in this experiment provide further evidence supporting the previous findings that indicated an increase in serum glucose levels in the Najdi breed during hot summer conditions (Al-Haidary *et al.,* 2012). Furthermore, the present short-term heat stress did not affect the PCV and the serum total protein levels of sheep breeds in this experiment. The overall PCV mean values ranged between 28.8% to 30.70%, and the overall serum total protein mean values were altered only by 0.24 g/dL from 4.50 to 4.26, which were within the normal range reported by Ghanem *et al.* (2008). These outcomes contradict the findings of McManus *et al.* (2009) and Al-Haidary *et al.* (2012), who reported an increase in PCV and total protein levels during heat stress.

Notably, the present experiment identified several genes that showed increased expression levels in response to heat stress in both breeds at 30 min and 120 min of heat exposure. A comparison was made between the two breeds regarding the expression patterns of these genes. These genes have previously been linked to heat tolerance traits in cattle and other ruminant species (Sajjanar *et al.,* 2015; Zeng *et al.,* 2022; Rawash *et al.,* 2022). The obtained finding of gene expression analysis indicated that most of the genes examined were activated in both breeds under heat stress conditions. However, Najdi lambs exhibited higher levels of gene expression than Naimi lambs (Table 3), therefore suggesting that the Najdi breed has a better heat tolerance than the Naimi breed. Nevertheless, a thorough investigation should encompass the relative long-term thermotolerance of Najdi and Naimi breeds across factors such as gender, age, production phases, and specific production purposes.

On the other hand, HSPs are a family of proteins that help cells cope with various types of stress, including heat, cold, oxidative damage, and infection (Parsell & Lindquist, 1993). They act as molecular chaperones that play essential roles in protein folding, stability, and degradation under normal and stressful conditions, where they protect cells from damage and promote their survival and recovery. These proteins are categorized into different families based on their molecular weight, such as HSP10, HSP40, HSP70, HSP90, HSPB1, and HSPH1 (Kregel, 2002), and produced by genes that are controlled by the HSFs. These factors are transcription factors that bind to the heat shock elements found in the promoters of HSP genes. In sheep, the primary HSF is HSF1, a conserved gene present in all eukaryotes and regulates various aspects of development and metabolism besides stress (Vihervaara & Sistonen, 2014). It is triggered by proteotoxic stress and stimulates the production of HSP70 and HSP90, two crucial chaperones that safeguard the proteome from damage and aggregation. This factor was found herein to up-regulated in both breeds of sheep. In fact, HSF1 gene activity influences the level of heat tolerance, with higher gene activity resulting in greater protection against heat (Sonna *et al.,* 2002); thus, HSF1 gene is a potential modulator of heat shock.

Additionally, the obtained findings in the present experiment revealed that the expression of HSP70 and HSP90 genes in sheep was influenced by the breed. One of the most studied HSPs in sheep is the HSP70, which is activated by heat stress and functions as a molecular chaperone that aids in protein folding and refolding inhibits protein aggregation, and speeds

up the breakdown of damaged proteins. This protein modulates as well the inflammatory response by interacting with various cytokines such as TNF-α and Interleukins. Another important HSP in sheep and other animals is HSP90, which also participates in protein folding and stability, as well as in the regulation of steroid hormone receptors such as glucocorticoid receptors. This experiment examined the mRNA expression of HSP70 in response to heat stress and found similar results to earlier studies on sheep and goats (Agnew & Colditz, 2008; Dangi *et al.,* 2016). In fact, the Najdi breed of sheep expressed more HSP70 mRNA than the Naimi breed of sheep, while the Naimi breed of sheep expressed more HSP90 mRNA than the Najdi breed of sheep. Therefore, both breeds, Najdi and Naimi, were found to have higher heat tolerant activity. These findings are consistent with an earlier report (Rout *et al.,* 2016). An earlier animal investigation has found an increase in HSP70 and HSP90 expression after heat exposure (Dangi *et al.,* 2012). The observed differences in HSP70 and HSP90 responses of the examined breeds could be attributed to the genetic origin and reproductive characteristics of these breeds. As a result, the differential expression of HSP70 and HSP90 may be an effective biological marker for assessing the impact of heat stress in sheep.

