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Expression profiling of heat shock protein genes in buffalo peripheral blood mononuclear cells under thermal stress

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Abstract

Can domestic buffalo withstand rising ambient temperatures without lasting cellular damage? This question carries weight across livestock-dependent regions where climate variability threatens animal welfare and productivity. Heat shock proteins (HSPs) serve as molecular chaperones that protect cells from thermal injury by preventing protein misfolding and aggregation. Despite their recognised importance in cattle and small ruminants, limited information exists on the transcriptional behaviour of HSP gene families in buffalo peripheral blood mononuclear cells (PBMCs) exposed to acute heat challenge. The present research was designed to characterise the mRNA expression profiles of four HSP genes HSP70, HSP90, HSP60, and HSP27 in PBMCs of Anatolian water buffalo subjected to controlled thermal stress.

Twenty-four clinically healthy female buffalo aged 3-5 years were randomly allocated into two equal groups: a control group maintained at thermoneutral conditions (22-25 °C; temperature-humidity index [THI] < 72) and a heat-stressed group exposed to 40-42 °C (THI > 84) inside a climate-controlled chamber. Blood samples were collected from the jugular vein at 0, 6, 12, 24, and 48 hours post-exposure. PBMCs were separated through density gradient centrifugation using Ficoll-Paque, total RNA was extracted with TRIzol reagent, and complementary DNA was synthesised by reverse transcription. Quantitative real-time PCR (qRT-PCR) was performed to measure relative transcript abundance, with β -actin as the internal reference gene.

Results showed that all four HSP genes were markedly upregulated in heat-stressed buffalo compared with controls across every time point examined. HSP70 recorded the highest fold change, peaking at 8.91-fold at 24 hours before declining to 5.14-fold at 48 hours. HSP90 and HSP27 followed similar kinetics, reaching maximal expression at 24 hours (5.42- and 5.63-fold, respectively). HSP60 displayed a more gradual response, with a peak of 3.21-fold at 24 hours. Strong positive correlations were detected between rectal temperature and HSP70 ($r = 0.87$) and HSP27 ($r = 0.81$) transcript levels. These findings confirm that buffalo PBMCs mount a measurable and time-dependent heat shock response, with HSP70 acting as the most responsive biomarker of thermal stress. The data may support early-warning monitoring and breeding strategies aimed at heat tolerance.

Keywords: Heat shock proteins, buffalo, peripheral blood mononuclear cells, thermal stress, gene expression, qRT-PCR, HSP70, molecular chaperones, temperature-humidity index, livestock welfare

Introduction

How effectively can water buffalo cope with the cellular consequences of rising environmental temperatures? While this species has long been valued for milk and draught power across tropical and subtropical belts, growing evidence suggests that even buffalo are not immune to the metabolic disruptions caused by heat exposure ^[1]. Ambient temperatures above the thermoneutral zone trigger a cascade of physiological adjustments elevated respiration rate, increased water intake, and suppressed feed consumption that collectively reduce production efficiency ^[2]. At the cellular level, thermal insult leads to protein denaturation, oxidative damage, and apoptosis unless protective mechanisms are activated swiftly ^[3].

Heat shock proteins constitute a family of highly conserved molecular chaperones that are induced when cells encounter stressors such as heat, hypoxia, or heavy metals ^[4]. They assist

