

Investigations of Heat Shock Protein 70 for Non-Invasive Detection of Heat Stress in Dairy Cows

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STATEMENT OF ORIGINALITY

This thesis is submitted to The University of Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all assistance received in preparing this thesis and all sources have been acknowledged.

Md Rezaul Hai Rakib

22 April 2026

Dedicated to Amma

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ABSTRACT

Heat stress (HS) is a major constraint on dairy production, negatively affecting animal welfare, milk yield, and reproductive performance. Although environmental and physiological indicators such as rectal temperature (RT), infrared thermography (IRT), and reticulorumen temperature (RRT) are commonly used to detect HS in dairy cows, they provide limited insight into the cellular responses underlying this condition. This thesis evaluated the potential of heat shock protein 70 (HSP70) as a non-invasive indicator of HS in dairy cows by investigating its analytical reliability, biological variability, and temporal behaviour in relation to established indicators in pasture-based dairy production systems. This study comprised a critical literature review, analytical validation, methodological optimisation, seasonal and time-lag analyses in a pasture-based dairy system. [Chapter 2](#) reviewed current knowledge of HS detection in dairy cows, focusing on the potential and practical limitations of HSP70 as a molecular biomarker and identifying the key knowledge gaps that were addressed by this research. The first two experimental chapters addressed the analytical and sample-handling knowledge gaps. [Chapter 3](#) compared an in-house competitive ELISA with a commercial assay using milk, blood, and saliva, demonstrating strong agreement in relative quantification, greater precision, a wider detection range, and lower analytical cost. [Chapter 4](#) showed that milk handling affects HSP70 stability, with skimming before storage reducing degradation and refrigerated or frozen skim milk preserving HSP70 concentrations in the short term. Seasonal investigations in [Chapter 5](#) indicated that HSP70 levels increased during periods of elevated heat load in milk, blood, and saliva. However, milk HSP70 exhibited greater variability and was associated with lactation stage, indicating that its variation might not be driven solely by heat exposure. [Chapter 6](#) demonstrated that milk HSP70 exhibited a significant delay relative to environmental and physiological indicators. While RT and IRT reflected short-term heat load and RRT captured slower internal heat accumulation, milk HSP70 indicated delayed cellular stress activation, a pattern confirmed during a heatwave in [Chapter 7](#). This delayed response is consistent with the biological role of HSP70 as a stress-induced molecular chaperone, requiring transcriptional activation and intracellular accumulation before extracellular detection. Overall, the findings demonstrate that milk HSP70 is not a direct, real-time HS indicator, but rather a complementary, non-invasive indicator reflecting cellular stress associated with prior heat exposure. By integrating physiological indicators with molecular biomarkers, this thesis advances understanding of cellular stress dynamics in dairy cows and emphasises the importance of integrated HS assessment frameworks for more robust HS assessment.

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PREFACE

This thesis is presented in the format of ‘thesis with publications’ and is written in Australian English. For e-readers, hyperlinks are enabled to facilitate navigation.

Some chapters of this thesis have either been published or are under review for publication. Published papers have been included respecting their original Journal format. The work of this thesis has also formed the basis of presentations at scientific and institutional research showcases, seminars, and symposia.

LIST OF PUBLICATIONS, PRESENTATIONS, AND ACHIEVEMENTS

Publications in peer-reviewed journals

1. **Rakib, M.R.H.**, V. Messina, J.I. Gargiulo, N.A. Lyons, and S.C. Garcia. 2024. Graduate student literature review: Potential use of HSP70 as an indicator of heat stress in dairy cows-A review. *J. Dairy Sci.* 107:11597–11610. <https://doi.org/10.3168/jds.2024-24947>
2. **Rakib, M.R.H.**, V. Messina, J.I. Gargiulo, N.A. Lyons, I.N. Pathirana, P.C. Thomson, and S.C. Garcia. 2025. Skimming and storage factors affect the detection of heat shock protein 70 in raw bovine milk. *JDS Commun.* 6:578–582. <https://doi.org/10.3168/jdsc.2025-0759>
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Abstracts and conference presentations

1. **Rakib, M.R.H.**, V. Messina, J.I. Gargiulo, N.A. Lyons, and S.C. Garcia. 2023. Can heat shock protein 70 be monitored in dairy cows under heat stress? *SOLES HDR Showcase (Introductory PhD Presentation)*, School of Life and Environmental Sciences, The University of Sydney, Camperdown, NSW, Australia.
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3. **Rakib, M.R.H.,** V. Messina, J.I. Gargiulo, N. Lyons, I. Pathirana, and S.C. Garcia. 2024. Influence of management and storage factors on detection of HSP70 in raw bovine milk. In: *Proceedings of the American Dairy Science Association Annual Meeting*, FL, USA. https://www.adsa.org/Portals/0/SiteContent/Docs/Meetings/2024ADSA/Abstracts_BOOK_2024_FINAL.pdf
4. **Rakib, M.R.H.,** V. Messina, J.I. Gargiulo, N. Lyons, I. Pathirana, and S.C. Garcia. 2024. Influence of management and storage factors on detection of HSP70 in raw bovine milk. *SOLES HDR Showcase (Intermediate Poster Presentation)*, School of Life and Environmental Sciences, The University of Sydney, Camperdown, NSW, Australia.
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6. **Rakib, M.R.H.,** V. Messina, J.I. Gargiulo, N. Lyons, I. Pathirana, and S.C. Garcia. 2025. Potential of HSP70 as a non-invasive biomarker for detecting heat stress in dairy cows. In: *Proceedings of the American Dairy Science Association Annual Meeting*, Louisville, KY, USA. https://dairyup.com.au/wp-content/uploads/2025/07/Potential-of-HSP70-as-a-non-invasive-biomarker_R-Rakib.pdf
7. **Rakib, M.R.H.,** V. Messina, J.I. Gargiulo, N.A. Lyons, I. Pathirana, P.C. Thomson, and S.C. Garcia. 2025. Investigations of heat shock protein 70 for non-invasive detection of heat stress in dairy cows. *SOLES HDR Showcase (Final PhD presentation)*, School of Life and Environmental Sciences, The University of Sydney, Camperdown, NSW, Australia.
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Media articles and other presentations

1. Gargiulo, J.I., V. Messina, and **M.R.H. Rakib**. 2023. Milk as an indicator of heat load. Dairy UP communication article. Dairy UP Program, Camden, NSW, Australia. <https://dairyup.com.au/wp-content/uploads/2024/07/P9b-Milk-as-an-indicator-of-heat-load.pdf>
2. **Rakib, M.R.H.**, V. Messina, J.I. Gargiulo, N. Lyons, I. Pathirana, and S.C. Garcia. 2024. Milk as an indicator of heat stress in dairy cows. *Presentation delivered to delegates from China Agricultural University, organised by the Sydney Institute of Agriculture, The University of Sydney, Camperdown, NSW, Australia.*
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Scholarships

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- University of Sydney (USYD) Tuition Fee Scholarship
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AUTHOR ATTRIBUTION STATEMENT

This thesis comprises six research chapters, which are the result of my own investigations.