Moreover, the present experiment revealed an increased expression of one of the HSP90 isoform (HSP90AB1) in Najdi and Naimi breeds. This isoform plays a role in several cellular functions, such as protein folding, degradation, signal transduction, survival, and evolution, and is linked to adaptation and heat tolerance traits in sheep (Singh *et al.,* 2017) and cattle (Charoensook *et al.,* 2012). Besides, heat stress substantially increased the expressions of HSPB1 and HSPB6 in both sheep breeds. The HSPB1 protein is a small HSP that safeguards cells against oxidative stress, involved in numerous cellular processes, and normalizes quickly after homeostasis is restored (Matsumoto *et al.,* 2015), while the HSPB6 protein is associated with heat resistance features in cattle (Kumar *et al.,* 2021). Studies on animals' responses to external stresses have revealed that such stimuli cause highly significant alterations in the expression patterns of these proteins (Kamboh *et al.,* 2013). Therefore, understanding the expression patterns and functions of HSPs in sheep can help to identify genetic markers and breeding strategies for improving the heat tolerance and performance of sheep under heat stress conditions. Nevertheless, further research is required to examine the modulation of HSPs by pharmacological or nutritional interventions as a potential strategy to enhance the adaptive capacity of sheep to heat stress.

The expression of stress induced phosphoprotein 1 (STIP1) is an important antioxidant factor that controls the oxidative stress response by triggering the genes that encode for antioxidant enzymes. In this experiment, we noticed an increased expression of STIP1 in both Najdi and Naimi breeds, signifying that STIP1 could be a useful biomarker for heat stress in sheep. A similar increase in STIP1 expression was reported in bovine under heat stress (Garner *et al.,* 2020). Additionally, Mitogen activated protein kinase (MAPK) is differentially expressed in both breeds. The MAPK is a member of MAPK subfamily called Stress Activated Protein Kinase (SAPKs) that regulates the cell cycle progression and cell survival or death responses to various environmental stresses (Nguyen & Shiozaki, 1999). According to Gorostizaga *et al.* (2005), heat stress triggers MAPK activation.

Furthermore, CAMKK1 (calcium/calmodulin dependent protein kinase) is a key enzyme involved in the regulation of cellular energy metabolism and stress response was differentially expressed herein. It is triggered by increased intracellular calcium levels and modulates the activity of AMP-activated protein kinase (AMPK), a master regulator of energy homeostasis (Darling & Cook, 2014). This enzyme may play an important role in arbitrating the adaptive responses of ruminants to heat stress by modulating the AMPK signaling pathway and enhancing cellular survival and protection. In addition, CCAAT enhancer binding protein beta (CEBPB) is a transcription factor that regulates the expression of genes involved in inflammation, immunity, and metabolism and may play a role in modulating these responses and protecting ruminants from heat stress-induced damage (Kim *et al.,* 2011). This factor was responsive to heat stress, as it is differentially expressed herein in both breeds.

Likewise, one of the genes that showed the highest expression change in both breeds after the heat stress was Bradykinin receptor B1 (BDKRB1). Garner *et al.* (2020) reported a similar finding in cattle under thermal stress. This gene is involved in the inflammatory response and its upregulation suggests that the heat stress increased the inflammation level. In fact, BDKRB1 is normally expressed only when there is tissue damage or inflammation, such as when pro-inflammatory cytokines are released, immune cells migrate, and blood vessels become more permeable (Raslan *et al.,* 2010). For the Interleukin gene-2 (IL2), it was differentially expressed in two breeds with high heat tolerance, indicating its important role in this trait. This is consistent with Bharati *et al.* (2017), who found that IL2 expression increased significantly after heat stress acclimation in Tharparkar cattle and Barki sheep, respectively. These animals were exposed to a temperature of 42 °C. In fact, according to Rawash *et al.* (2002), IL2 may help to activate the immune system and reduce the damage caused by heat stress.