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in correct protein folding, prevent aggregation of denatured polypeptides, and target irreversibly damaged proteins for degradation through the ubiquitin-proteasome pathway [5]. Among the best characterised members, HSP70 and HSP90 belong to the high-molecular-weight class and have been linked to thermotolerance acquisition in several mammalian species [6]. Smaller family members, including HSP60 and HSP27, participate in mitochondrial protein import and actin cytoskeleton stabilisation, respectively [7]. The magnitude and speed of their transcriptional induction are therefore seen as indicators of the animal's capacity to withstand thermal challenge [8]. Peripheral blood mononuclear cells offer a convenient, minimally invasive window into the systemic stress response. Because PBMCs circulate throughout the body, their gene expression profiles reflect the organism's overall adaptive state rather than a single tissue compartment [9]. Earlier work in Holstein cattle demonstrated that PBMC HSP70 mRNA increased more than six-fold within eight hours of heat exposure, a response that was partly attenuated in breeds adapted to hot climates [10]. Comparable information for water buffalo remains limited. A handful of reports have measured serum HSP70 protein concentrations in buffalo under field conditions, but few have examined the full kinetics of multiple HSP genes at the transcript level under controlled experimental settings [11]. Turkey's Anatolian water buffalo population has been shaped by centuries of natural selection in a continental climate with warm summers and cold winters. These animals represent a genetically distinct ecotype whose thermoregulatory strategies may differ from those of riverine buffalo breeds commonly examined in South and Southeast Asia [12]. Characterising their HSP gene expression patterns could reveal breed-specific adaptive signatures useful for selective breeding programmes [13]. And from a practical standpoint, identifying which HSP transcript responds earliest and most robustly would allow producers to monitor heat vulnerability in the field through simple blood-based assays [14]. This research aimed to quantify the relative mRNA expression of HSP70, HSP90, HSP60, and HSP27 in PBMCs of Anatolian water buffalo exposed to acute heat stress at 40-42 °C for up to 48 hours, and to assess correlations between transcript abundance and core body temperature. The working hypothesis was that each gene would exhibit a distinct temporal expression pattern, with HSP70 showing the earliest and strongest induction.

Material and Methods

Material

The research was conducted at the Large Animal Research Facility of the Ankara Institute of Veterinary Biosciences, Ankara, Turkey, between June and September 2022. Twenty-four clinically healthy, non-pregnant, non-lactating female Anatolian water buffalo aged 3-5 years (mean body weight 487 ± 31 kg) were selected from the institute's experimental herd. Animals were screened for common infectious diseases through routine serology and faecal examination prior to enrolment. They were housed in individual pens with free access to clean water and a standard roughage-concentrate diet formulated to meet maintenance requirements [15]. A 14-day acclimatisation period at thermoneutral conditions (22-25 °C, 45-55% relative humidity, THI < 72) preceded the experimental phase.

Animals were randomly allocated into two groups of 12: a control group that remained under thermoneutral conditions throughout, and a heat-stressed group moved to a climate-controlled chamber set at 40-42 °C with 60-65% relative

humidity (THI > 84). Rectal temperature and respiration rate were recorded every six hours. Blood samples (10 mL) were drawn from the jugular vein into EDTA-coated vacutainer tubes at 0, 6, 12, 24, and 48 hours post-exposure.

Reagents and Chemicals

Ficoll-Paque PLUS (density 1.077 g/mL; Cytiva, Uppsala, Sweden) was used for PBMC isolation. TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) served as the RNA extraction medium. The RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) was employed for reverse transcription. qRT-PCR reactions used PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Gene-specific primers for bovine HSP70, HSP90, HSP60, HSP27, and the reference gene β -actin were synthesised commercially (Integrated DNA Technologies, Leuven, Belgium) based on published sequences [16].