Chapter 2 of this thesis has been published as Rakib, M. R. H., Messina, V., Gargiulo, J. I., Lyons, N. A., & Garcia, S. C. (2024). *Graduate Student Literature Review: Potential use of HSP70 as an indicator of heat stress in dairy cows-A review*. *Journal of Dairy Science*, 107(12), 11597-11610. <https://doi.org/10.3168/jds.2024-24947>. I, Md Rezaul Hai Rakib (RR), designed and conducted the review and prepared the original draft under the supervision of Professor Sergio (Yani) Garcia (YG), Dr Nicolas Lyons (NL), Dr Juan Ignacio Gargiulo (JG) and Dr Valeria Messina (VM). All authors contributed to the conceptualisation and participated in the review and editing process.

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Chapter 6 of this thesis has been prepared for submission to the Journal of Dairy Science and is titled ‘Comparative evaluation of environmental, physiological, and milk HSP70 indicators for detecting heat stress in dairy cows’. I designed and conducted the research under the supervision of YG, JG and VM. Data collection and curation were done by RR, Jade Nguyen (JN) and VM. Data validation, visualisation, and statistical analyses were conducted by RR under the supervision of JG. The original draft was written by RR and reviewed and edited by YG, NL, JG and VM.

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In addition to the authorship attribution statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Md Rezaul Hai Rakib

22 April 2026

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Professor Sergio (Yani) Garcia

22 April 2026

ARTIFICIAL INTELLIGENCE

During the preparation of this thesis, the author used Microsoft Copilot to assist with understanding data analysis methods, minor sentence restructuring, and clarity enhancement. The author confirms that where text was modified by generative AI, the content was reviewed for possible errors, inaccuracies, and bias. The author takes full responsibility for the submitted thesis and ensures the work is their own and has used generative AI in accordance with the University guidelines and policies (refer to the University of Sydney generative AI guide for researchers).

ABBREVIATIONS

Below is a list of alphabetised abbreviations used throughout this thesis. Abbreviations are also defined at first use within respective chapters.

%	Percent
°C	Degrees Celsius
AIC	Akaike information criterion
ANOVA	Analysis of Variance
AUD	Australian dollar
BIC	Bayesian information criterion
BOM	Bureau of Meteorology
BTSCC	Bulk tank somatic cell count
CBT	Core body temperature
CI	Confidence interval
conc	Concentration
CV	Coefficient of variation
d	Day
DIM	Days in milk
DM	Dry matter
ELISA	Enzyme-linked immunosorbent assay
EMM	Estimated marginal means
GHLI	Grazing heat load index
GLS	Generalised least squares
h	Hour
HLI	Heat load index
HRP	Horseradish peroxidase
HrHSP70	Human recombinant HSP70 solution
HS	Heat stress
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein

HSP70	Heat shock protein 70
HW	Heat wave
ID	Identification
IRT	Infrared thermography
L	Litre
LMM	Linear mixed model
MAE	Mean absolute error
mL	Millilitre
μL	Microlitre
n	Number
ng	Nanogram
<i>P</i>	P value
PBMC	Peripheral blood mononuclear cell
R^2	Coefficient of determination
<i>r</i>	Correlation coefficient
RH	Relative humidity
RMSE	Root mean square error
ROC	Receiver operating characteristic
RRT	Reticulorumen temperature
RT	Rectal temperature
R^2_c	Conditional R-squared
R^2_m	Marginal R-squared
SAS	Skimmed after storage
SBS	Skimmed before storage
SCC	Somatic cell count
SD	Standard deviation
SE	Standard error
SNF	Solids not fat
THI	Temperature humidity index
TNZ	Thermoneutral zone
TMB	3,3',5,5'-tetramethylbenzidine
USD	United States dollar

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CHAPTER 1

General Introduction

Introduction

The Australian dairy industry is the country's third-largest rural industry, producing over eight billion litres of milk each year from around 3,800 farms, with average herd sizes exceeding 300 cows (Dairy Australia, 2025). Most dairy farms in Australia rely on pasture-based systems, where cows are exposed to external environmental conditions and have limited options to regulate their temperature mitigation (Chang-Fung-Martel et al., 2017). Therefore, heat stress (HS) represents an ongoing and substantial challenge to animal welfare, productivity, and the economic sustainability of Australian dairy systems (Osei-Amponsah et al., 2020).

Heat stress occurs when elevated ambient temperature and humidity exceed the cow's capacity to dissipate body heat, leading to an increase in core body temperature (CBT) (Dahl et al., 2020). Dairy cows tend to regulate their body temperature within a defined thermoneutral zone (TNZ), enabling efficient use of energy for milk production and reproduction. When conditions surpass this TNZ, cows divert energy towards thermoregulatory responses, including increased respiration, sweating, and reduced dietary intake (Oliveira et al., 2025). If these responses are insufficient, the natural physiological balance is disrupted to maintain CBT (Dahl et al., 2020; Oliveira et al., 2025). This imbalance results in significant implications, including reduced feed intake (Chen et al., 2024), decreased milk production (West, 2003), compromised reproductive capabilities (Sammad et al., 2020), impaired immune response (Bagath et al., 2019), and increased susceptibility to disease and mortality (Polsky and Von Keyserlingk, 2017). The economic impact of HS on dairy sector is substantial, accounting for approximately 64% of overall livestock HS-related losses in the United States, corresponding to ~USD 1.5 billion annually (Cartwright et al., 2023). In Australia, HS has been estimated to cost ~AUD 12,000 per year for a 100-cow herd, although the overall losses are challenging to quantify due to its interacting effects on feed intake, milk production and quality, as well as reproductive performance (Lees, 2018).