CONCLUSION

The current experiment examined the thermo-physiological and molecular responses of two indigenous purebred sheep breeds, Najdi and Naimi, under acute heat stress conditions, revealing noticeable differences in thermotolerance, with the Najdi breed demonstrating superior resilience and distinctive patterns in the expression levels of heat stress-responsive genes. These findings underscore the evolutionary adaptation of the Najdi breed to the hot climate of the KSA, positioning it as inherently more heat-tolerant compared to the Naimi breed. Future

research should delve into the genetic response to whole-genome variations among these breeds, focusing on additional heat tolerance-associated genes in various tissues to broaden our understanding of evaporative heat dissipation mechanisms.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

REFERENCES

Abdoun, K. A., E. M. Samara, A. B. Okab, & A. A. Al-Haidary. 2012. A comparative study on seasonal variation in body temperature and blood composition of camels and sheep. J. Anim. Vet. Adv. 11:769–763. https://doi.org/10.3923/javaa.2012.769.773

Agnew, L. L. & I. G. Colditz. 2008. Development of a method of measuring cellular stress in cattle and sheep. Vet. Immunol. Immunopathol. 123:197–204. https://doi.org/10.1016/j.vetimm.2008.01.038

Al-Haidary, A. A., R. S. Aljumaah, M. A. Alshaikh, E. M. Samara, K. A. Abdoun, A. B. Okab, & M. M. Alfuraiji. 2012. Thermoregulatory and physiological responses of Najdi sheep exposed to environmental heat load prevailing in Saudi Arabia. Pak. Vet. J. 32:515–519.

Al-Haidary, A. A., Y. Al-Dosari, A. Abd-Elwahab, E. M. Samara, M. A. Al-Badwi, & K. A. Abdoun. 2021. White hair coat color does not influence heat tolerance of sheep grazing under a hot arid environment. Small Rumin. Res. 201:106410. https://doi.org/10.1016/j.smallrumres.2021.106410

Ammar, M. H., A. M. Khan, H. M. Migdadi, S. M. Abdelkhalek, & S. S. Alghamdi. 2017. Faba bean drought responsive gene identification and validation. Saudi J. Biol. Sci. 24:80–89. https://doi.org/10.1016/j.sjbs.2016.05.011

Andersson, B. E. & H. Jónasson. 2006. Regulação da Temperatura e Fisiologia Ambiental. In: M. J. Swenson & W. O. Reece (eds.). Dukes –Fisiologia dos Animais Domésticos. 12th ed. Guanabara Koogan S. A., Rio de Janeiro. pp. 805–813.

Bai, H., H. Ukita, M. Kawahara, T. Mitani, E. Furukawa, Y. Yanagawa, N. Yabuuchi, H. Kim, & M. Takahashi. 2020. Effect of summer heat stress on gene expression in bovine uterine endometrial tissues. Anim. Sci. J. 91:e13474. https://doi.org/10.1111/asj.13474

Bharati, J., S. S. Dangi, S. R. Mishra, V. S. Chouhan, V. Verma, O. Shankar, M. K. Bharti, A. Paul, D. K. Mahato, G. Rajesh, G. Singh, V. P., Maurya, S. Bag, P. Kumar, & M. Sarkar. 2017. Expression analysis of Toll like receptors and interleukins in Tharparkar cattle during acclimation to heat stress exposure. J. Therm. Biol. 65:48–56. https://doi.org/10.1016/j.jtherbio.2017.02.002

Brown-Brandl, T. M. 2018. Understanding heat stress in beef cattle. Rev. Bras. Zootecn 47:e20160414. https://doi.org/10.1590/rbz4720160414

Charoensook, R., K. Gatphayak, A. R. Sharifi, C. Chaisongkram, B. Brenig, & C. Knorr. 2012. Polymorphisms in the bovine HSP90AB1 gene are associated with heat tolerance in Thai indigenous cattle. Trop. Anim. Health Prod. 44:921–928. https://doi.org/10.1007/s11250-011-9989-8