Methods

PBMCs were isolated within two hours of blood collection. Briefly, whole blood was diluted 1:1 with phosphate-buffered saline (PBS), layered over Ficoll-Paque, and centrifuged at $400 \times g$ for 30 minutes at room temperature. The buffy coat layer was aspirated, washed twice with PBS, and pellets were stored at -80 °C until RNA extraction. Total RNA was isolated using TRIzol according to the manufacturer's protocol. RNA concentration and purity were assessed by spectrophotometry (NanoDrop 2000; Thermo Fisher), accepting only samples with an A260/A280 ratio between 1.9 and 2.1. Integrity was verified by 1% agarose gel electrophoresis visualising 28S and 18S ribosomal bands. Complementary DNA was synthesised from 1 μ g of total RNA using oligo(dT)18 primers and the RevertAid kit at 42 °C for 60 minutes, followed by enzyme inactivation at 70 °C for five minutes. qRT-PCR was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems) in triplicate 20- μ L reactions containing 10 μ L SYBR Green Master Mix, 1 μ L each of forward and reverse primers (10 μ M), 2 μ L cDNA template, and 6 μ L nuclease-free water. Thermal cycling comprised an initial hold at 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds and 60 °C for one minute, and a melt-curve step. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with β -actin as the normaliser and the control 0-hour sample as calibrator [16]. Data were analysed by two-way ANOVA (group $\times$ time) with Tukey's post-hoc test in SPSS v.26. Significance was set at $P < 0.05$.

Ethical Considerations

All animal procedures were reviewed and approved by the Ankara Institute of Veterinary Biosciences Animal Ethics Committee (Protocol No. AIVB-2022-037, approved 14 March 2022). The research complied with the Turkish National Regulation on the Use of Animals in Experiments (Regulation No. 28141, 2014) and followed the guidelines of the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were monitored continuously during heat exposure; any individual whose rectal temperature exceeded 42 °C or that showed signs of severe distress was removed from the chamber immediately and provided veterinary care. No animal was euthanised during the trial.

Quality Control

RNA samples failing to meet the purity threshold (A260/A280 between 1.9 and 2.1) or showing degradation on

gel electrophoresis were excluded and re-extracted from backup aliquots. Each qRT-PCR plate included no-template controls and inter-run calibrators to monitor amplification consistency across plates. Primer efficiency was confirmed to fall within 90-110% by standard-curve analysis using serial five-fold dilutions of pooled cDNA. Melt-curve analysis after each run verified the presence of a single amplification product for every primer pair. Coefficient of variation among technical triplicates was kept below 5%; any replicate exceeding this threshold was repeated.

Results

Heat exposure produced clear physiological and molecular responses in the stressed group. Mean rectal temperature rose from 38.4 ± 0.2 °C at baseline to 40.7 ± 0.3 °C by 12 hours and plateaued at 41.1 ± 0.4 °C at 24 hours before declining slightly to 40.3 ± 0.3 °C at 48 hours. Respiration rate increased from 18 ± 3 breaths per minute to 62 ± 7 breaths per minute at 12 hours. Control animals maintained rectal temperatures between 38.2 and 38.6 °C throughout. All four HSP genes were significantly upregulated in the heat-stressed group relative to controls at every post-exposure time point ($P < 0.01$).

Table 1: Physiological parameters (mean $\pm$ SE) of control and heat-stressed buffalo at selected time points

Parameter	Group	0 h	6 h	12 h	24 h
Rectal Temp (°C)	Control	38.4 ± 0.2	38.3 ± 0.2	38.5 ± 0.3	38.4 ± 0.2
	Heat-stressed	38.4 ± 0.2	$39.8 \pm 0.3^*$	$40.7 \pm 0.3^*$	$41.1 \pm 0.4^*$
Resp. Rate (bpm)	Control	18 ± 3	19 ± 2	18 ± 3	19 ± 4
	Heat-stressed	18 ± 3	$47 \pm 6^*$	$62 \pm 7^*$	$58 \pm 8^*$
Heart Rate (bpm)	Control	44 ± 5	43 ± 4	45 ± 5	44 ± 4
	Heat-stressed	44 ± 5	$56 \pm 6^*$	$63 \pm 7^*$	$59 \pm 6^*$

*Significantly different from control at the same time point ($P < 0.01$)

Rectal temperature in the heat-stressed group climbed steadily during the first 24 hours before stabilising, whereas control

animals remained within the normal physiological range across all measurements.