Global temperatures are gradually rising due to climate change, leading to more frequent, intense, and prolonged extreme heat events that increase thermal exposure in both natural and controlled ecosystems (IPCC, 2018; 2021; Bolan et al., 2024). Australia is particularly vulnerable to these heat extremes because of its predominantly warm environment with substantial seasonal variation in temperature and precipitation, with heat exposure posing significant risks to both human and animal health (Xu et al., 2023). Over the past seven decades,

heatwaves across Australia have become increasingly frequent, intense, and long-lasting, with a significant increase observed in recent years (Trancoso et al., 2020). The average duration of heatwaves in southern Australia increased from about two to four consecutive days between 1960 and 2008 (Nidumolu et al., 2014). These trends are particularly concerning for pasture-based dairy systems, where cows are directly exposed to variable and often extreme environmental conditions (Chang-Fung-Martel et al., 2017; Anuta et al., 2025).

In addition to environmental exposure, genetic selection for increased milk production has enhanced metabolic heat generation, making modern dairy cows more vulnerable to HS (Brito et al., 2021). These factors underscore the need for practical, welfare-focused HS detection and management in commercial dairy production systems, particularly in pasture-based systems common in Australia.

Several techniques have been developed to detect and monitor HS in dairy cows, utilising environmental, physiological, behavioural, and molecular markers (Rakib et al., 2024). Among these, the temperature humidity index (THI) remains the most widely used indicator of environmental HS and is considered the industry standard in both research and on-farm settings due to its simplicity and comprehensive historical validation (Polsky and Von Keyserlingk, 2017; Islam et al., 2020). However, THI does not account for individual cow variability or additional climatic factors such as wind speed and solar radiation, which can significantly influence the accuracy of HS assessment (Dunshea et al., 2013). To address some of these limitations, composite indices such as the Grazing Heat Load Index (GHLI) have been developed (Bryant et al., 2023) and recently updated for New Zealand dairy systems (Hitchman et al., 2025). However, its wider application across various climatic and management systems has not been fully established yet.

Animal-based indicators are also used to detect HS and enhance environmental assessments. Visual evaluation of respiratory rate, panting, sweating, and reduced activity is common in commercial settings due to its simplicity and cost-effectiveness. However, these signs are subjective and generally represent moderate to severe HS rather than immediate physiological changes (Tresoldi et al., 2018; Shu et al., 2021). Reductions in milk production are often used as indirect indicators of HS; however, these changes reflect cumulative responses and are therefore inadequate for immediate detection or timely intervention (Tao et al., 2020). Research reveals that there is a lag between elevated temperatures and a decrease in milk yield

(Wildridge et al., 2018), suggesting that milk yield reflects prior heat exposure rather than current HS (Polsky and Von Keyserlingk, 2017).

Rectal temperature is considered the gold standard for measuring CBT in individual cows (Idris et al., 2021), but its invasive nature, labour intensity and susceptibility of cows to handling stress, limit its routine application on farms (Islam et al., 2021). Advancements in sensor technology, including ruminal boluses, ear tags, neck collars, and accelerometer-based systems, have enabled near-real-time monitoring of temperature and behaviour (Knight, 2020). However, high costs, infrastructure requirements and insufficient external validation remain barriers to widespread implementation, especially in pasture-based systems (Islam et al., 2020; Stygar et al., 2021). Infrared thermography provides a non-invasive method of measuring surface temperature; although its accuracy is significantly affected by environmental factors, including wind speed, humidity, and solar radiation (Church et al., 2014).

Indicators of HS at the tissue or cellular level remain underexplored in dairy cows, despite their potential to provide insights into cellular stress responses. Heat shock proteins (HSPs), especially heat shock protein 70 (HSP70), are highly conserved molecular chaperones induced by elevated temperature and other stresses (Hyder et al., 2017; Rakib et al., 2024). HSP70 promotes protein homeostasis by helping proper protein folding, stabilisation, and prevention of aggregation of denatured or misfolded proteins, thereby mitigating cellular dysfunction under HS (Mosser and Morimoto, 2004; Mayer and Bukau, 2005). In response to HS, HSP70 is upregulated and has been detected in both intracellular compartments and extracellular fluids across multiple livestock species (Gaughan et al., 2013; Lamy et al., 2017; Pathirana and Garcia, 2022). However, the potential lag-time in the reaction at cellular level, and whether the up-regulation of HSP70 due to HS can be detected and quantified through milk, remain unelucidated.

In dairy cattle, HSP70 expression is typically low under thermoneutral conditions but significantly increases during HS, often rising two- to three-fold across biological matrix and analytical methods (Li et al., 2021; Altyeb et al., 2025), particularly when CBT exceeds $\sim 38.6^{\circ}\text{C}$ (Gaughan et al., 2013). Previous studies on HSP70 in dairy cattle have focused on blood-based measurements, including plasma and peripheral blood mononuclear cells (PBMCs) (Haque et al., 2012; Bhanuprakash et al., 2016). Blood is commonly used as a reference matrix because it reflects systemic physiological status; however, sample collection is invasive,

labour-intensive, and itself a potential source of stress, limiting its practicality for routine monitoring (Lamy et al., 2017). Therefore, increasing attention has been directed towards non-invasive or minimally invasive matrices such as saliva (Rajamanickam et al., 2025) and milk (Pathirana and Garcia, 2022). Milk samples are especially attractive as a source, as they are already included in modern routine farm management practices of lactating dairy cows and do not require additional animal handling (Rakib et al., 2025). Thus, milk samples have the potential to provide an animal-friendly and practical method for HS monitoring (Pathirana and Garcia, 2022; Rakib et al., 2024).