Corazzin, M., E. Saccà, G. Lippe, A. Romanzin, V. Foletto, F. Da Borso, & E. Piasentier. 2020. Effect of heat stress on dairy cow performance and on expression of protein metabolism genes in mammary cells. Animals 10:2124. https://doi.org/10.3390/ani10112124

da Silva, R. G. & A. S. C. Maia. 2013. Principles of Animal Biometeorology. Springer, New York. https://doi.org/10.1007/978-94-007-5733-2

Dangi, S. S., M. Gupta, D. K. Maurya, V. P. Yadav, R. P. Panda, G. Singh, N. H. Mohan, S. K. Bhure, B. C. Das, S. Bag, R. Mahapatra, G. T. Sharma, & M. Sarkar. 2012. Expression profile of HSP genes during different seasons in goats (*Capra hircus*). Trop. Anim. Health. Prod. 44:1905–1912. https://doi.org/10.1007/s11250-012-0155-8

Dangi, S. S., S. K. Dangi, V. S. Chouhan, M. R. Verma, P. Kumar, G. Singh, & M. Sarkar. 2016. Modulatory effect of betaine on expression dynamics of HSPs during heat stress acclimation in goat (*Capra hircus*). Gene 575:543–550. https://doi.org/10.1016/j.gene.2015.09.031

Darling, N. J. & S. J. Cook. 2014. The role of MAPK signaling pathways in the response to endoplasmic reticulum stress. Biochim. Biophys Acta 1843:2150-2163. https://doi.org/10.1016/j.bbamcr.2014.01.009

El-Zarei, M. F., A. M. Alseaf, A. A. Alhaidary, E. F. Mousa, A. B. Okab, E. M. Samara, & K. A. Abdoun. 2019. Short-term heat shock proteins 70 and 90 mRNA expression profile and its relation to thermo-physiological parameters in goats exposed to heat stress. Int. J. Biometeorol. 63:459–465. https://doi.org/10.1007/s00484-019-01677-2

Fonsêca, V. F. C., A. S. C. Maia, E. P. Saraiva, C. C. M. Costa, R. G. da Silva, K. A. Abdoun, A. A. Al-Haidary, E. M. Samara, & A. Fuller. 2019. Bio-thermal responses and heat balance of a hair coat sheep breed raised under an equatorial semi-arid environment. J. Therm. Biol. 84:83–91. https://doi.org/10.1016/j.jtherbio.2019.05.024

Fuller, A., D. Mitchell, S. K. Maloney, & R. S. Hetem. 2016. Towards a mechanistic understanding of the responses of large terrestrial mammals to heat and aridity associated with climate change. Clim. Change Rep. 3:1–19. https://doi.org/10.1186/s40665-016-0024-1

Gaughan, J. B., V. Sejian, T. L. Mader, & F. R. Dunshea. 2018. Adaptation strategies: Ruminants. Anim. Front. 9:47-53. https://doi.org/10.1093/af/vfy029

Ghanem, A. M., L. S. Jaber, M. A. Said, E. K. Barbour, & S. K. Hamadeh. 2008. Physiological and chemical responses in water-deprived Awassi ewes treated with vitamin C. J. Arid Environ. 72:141–149. https://doi.org/10.1016/j.jaridenv.2007.06.005

Gorostizaga, A., L. Brion, P. Maloberti, F. C. Maciel, E. J. Podestá, & C. Paz. 2005. Heat shock triggers MAPK activation and MKP-1 induction in Leydig testicular cells. Biochem. Biophys. Res. Commun. 327:23–28. https://doi.org/10.1016/j.bbrc.2004.11.129

Grewal, S., A. Aggarwal, & M. N. Alhussien. 2021. Seasonal alterations in the expression of inflammatory cytokines and cortisol concentrations in periparturient sahiwal cows. Biol. Rhythm. Res. 52:1229–1239. https://doi.org/10.1080/09291016.2019.1670971