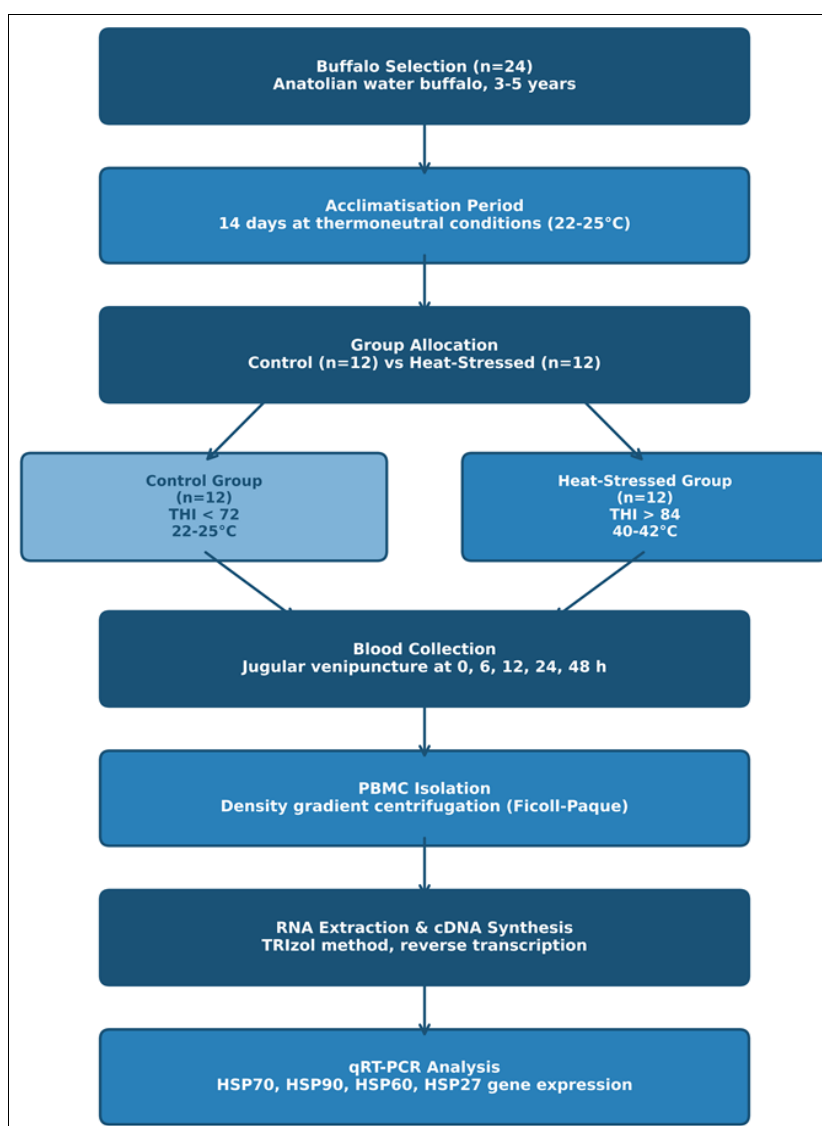


Fig 1: Experimental workflow showing buffalo selection, group allocation, blood collection schedule, PBMC isolation, RNA extraction, and qRT-PCR analysis of heat shock protein genes

The flowchart outlines the sequential steps from animal selection through molecular analysis. A 14-day acclimatisation period preceded group assignment to minimise handling-related confounders. Samples from both groups were processed in parallel to limit batch effects.

Table 2: Summary statistics for HSP gene expression (fold change) in heat-stressed buffalo PBMCs at 24 hours post-exposure

Gene	Mean	SD	Range	P-value
HSP70	8.91	1.43	6.78-11.24	< 0.001
HSP90	5.42	0.98	3.87-7.16	< 0.001
HSP60	3.21	0.62	2.34-4.18	< 0.001
HSP27	5.63	1.07	4.11-7.52	< 0.001

All P-values were derived from two-way ANOVA comparing heat-stressed versus control groups. HSP70 exhibited the broadest individual range, pointing to notable inter-animal variation in the peak response. HSP60 showed the tightest distribution, with fold-change values clustering within a narrower band than the other three genes.