However, some important gaps remain in the understanding and application of HSP70 for HS detection. The analytical performance of emerging in-house assay systems compared to commercially available methods has not been comprehensively assessed across various biological matrices. Before practical application, it is also essential to understand how to handle milk samples, determine acceptable storage durations, and evaluate protein stability under various conditions, as these factors may significantly influence the practicality of the method and measurement reliability. Moreover, there is insufficient data about the behaviour of HSP70 across various body fluids under different seasonal and environmental conditions. There is also a lack of comprehensive evaluations comparing molecular biomarkers with established environmental and physiological indicators of HS, particularly in terms of their relative sensitivity, temporal behaviour, and potential lag effects. In addition, short-term variability in milk HSP70 concentrations under field conditions remains unexplored, which limits the understanding of its consistency and possible applicability in field conditions. Addressing these research gaps provides the foundation for the studies conducted in this thesis, which aim to systematically evaluate the analytical performance, biological variability, and temporal behaviour of HSP70 in relation to recognised indicators of HS under practical dairy production settings.

Thesis objectives and outline

Based on the knowledge gaps outlined above, the overall aim of this research was to evaluate the potential of HSP70 as a non-invasive indicator of HS in dairy cows by investigating its analytical reliability, biological variability, and temporal behaviour in relation to established environmental and physiological indicators in pasture-based dairy production systems.

The primary hypothesis of this thesis was that HSP70, particularly when measured in milk, reflects cumulative cellular responses to HS and may provide complementary information alongside established environmental and physiological indicators, thereby offering potential as a non-invasive biomarker for HS detection and interpretation in pasture-based dairy systems.

In accordance with this aim and hypothesis, the specific objectives of this thesis were to:

1. Critically review the current understanding of HS in dairy cows, with a particular emphasis on cellular stress responses and the role of HSP70 in promoting thermotolerance.
2. Evaluate the analytical performance and reproducibility of an in-house competitive ELISA for measuring HSP70 levels in comparison with a commercial ELISA across various biological matrices.
3. Assess the influence of milk handling and storage practices on the reliability and consistency of milk HSP70 measurements.
4. Examine the biological and seasonal variations in HSP70 concentrations in milk, blood, and saliva, and assess their responsiveness to environmental heat load.
5. Compare environmental, physiological, and milk HSP70 indicators to evaluate their relative sensitivity, temporal behaviour, and practical applicability in HS detection.
6. Examine the daily variation in milk HSP70 levels under heatwave conditions and investigate threshold-based interpretation for HS detection.

This thesis includes a review of existing literature ([Chapter 2](#)), five individual studies ([Chapter 3](#), [Chapter 4](#), [Chapter 5](#), [Chapter 6](#) and [Chapter 7](#)), and a general discussion and conclusion ([Chapter 8](#)). The experimental chapters are presented as independent scientific manuscripts, each comprising an abstract, introduction, materials and methods, results, discussion, and conclusion. Any additional supporting information is included as supplementary material in the [Appendix](#).

The research presented in this thesis is part of the [Dairy UP](#) program, an industry-driven initiative led by the University of Sydney's Dairy Research Foundation and co-delivered together with the New South Wales Department of Primary Industries and Regional Development, Scibus, and Dairy Australia, with the aim of supporting innovation and sustainability in the New South Wales dairy industry.

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CHAPTER 2

Literature Review

Heat stress detection in dairy cows has traditionally relied on environmental indicators that quantify external heat load and physiological measures that reflect animal-level responses. However, these approaches offer limited insight into the underlying cellular stress processes. [Chapter 2](#) critically reviews the current understanding of HS detection in dairy cows, focusing on the biological basis and practical limitations of heat shock protein 70 (HSP70) as a molecular biomarker. By highlighting critical gaps in validation, interpretation, and non-invasive application, this chapter establishes the foundation for the subsequent experimental investigations.

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Graduate Student Literature Review: Potential use of heat shock protein 70 as an indicator of heat stress in dairy cows—A review*

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ABSTRACT

Heat stress (HS) poses significant challenges to the dairy industry, resulting in reduced milk production, impaired reproductive performance, and compromised animal welfare. Therefore, understanding the molecular mechanisms underlying cellular responses to HS is crucial for developing effective strategies to mitigate its adverse effects. Heat shock protein 70 (HSP70) has emerged as a potential player involved in cellular thermotolerance in dairy cows. This review provides a comprehensive overview of the role of HSP70 as a molecular chaperone in cellular thermotolerance in dairy cows under HS. HSP70 facilitates proper protein folding and prevents the aggregation of denatured proteins. By binding to misfolded proteins, it helps maintain protein homeostasis and prevents the accumulation of damaged proteins during HS. Additionally, HSP70 interacts with various regulatory proteins and signaling pathways, contributing to the cellular adaptive response to HS. The upregulation of HSP70 expression in response to HS is regulated by a complex network involving heat shock factors (HSF), heat shock element-binding proteins, and HSF co-chaperones. Therefore, HSP70 holds the potential to be a useful indicator of tissue stress due to its role in maintaining cellular balance and because it is released both inside and outside cells in response to stress. Traditional methods of measuring HSP70 in blood samples are labor intensive, and because the process is potentially stressful for the animals, this may subsequently affect the results. Therefore, measuring HSP expression in cow milk has shown promise as an easy, noninvasive, and accurate way to detect HS in dairy cows. Monitoring HSP70 levels in milk can be applied as a supplementary

approach to identify HS or HS resistance of individual cows, select suitable animals, and guide targeted management strategies. However, despite the potential advantages of using HSP70 as a biomarker for monitoring HS on dairy cows, challenges remain in standardizing measurement protocols, establishing species-specific reference ranges, addressing interindividual variations, and determining the specificity of changes in HSP70 due to HS. Future research should focus on developing non-invasive techniques for HSP70 detection, with consideration of climatic conditions and unraveling the molecular interactions and regulatory networks involving HSP70.

Key words: animal welfare, biomarker, ELISA, heat shock protein, milk

INTRODUCTION

Heat stress (HS) in dairy cows refers to an environment that raises the body temperature of cows due to exposure to high temperatures and humidity levels beyond their ability to dissipate heat effectively (Dunshea et al., 2013; Hyder et al., 2017). This can lead to reduced feed intake, lower milk production, and compromised reproductive performance in cows (Bernabucci et al., 2014; Polsky and Von Keyserlingk, 2017; Becker et al., 2020; Rakib et al., 2020). To cope with HS, cows attempt to regulate their body temperature through panting and sweating, leading to increased water consumption and dehydration (Islam et al., 2021). A combination of observed behavioral changes and physiological indicators are used to diagnose HS in cows, including monitoring increased respiration rates, rectal temperature, panting, drooling, and reduced activity (Tresoldi et al., 2018).