Haire, A., J. Bai, X. Zhao, Y. Song, G. Zhao, A. Dilixiati, J. Li, W. Sun, P. Wan, X. Fu, & A. Wusiman. 2022. Identifying the heat resistant genes by multi-tissue transcriptome sequencing analysis in Turpan Black sheep. Theriogenology 179:78–86. https://doi.org/10.1016/j.theriogenology.2021.11.008

Kalds, P., S. Zhou, Y. Gao, B. Cai, S. Huang, Y. Chen, & X. Wang. 2022. Genetics of the phenotypic evolution in sheep: A molecular look at diversity-driving genes. Genet. Sel. Evol. 54:61. https://doi.org/10.1186/s12711-022-00753-3

Kamboh, A. A., S. Q. Hang, M. Bakhetgul, & W. Y. Zhu. 2013. Effects of genistein and hesperidin on biomarkers of heat stress in broilers under persistent summer stress. Poult. Sci. 92:2411–2418. https://doi.org/10.3382/ps.2012-02960

Kelly, C. F. & T. E. Bond. 1971. Bioclimatic Factors and their Measurement: A Guide to Environmental Research on Animals. Natl. Acad. Sci., Washington, DC.

Kim, N. K., D. Lim, S. H. Lee, Y. M. Cho, E. W. Park, C. S. Lee, B. S. Shin, T. H. Kim, & D. Yoon. 2011. Heat shock protein b1 and its regulator genes are negatively correlated with intramuscular fat content in the longissimus thoracis muscle of hanwoo (Korean cattle) steers. J. Agric. Food Chem. 59:5657–64. https://doi.org/10.1021/jf200217j

Kregel, K. C. 2002. Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolerance. J. Appl. Physiol. 92:2177–2186. https://doi.org/10.1152/japplphysiol.01267.2001

Kumar, R., I. D. Gupta, A. Verma, N. Verma, & M. R. Vineeth. 2017. Single nucleotide polymorphisms in Heat Shock Protein (HSP) 90AA1 gene and their association with heat tolerance traits in Sahiwal cows. Indian J. Anim. Res. 51:64–69.

Kumar, R., I. D. Gupta, A. Verma, S. Singh, R. Kumari, & N. Verma. 2021. Genetic polymorphism in HSPB6 gene and their association with heat tolerance traits in Indian Karan Fries (*Bos aurus* x *Bos indicus*) cattle. Anim. Biotechnol. 29:1–12. https://doi.org/10.1080/10495398.2021.1899939

Li, Y., L. Kong, M. Deng, Z. Lian, Y. Han, B. Sun, Y. Guo, G. Liu, & D. Liu. 2019. Heat stress-responsive transcriptome analysis in the liver tissue of Hu sheep. Genes 100:395. https://doi.org/10.3390/genes10050395

Livak, K. J. & T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−deltadeltaCt method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

Lu, Z., M. Chu, Q. Li, M. Jin, X. Fei, L. Ma, L. Zhang, & C. Wei. 2019. Transcriptomic analysis provides novel insights into heat stress responses in sheep. Animals 9:387. https://doi.org/10.3390/ani9060387

Luna-Ramirez, R. I., S. W. Limesand, R. Goyal, A. L. Pendleton, G. Rincón, X. Zeng, G. Luna-Nevárez, J. R. Reyna-Granados, & P. Luna-Nevárez. 2023. Blood transcriptomic analyses reveal functional pathways associated with thermotolerance in pregnant ewes exposed to environmental heat stress. Genes 14:1590. https://doi.org/10.3390/genes14081590

Marai, I. F. M., A. A. El-Darawany, A. Fadiel, & M. A. M. Abdel-Hafez. 2007. Physiological traits as affected by heat stress in sheep – A review. Small Rumin. Res. 71:1–12. https://doi.org/10.1016/j.smallrumres.2006.10.003

Martins Júnior, L. M., A. P. R. Costa, D. M. R. Azevedo, S. H. N. Turco, J. E. G. Campelo, & M. C. S. Muratori. 2007. Adaptabilidade de caprinos Boer e Anglo-nubiana às condições climáticas do meio-norte do Brasil. Arch. Zootec. 56:103–113.