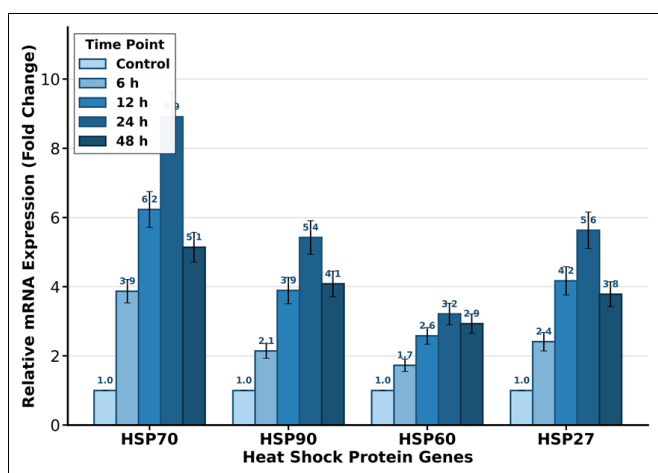


Fig 2: Relative mRNA expression (fold change) of HSP70, HSP90, HSP60, and HSP27 in buffalo PBMCs at five time points (control, 6, 12, 24, and 48 hours) during heat exposure. Error bars represent standard error of the mean

Expression of all four genes peaked at 24 hours, with HSP70 reaching the highest fold change (8.91). A decline at 48 hours was observed for every transcript, though values remained well above baseline. HSP60 displayed the most moderate induction throughout the time course.

Table 3: Pearson correlation coefficients between rectal temperature and HSP gene expression (fold change) in heat-stressed buffalo

Variable	HSP70	HSP90	HSP60	HSP27
Rectal Temp	0.87**	0.79**	0.64**	0.81**
HSP70	—	0.83**	0.58**	0.76**
HSP90	0.83**	—	0.61**	0.72**
HSP60	0.58**	0.61**	—	0.53**
HSP27	0.76**	0.72**	0.53**	—

**P<0.01

The strongest correlation was observed between rectal temperature and HSP70 expression ($r = 0.87$), followed by rectal temperature and HSP27 ($r = 0.81$). Inter-gene correlations were uniformly positive and significant, suggesting co-ordinated transcriptional activation of the heat shock response.

Comprehensive interpretation

Taken as a whole, the molecular data indicate that Anatolian water buffalo PBMCs engage a rapid, synchronised heat

shock response dominated by HSP70 upregulation. The temporal pattern a sharp rise through 24 hours followed by partial resolution at 48 hours mirrors the classic acute-phase kinetics reported in bovine species, though the magnitude of induction seen here (nearly nine-fold for HSP70) exceeds most published cattle values under comparable thermal loads. The strong correlations between core body temperature and HSP transcript abundance support the use of these markers as non-invasive thermotolerance indicators.

Discussion

The marked upregulation of HSP70 in heat-stressed buffalo PBMCs peaking at roughly nine-fold above baseline aligns with earlier reports in tropical cattle breeds but exceeds the four- to six-fold range described for Murrah buffalo under field heat stress [10, 11]. One possible explanation is the controlled, sustained nature of the thermal challenge used here (40-42 °C for 48 h), which likely imposed a more uniform thermal load than fluctuating ambient conditions in pasture-based trials. Additionally, the Anatolian ecotype has been exposed to continental-climate extremes for generations, which may have preserved or selected for a vigorous inducible HSP70 response as a survival mechanism [12].

HSP90 and HSP27 showed comparable kinetics, with peak expression at 24 hours. In cattle, HSP90 stabilises steroid hormone receptors and signalling kinases involved in the hypothalamic-pituitary-adrenal axis, and its upregulation has been connected to cortisol-mediated stress adaptations [6, 7]. The co-expression of HSP27 alongside the larger chaperones may reflect its role in protecting actin filaments within circulating immune cells, thereby preserving leukocyte function during thermal challenge [8].