The ability of an animal to maintain homeostasis in response to thermal stress is known as thermotolerance, and it is essential for survival under these conditions. Heat shock proteins (HSP) are a class of molecular chaperones (i.e., assistants or helpers) that play a crucial role in maintaining cellular homeostasis and promoting thermotolerance in cells exposed to high temperatures (Mayer and

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

Bukau, 2005). Among the HSP family, HSP70 is the most extensively studied, as it is a widespread protein that acts as a critical regulator of protein folding, stabilization, and degradation under various environmental stresses (Mosser and Morimoto, 2004; Mayer and Bukau, 2005). The HSP70 is known to be induced in response to HS, and its expression levels have been shown to increase in various tissues of dairy cows under HS conditions (Aggarwal et al., 2012). The increased expression of HSP70 has been suggested to play a role in the cellular response to thermal stress in dairy cows, protecting proteins against damage and reducing the risk of cellular dysfunction (Gaughan et al., 2013; Hassan et al., 2019). Although the functions of HSP70 have been thoroughly examined by Hyder et al. (2017) and Archana et al. (2017), recent efforts to understand the role of HSP70 in the cellular response of cows to HS are limited. Additionally, there is a significant gap in the literature regarding methods for detecting HSP70. Several studies have investigated HSP70 detection using different sample types. Although some focused primarily on blood plasma (Aggarwal et al., 2012; Haque et al., 2012; Gaughan et al., 2013; Kumar et al., 2018; Kumar et al., 2020), others reported HSP70 detection through salivary concentration (Lamy et al., 2017). Furthermore, Pathirana and Garcia (2022) developed a competitive ELISA test for detecting HSP70 in milk samples, which presents promising avenues for further exploration. This suggests the potential for noninvasive sampling methods to monitor the presence of HS response biomarkers in cattle, which could offer practical advantages in field settings.

This review aims to present a comprehensive overview of the existing literature concerning the role of HSP70 as a molecular chaperone for cellular thermotolerance in dairy cows during HS. Heat stress and its adverse impacts on the health and productivity of dairy cattle is explored, along with the currently available methods for detecting HS. It then focuses on HSP, particularly HSP70, providing insights into the structure, function, and regulatory mechanisms of the protein. The current understanding the role of HSP70 in the cellular response to thermal stress in dairy cows is investigated, encompassing its impact on cellular protein folding, degradation, and apoptosis. Finally, the potential mechanisms through which HSP70 may enhance thermotolerance in dairy cows are reviewed. This includes its possible role in regulating cellular signaling pathways, metabolism, and immune function, along with discussing available HSP70 detection methods to highlight the possibilities for using noninvasive approaches to detect HS in dairy cattle.

HEAT STRESS IMPACT ON THE PERFORMANCE OF DAIRY COWS

Heat stress in lactating dairy cattle trigger physiological responses that leads to reduced feed intake and decreased milk production, lower reproductive efficiency, and increased susceptibility to disease, which can have significant economic consequences for the dairy industry (Becker et al., 2020; Rakib et al., 2020; Figure 1). Homeothermic animals, such as cattle, have a thermoneutral zone (TNZ), defined as a temperature range in which

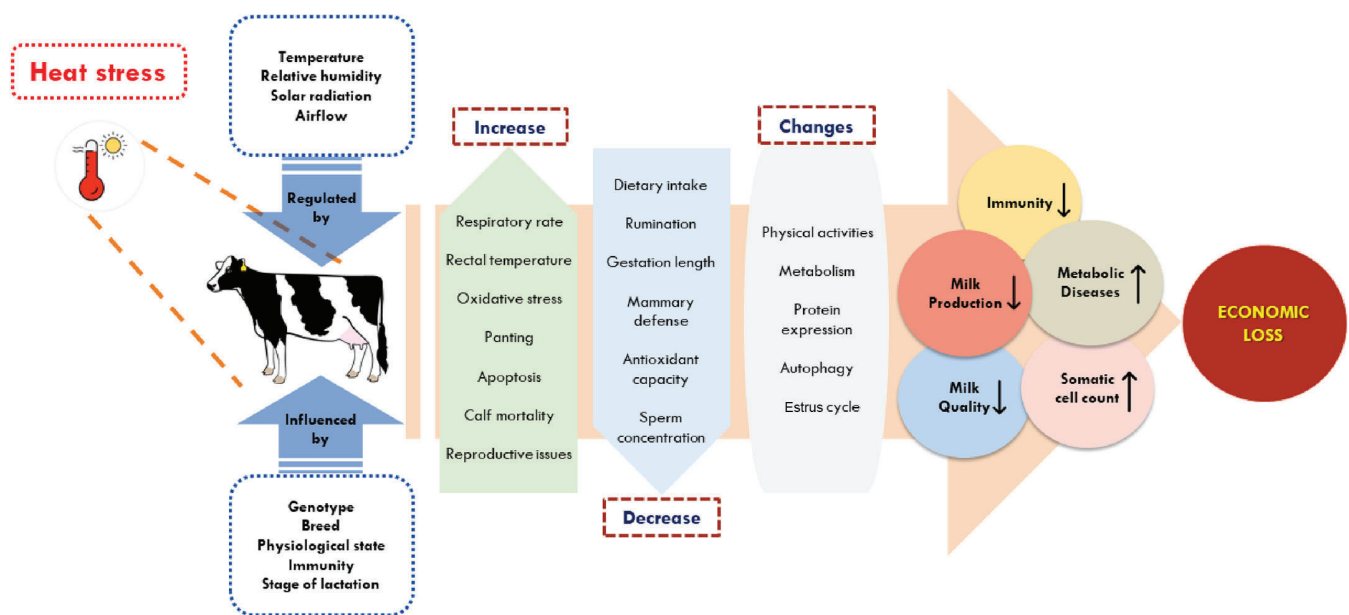


Figure 1. Diagram summarizing the impact of HS on the health and productivity of dairy cows. Expanded from Rakib et al. (2020).