Matsumoto, T., M. Urushido, H. Ide, M. Ishihara, K. Hamada-Ode, Y. Shimamura, K. Ogata, K. Inoue, Y. Taniguchi, & T. Taguchi. 2015. Small heat shock protein beta-1 (HSPB1) is upregulated and regulates autophagy and apoptosis of renal tubular cells in acute kidney injury. PLoS One 10:e0126229. https://doi.org/10.1371/journal.pone.0126229

McManus, C., G. R. Paludo, H. Louvandini, R. Gugel, L. C. B. Sasaki, & S. R. Paiva. 2009. Heat tolerance in Brazilian sheep: Physiological and blood parameters. Trop. Anim. Health. Prod. 4:95–101. https://doi.org/10.1007/s11250-008-9162-1

McManus, C. M., C. M. Lucci, A. Q. Maranhão, D. Pimentel, F. Pimentel, & S. R. Paiva. 2022. Response to heat stress for small ruminants: Physiological and genetic aspects. Livest. Sci. 263:105028. https://doi.org/10.1016/j.livsci.2022.105028

Mishra, S. R. 2021. Behavioural, physiological, neuro-endocrine and molecular responses of cattle against heat stress: An updated review. Trop. Anim. Health Prod. 53:400. https://doi.org/10.1007/s11250-021-02790-4

Nguyen, A. N. & K. Shiozaki. 1999. Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. Genes Dev. 13:1653-63. https://doi.org/10.1101/gad.13.13.1653

Parsell, D. A. & S. Lindquist. 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27:437–496. https://doi.org/10.1146/annurev.ge.27.120193.002253

Pugh, D. G., N. N. Baird, M. Edmondson, & T. Passler. 2020. Sheep, Goat, and Cervid Medicine-E-Book. Elsevier Health Sciences. https://doi.org/10.1016/C2017-0-02021-9

Raslan, F., T. Schwarz, S. Meuth, M. Austinat, M. Bader, T. Renné, K. Roosen, G. Stoll, A.L. Sirén, & C. Kleinschnitz. 2010. Inhibition of bradykinin receptor B1 protects mice from focal brain injury by reducing blood-brain barrier leakage and inflammation. J. Cereb. Blood Flow Metab. 30:1477–1486. https://doi.org/10.1038/jcbfm.2010.28

Rawash, R. A. A., M. A. Sharaby, & G. Hassan. 2022. Expression profiling of HSP 70 and interleukins 2, 6 and 12 genes of Barki sheep during summer and winter seasons in two different locations. Int. J. Biometeorol. 66:2047–2053. https://doi.org/10.1007/s00484-022-02339-6

Renaudeau, D., A. Collin, S. Yahav, V. de Basilio, J. L. Gourdine, & R. J. Collier. 2012. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animals 6:707–728. https://doi.org/10.1017/S1751731111002448

Rhoad, A. O. 1944. The Iberia heat tolerance test for cattle. Trop. Agric. 21:162–164.

Rong, Y., M. Zeng, X. Guan, K. Qu, J. Liu, J. Zhang, H. Chen, B. Huang, & C. Lei. 2019. Association of HSF1 genetic variation with heat tolerance in Chinese cattle. Animals 9:1027. https://doi.org/10.3390/ani9121027

Rout, P., R. Kaushik, & N. Ramachandran. 2016. Differential expression pattern of heat shock protein 70 gene in tissues and heat stress phenotypes in goats during peak heat stress period. Cell Stress Chaperon. 21:645–651. https://doi.org/10.1007/s12192-016-0689-1

Sajjanar, B., R. Deb, U. Singh, S. Kumar, M. Brahmane, A. Nirmale, S. K. Bal, & P. S. Minhas. 2015. Identification of SNP in HSP90AB1 and its association with the relative thermotolerance and milk production traits in Indian dairy cattle. Anim. Biotechnol. 26:45–50. https://doi.org/10.1080/10495398.2014.882846