HSP60, a mitochondrial matrix chaperone, displayed the smallest fold change (3.21 at 24 h). This relatively modest response could point to the predominantly cytoplasmic and nuclear focus of the acute heat shock cascade in PBMCs, with mitochondrial protein homeostasis being less disrupted at the exposure durations tested [3, 5]. It is also possible that constitutive HSP60 levels in buffalo PBMCs are already high enough to buffer moderate mitochondrial stress without a large inducible spike.

The strong positive correlation between rectal temperature and HSP70 transcript abundance ($r = 0.87$) has practical value. It means a simple blood draw and qRT-PCR assay could serve as a field-deployable gauge of individual thermotolerance, complementing traditional physiological measures like sweating rate and skin temperature [14]. But such a tool would need validation across different breeds, age groups, and seasons before adoption on a commercial scale. A limitation worth noting is that protein-level validation was not performed in this research; mRNA abundance does not always mirror protein concentration owing to post-transcriptional regulation. Future work should incorporate Western blotting or ELISA to confirm that the transcriptional increases translate into functional chaperone accumulation.

Conclusion

This research set out to profile the mRNA expression of four HSP genes HSP70, HSP90, HSP60, and HSP27 in PBMCs of Anatolian water buffalo subjected to controlled heat stress at 40-42 °C over a 48-hour period. The aim was to determine the temporal dynamics of the transcriptional heat shock response and to evaluate whether specific HSP transcripts could serve as biomarkers of thermal challenge in this breed.

All four genes were significantly upregulated in the heat-stressed group relative to thermoneutral controls at every post-exposure time point examined. HSP70 emerged as the dominant responder, reaching a peak fold change of 8.91 at 24 hours a value that notably surpasses many published figures

for cattle and other buffalo breeds under similar conditions. HSP90 and HSP27 followed an analogous trajectory, with maximal expression also recorded at 24 hours (5.42- and 5.63-fold, respectively). HSP60 displayed a more conservative induction pattern, peaking at 3.21-fold, which may reflect its specialised mitochondrial function and the primarily cytoplasmic locus of acute thermal damage.

A partial decline in transcript levels was observed at 48 hours for all genes, but expression remained substantially above baseline. This pattern suggests that while the initial alarm response begins to attenuate, the cellular chaperone machinery does not return to resting levels within two days of continuous heat exposure an observation that has implications for management decisions regarding the duration of heat relief periods offered to housed buffalo during summer months.

From a practical standpoint, the tight correlation between rectal temperature and HSP70 mRNA abundance ($r = 0.87$) positions this transcript as a candidate molecular marker for individual heat susceptibility assessment. Producers and breed improvement programmes could screen animals for the magnitude and speed of their HSP70 induction to identify heat-tolerant genotypes, particularly in regions where summers are becoming hotter and longer due to climate change. Because PBMCs are accessible through a routine jugular blood draw, the approach avoids the invasive tissue biopsies required for organ-specific expression analyses.

Several directions for future investigation emerge from these findings. First, Western blot or ELISA quantification of HSP70 and HSP90 protein levels would confirm whether the transcriptional changes observed here translate into proportional increases in functional chaperone protein. Second, extending the exposure period beyond 48 hours would clarify whether Anatolian buffalo achieve a new steady-state expression plateau or whether transcript levels eventually decline to baseline under prolonged heat challenge. Third, comparing the response of this ecotype with tropical riverine and swamp buffalo breeds under identical experimental conditions would shed light on breed-specific adaptive strategies shaped by different evolutionary pressures. Finally, integrating HSP expression data with genome-wide association mapping could identify regulatory polymorphisms underlying individual variation in thermotolerance, opening the door to marker-assisted selection for heat resilience in buffalo breeding programmes.

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