they do not spend extra energy to maintain their core body temperature, allowing more energy to be diverted toward production (Hyder et al., 2017). For most dairy cattle, the TNZ is between 4°C and 25°C, with some variation based on age, species, breed, lactation stage, dietary intake and composition, housing facilities and management, temperature and humidity of the barns, preceding temperature, acclimation, productivity, and behavior of the animal (West, 2003). To regulate internal temperature, animals must balance the heat they acquire from the environment and the heat they generate through metabolism, and release excess heat to the environment (Dunshea et al., 2013). The challenge of managing HS has become more complex due to the increasing number of animals with higher genetic merit for production, greater metabolic activity, and the impact of climate change, including an increased frequency of extreme heatwaves (Polsky and Von Keyserlingk, 2017). Since the 1800s, average global temperatures have increased by 1.0°C, and they are expected to exceed pre-industrial levels by 1.5°C as early as 2030 (IPCC, 2023). Nidumolu et al. (2014) reported that in Southern Australia, the average duration of HS events doubled from 2 to 4 consecutive days between 1960 and 2008, where a HS event refers to a period of sustained high temperatures that exceed the threshold for what is considered normal or comfortable for the region.

Dairy cows are highly sensitive to temperature and humidity changes, making them susceptible to HS (Polsky and Von Keyserlingk, 2017). The diurnal temperature range is crucial for preventing HS by enabling animals to cool down during the lower nighttime temperatures. This natural cooling cycle effectively regulates body temperature, reducing the risk of HS (Veissier et al., 2017).

In intensive indoor or contained housing systems, optimal indoor climate conditions, such as temperature, relative humidity, light, and ventilation, can be controlled more easily than in pasture-based systems to ensure the wellbeing of the dairy cattle (Veissier et al., 2017). Farmers can implement various strategies to increase cow comfort, including decreased animal density in the barn, active cooling with sprinklers, air movement via fan and proper ventilation, dietary changes, feed supplementation, and adjusting feeding schedules. These strategies can help mitigate the impact of HS and ensure the wellbeing and productivity of their cows during periods of hot weather (Rakib et al., 2020).

In Australia, most dairy cows graze pasture throughout the year and receive low to moderate levels of concentrate and supplements (Garcia et al., 2013). Under typical seasonal conditions, Dairy Australia (2022) reports that ~60% to 65% of cattle feed requirements are fulfilled through grazing. Dairy cows fed with pasture are also influenced by the effect of the temperature-humidity in-

dex (THI) which is considered the most appropriate and straightforward parameter for assessing environmental HS in dairy cattle (Polsky and Von Keyserlingk, 2017). Although THI serves as a reliable indicator in indoor or barn settings, its effectiveness diminishes when applied to cows grazing in pastures. A study conducted by Bryant et al. (2023) in the Waikato region of New Zealand developed a grazing heat load index (HLI) incorporating ambient temperature, solar radiation, and wind speed to predict respiration rates in extensively grazed dairy cattle. The study demonstrated increased accuracy compared with existing indices such as THI, with observations indicating that a grazing HLI exceeding 70 may indicate compromised welfare due to HS, although the respiration rate begins to steeply increase before this threshold. However, Wildridge et al. (2018) reported a significant correlation between THI and both milk yield and milking frequency in dairy cows within pasture-based automatic milking systems. Dairy cows with high milk yields experience a reduction in milk production when the THI reaches approximately 68 (Collier et al., 2012). A significant correlation was reported between the THI and the physiological responses of Australian dairy cows during summer, with notable increases observed in respiratory rate (66.7, 84.7, and 109.1 breaths per minute), panting scores (1.4, 1.9, and 2.3), and average body temperatures (38.4, 39.4, and 41.5 °C) as THI levels increased from low (≤ 72) to moderate (73–82) to high (≥ 83) levels, respectively (Osei-Amponsah et al., 2020). Moreover, during periods of moderate and high THI, the cows tended to seek shade, spend more time around watering points, and exhibit signs of distress, such as excessive salivation and open-mouth panting (Wildridge et al., 2018; Osei-Amponsah et al., 2020). Although THI is widely used, it has limitations because it only considers air temperature and humidity, ignoring other crucial factors such as wind speed and solar radiation that are important for assessing environmental conditions (Dunshea et al., 2013). Additionally, THI lacks animal-specific parameters, and the threshold for cow HS varies depending on the specific THI calculation used.

Heat stress also affects the cow's appetite, increasing metabolic maintenance requirements by 7% to 25% and resulting in prolonged negative effects on milk yield, composition, and quality (Bernabucci et al., 2014). Decreases in daily milk production (14%) and increases in milk temperature (3%), fat percentage (3%), and protein content (2%) were correlated with increases in THI observed by Osei-Amponsah et al. (2020). Dairy farms equipped with efficient cooling systems and located in temperate regions may encounter about 10% to 15% decline in milk production during HS, characterized by prolonged periods of elevated temperatures surpassing

the region's typical comfort threshold. Conversely, dairy operations lacking cooling infrastructure or situated in regions prone to severe heatwaves may face a more substantial reduction of 40% to 50% in milk yield (Dunshea et al., 2013).

Acclimation of the animal plays an important role in alleviating the effects of HS (Becker et al., 2020). In temperate climates, dairy cattle may exhibit lower levels of heat acclimation compared with cows in tropical, subtropical, and Mediterranean climates, as these latter regions often experience prolonged periods of HS, which can hinder the recovery of cattle from its detrimental effects (Becker et al., 2020). Additionally, when animals experience short bursts of HS, production is negatively affected for a recovery period of ~5 d (Ominski et al., 2002). Although HS-related performance decline is typically associated with summer, adverse effects can persist into the autumn months, even if cows are no longer exposed to HS (De Rensis and Scaramuzzi, 2003).