Samara, E. M., M. A. Bahadi, M. A. Al-Badwi, K. A. Abdoun, & A. A. Al-Haidary. 2023. A comparative thermophysiological study between two purebred Saudi sheep under biometeorologically-simulated environment. J. Saudi Soc. Agric. Sci. 22:283–287. https://doi.org/10.1016/j.jssas.2023.01.003

Samara, E. M., K. A. Abdoun, A. B. Okab, M. A. Al-Badwi, M. F. El-Zarei, A. M. Al-Seaf, & A. A. Al-Haidary. 2016. Assessment of heat tolerance and production performance of Aardi, Damascus, and their crossbred goats. Int. J. Biometeorol. 60:1377–1387. https://doi.org/10.1007/s00484-015-1131-6

Saudi National Center for Meteorology (SNCM). 2024. Historical Data Service. https://ncm.gov.sa/Ar/EService/met/Pages/ClimateDataRequest.aspx

Sejian, V., V. P. Maurya, & S. M. K. Naqvi. 2010. Adaptive capability as indicated by endocrine and biochemical responses of Malpura ewes subjected to combined

stresses (thermal and nutritional) in a semi-arid tropical environment. Int. J. Biometeorol. 54:653–661. https://doi.org/10.1007/s00484-010-0341-1

Silanikove, N. 2000. Effects of heat stress on the welfare of extensively managed domestic ruminants: A review. Livest. Prod. Sci. 67:1–18. https://doi.org/10.1016/S0301-6226(00)00162-7

Singh, K. M., S. Singh, I. Ganguly, R. K. Nachiappan, A. Ganguly, R. Venkataramanan, A. Chopra, & H. K. Narula. 2017. Association of heat stress protein 90 and 70 gene polymorphism with adaptability traits in Indian sheep (*Ovis aries*). Cell Stress Chaperones 22:675–684. https://doi.org/10.1007/s12192-017-0770-4

Sivakumar, A. V. N., G. Singh, & V. P. Varshney. 2010. Antioxidants supplementation on acid base balance during heat stress in goats. Asian-Australas. J. Anim. Sci. 23:1462-1468. https://doi.org/10.5713/ajas.2010.90471

Sonna, L. A., J. Fujita, S. L. Gaffin, & C. M. Lilly. 2002. Effects of heat and cold stress on mammalian gene expression. J. Appl. Physiol. 92:1725–1742. https://doi.org/10.1152/japplphysiol.01143.2001

Starling, J. M. C., R. G. Silva, M. Cerón-Muñoz, G. S. S. C. Barbosa, & M. J. R. P. Costa. 2002. Analysis of some physiological variables for the evaluation of the degree of adaptation in sheep submitted to heat stress. Rev. Bras. Zootecn. 32:2070–2077. https://doi.org/10.1590/S1516-35982002000800022

Vihervaara, A. & L. Sistonen. 2014. HSF1 at a glance. J. Cell Sci. 127:261–266. https://doi.org/10.1242/jcs.132605

West, J. W. 2003. Effects of heat-stress on production in dairy cattle. J. Dairy Sci. 86:2131–2144. https://doi.org/10.3168/jds.S0022-0302(03)73803-X

Woolley, S. A., M. Salavati, & E. L. Clark. 2023. Recent advances in the genomic resources for sheep. Mamm. Genome. 34:545–558. https://doi.org/10.1007/s00335-023-10018-z

Yang, H., Y. L. Yang, G. Q. Li, Q. Yu, & J. Yang. 2021. Identifications of immune-responsive genes for adaptative traits by comparative transcriptome analysis of spleen tissue from Kazakh and Suffolk sheep. Sci. Rep. 11:3157. https://doi.org/10.1038/s41598-021-82878-x

Zeng, L., K. Qu, J. Zhang, B. Huang, & C. Lei. 2022. Genes related to heat tolerance in cattle-a review. Anim. Biotechnol. 15:1–9. https://doi.org/10.1080/10495398.2022.2047995