In addition to reduced productivity, HS in livestock has negative effects on reproductive efficiency and disease susceptibility. According to Becker et al. (2020), it can have a detrimental impact on multiple aspects of livestock reproductive physiology, such as changes in estrus duration, uterine function, endocrine status, follicular growth and development, luteolytic mechanisms, early embryonic development and survival, fetal growth, and colostrum quality. Additionally, it reduces conception rates, dropping below 35% during periods of HS (De Rensis and Scaramuzzi, 2003). Heat stress especially in summer, also has adverse effects on the bulk tank SCC (BTSCC) and the incidence of clinical mastitis in dairy herds (Rakib et al., 2020). According to Nasr and El-Tarabany (2017), there is a positive linear relationship between HS and BTSCC, with BTSCC increasing up to 36% as the THI increases from low (≤ 70) to moderate (70–80) to high (80–85) levels and with the advancement of the cow's parity.

Although dry cows have lower feed requirements (do Amaral et al., 2011) and generate less metabolic heat than lactating cows, HS can still lead to adverse effects, including increased rectal temperature and respiration rate (West, 2003). In addition to these effects, HS can affect dry cows' immune function, particularly when cooling measures are absent, leading to a reduction in lymphocyte proliferation (do Amaral et al., 2011). According to Ferreira et al. (2016), if dry cows are not provided with cooling measures, HS results in annual economic losses of over \$800 million in the US dairy industry. The losses are expected to rise in the coming years due to ongoing global climate change.

DETECTION OF HS IN DAIRY COWS

Detection and prevention of HS is critical for the overall welfare of dairy cows and the economic viability of dairy farming. Several methods have been developed to detect and quantify HS in dairy cows in recent years, ranging from physiological and behavioral measurements to advanced technologies such as remote sensing and machine learning (Becker et al., 2021). In this context, understanding the principles and applications of HS detection methods is essential for dairy farmers and industry professionals to effectively detect, mitigate, and manage the adverse effects of HS and ensure sustainable dairy production.

Several parameters are currently being used to determine HS in dairy cattle. Among these, THI is widely considered as the most suitable and straightforward indicator for evaluating environmental HS in this context (Polsky and Von Keyserlingk, 2017). Researchers have used diverse THI formulas, depending on their unique assessments of humidity and temperature (Table 1). However, THI has limitations, as it only considers air temperature and relative humidity, neglecting factors such as wind speed or solar radiation, which are useful indicators to

Table 1. Summary of frequently used THI formulas for dairy cattle¹

THI equation	Reference
$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$	NRC (1971)
$THI = T_{db} + 0.36 \times T_{dp} + 41.2$	Yousef (1985)
$THI = (0.35 \times T_{db} + 0.65 \times T_{wb}) \times 1.8 + 32$	Bianca (1962)
$THI = (0.55 \times T_{db} + 0.2 \times T_{dp}) \times 1.8 + 32 + 17.5$	NRC (1971)
$THI = (0.15 \times T_{db} + 0.85 \times T_{wb}) \times 1.8 + 32$	Bianca (1962)
$THI = [0.4 \times (T_{db} + T_{wb})] \times 1.8 + 32 + 15$	Thom (1959)
$THI = (T_{db} + T_{wb}) \times 0.72 + 40.6$	NRC (1971)
$THI = (0.8 \times T_{db}) + [(RH/100) \times (T_{db} - 14.4)] + 46.4$	Mader et al. (2006)
$THI = 1.8T_{db} - (1 - RH)(T_{db} - 14.3) + 32$	Kibler (1964)
$THI = 0.8 \times T_{db} + (T_{db} - 14.4) \times RH + 46.4$	Johnson (1965)
$THI = T_{db} - [(0.31 - 0.31 RH)(T_{db} - 14.4)]$	Marai et al. (2001)

¹THI = temperature-humidity index; T_{db} = dry-bulb temperature; T_{wb} = wet bulb temperature; T_{dp} = dew point temperature; RH = relative humidity.

understand environmental conditions (Dunshea et al., 2013). Additionally, THI does not include any animal-specific parameter. Therefore, dairy cows with high milk yields experience a reduction in milk production when the THI reaches approximately 68 (Collier et al., 2012). However, it is important to note that the value at which cows experience HS depends on the specific THI calculation method used, as different formulas and approaches exist for calculating THI (Table 1).

When exposed to HS, animals exhibit various physiological and behavioral changes that can serve as indicators, including increased respiration rate, panting, open-mouth breathing, and standing time, as well as elevated body temperature (Tresoldi et al., 2018). Alterations in their behavior are also evident, such as reduced eating, rumination, and lying down, as well as an increase in drinking and seeking shade (Islam et al., 2021).

Measuring rectal temperature (**RT**) has been a widely accepted method for monitoring core body temperature (**CBT**) in animals. However, one of the challenges of using RT is the interference of defecation, which can affect the accuracy and reliability of RT measurements (Islam et al., 2020; Islam et al., 2021). As a result, alternative methods for measuring CBT in animals have been explored, including noninvasive techniques such as thermal imaging and implantable devices that can provide continuous temperature monitoring without the need for manual intervention.

Wearable sensor technology for individual-level HS monitoring has recently gained substantial popularity in the dairy industry, presenting a methodological advancement. This technology offers a promising approach to address HS management at the individual animal level, leading to improved animal welfare, increased productivity, and reduced heat management costs (Islam et al., 2020; Becker et al., 2021). Ongoing research focuses on evaluating various remote and automated monitoring techniques, some of which have already been validated for monitoring cattle behavior and health concerning HS. A study conducted by Islam et al. (2020) examined Australian feedlot cattle and employed ear tag-based sensors to monitor panting and individual variability of HS-related behaviors. The findings revealed that heat-susceptible cattle exhibited higher levels of panting and eating behavior and reduced resting time, particularly during hotter periods of the day. These sensors have also proven effective in detecting variations in panting behavior associated with the breed, coat color, and individual animals. However, Stygar et al. (2021) reported that among currently available commercial sensors, only 18 (14%) have been externally validated, with accelerometers demonstrating the highest validation rate (30%) and other sensor types having lower rates. These authors also highlighted the limited potential of existing sensors to

evaluate appropriate behavior in dairy cows, underscoring the importance of future validation research, particularly in commercial herds.

On the other hand, relatively less research has focused on cellular-level indicators of HS, especially the relationship between extracellular expression of HSP70 and HS. Hassan et al. (2019) reported that HSP, particularly HSP70 in bovines, provided a direct and quantitative measurement to assess the cellular stress response with enhanced sensitivity and precision. This underscores its potential as a biomarker, offering a supplementary method for identifying HS or HS resistance in dairy cows. The measurement of HSP70 can also enhance predictive models when combined with other physiological and behavioral variables, leading to more precise predictions of HS. Additionally, elevated levels of HSP70 have been linked to HS in cattle across various sampling methods, including blood, skin (dermal fibroblasts), mammary epithelial cells, milk, and saliva (Gaughan et al., 2013; Lamy et al., 2017; Pathirana and Garcia, 2022).

TYPES OF HSP ASSOCIATED WITH LIVESTOCK DURING HS

Heat shock proteins are classified based on their molecular weight and biological functions, and the different types include HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, HSP27, and HSP10. Among these, HSP110, HSP70, HSP90, HSP60, and HSP27 are significantly linked to thermotolerance in livestock species (Fujimoto and Nakai, 2010; Belhadj Slimen et al., 2016). These proteins are critical for maintaining cellular homeostasis and protecting cells from HS, making them essential for coping with HS in animal agriculture. HSP70 and HSP90 have been identified in several studies as important proteins associated with the development of thermotolerance in various farm animals, such as cattle, buffalo, sheep, goats, and broilers (Belhadj Slimen et al., 2016).

Cells detect heat and respond by increasing the expression of HSP through various mechanisms. Heat stress can lead to protein denaturation and misfolding, which triggers the activation of heat shock factors (**HSF**). Activated HSF then promote the transcription of HSP genes, aiding in the refolding of damaged proteins (Fujimoto and Nakai, 2010). Although not all cells have specialized thermal receptors, these mechanisms enable a wide range of cells to sense and respond to HS effectively, thereby protecting themselves from thermal damage (Doberentz and Madea, 2018).

The heat shock response is primarily regulated transcriptionally by 4 HSF (HSF1, HSF2, HSF3, and HSF4), which bind to heat shock elements (**HSE**) in DNA to increase the expression of HSP (Fujimoto and Nakai, 2010). The HSF1 is mainly associated with HSP70 induction

and directly correlates with livestock thermotolerance (Archana et al., 2017). The HSF2 acts as an inducible regulator for chaperones, ensuring the continued expression of misfolded proteins marked for degradation. Multiple HSF coordinate to protect unfolded proteins (Fujimoto and Nakai, 2010). During HS, HSF1 monomers dissociate and trimerize before nuclear translocation, increasing HSP mRNA expression (Collier et al., 2008). However, HSP expression requires a threshold temperature, which varies among livestock species in different environmental zones (including temperature fluctuations and the availability of resources such as food, water, and shelter) with distinct HSP expression patterns. According to Armstrong (1994) high-producing dairy cows are adversely affected when the THI exceeds 72. Additionally, Armstrong and Janni (2023) reported that dairy cattle begin to experience HS when the ambient temperature reaches ~22°C (72°F) with 50% humidity, which leads to the activation of HSF and the synthesis of HSP, the protective measure against HS. When denatured proteins accumulate in a cell due to heat or other stressors, HSP interact with them and prevent the formation of cytotoxic protein aggregates, thus maintaining this protein homeostasis of the cell (Mayer and Bukau, 2005).

In addition to their chaperone function, HSP also play a role in signaling to the immune system to encourage the increased killing of pathogenic bacteria by neutrophils, macrophages, and other innate immune cells (Yokota and Fujii, 2010). Moreover, HSP confer thermotolerance, enhancing a cell's ability to survive injury and oxidative stress induced by high temperatures (Wang et al., 2015). Overall, these highly conserved HSP are activated by heat and other stressors and act as molecular chaperones critical for cellular survival and protection under stress conditions.

HSP70

HSP70 represents ~2% to 15% of the total cellular proteins expressed by all living organisms (Hyder et al., 2017). This protein is encoded by 2 genes, *HSPA1A* and *HSPA1B*, which are inducible by various stressors, including heat shock, oxidative stress, and inflammation (Mayer and Bukau, 2005; Aggarwal et al., 2012). It is involved in numerous biological processes, including protein folding and trafficking, DNA repair, signal transduction, antigen processing and presentation, and apoptosis (Bagath et al., 2019). Moreover, HSP70 protects newly synthesized polypeptides by ensuring proper folding and packaging (Hassan et al., 2019). By binding to and stabilizing denatured proteins, HSP70 prevents their aggregation and facilitates their refolding or degradation, thereby maintaining cellular proteostasis and avoiding the accumulation of misfolded proteins that can lead to

cellular dysfunction and disease (Mosser and Morimoto, 2004; Mayer and Bukau, 2005).

In addition to its role in protein homeostasis, HSP70 has been implicated in several pathophysiological conditions, including cancer, neurodegenerative disorders, and infectious diseases. In cancer, HSP70 has been shown to promote tumor progression and resistance to chemotherapy by inhibiting apoptosis and promoting cell survival (Muchowski and Wacker, 2005). In neurodegenerative disorders, HSP70 has been shown to protect neurons from protein misfolding and aggregation, which are hallmark features of diseases such as Alzheimer and Parkinson (Sweeney et al., 2017). In infectious diseases, HSP70 is involved in the host immune response by facilitating antigen presentation, promoting the activity of immune cells, helping to transport antigenic peptides from the cytosol to the endoplasmic reticulum, and stimulating dendritic cell proliferation (Bagath et al., 2019). Heat shock proteins are released by damaged cells and activate dendritic cells to induce an inflammatory response, boosting innate immunity, and they are also involved in the degradation of ineffective or irreparable proteins (Yokota and Fujii, 2010). Overall, HSP70 is an essential protein with multiple functions in maintaining cellular homeostasis, and its dysregulation has been linked to various disease states (Mayer and Bukau, 2005). The following sections provide a more detailed discussion of the crucial functions of HSP70 in thermotolerance, cellular apoptosis, and immunoregulation.

Cellular Thermotolerance Associated with HSP70

Cellular thermotolerance is a phenomenon in which cells are able to survive exposure to higher-than-normal temperatures after being exposed to a short period of HS (Wang et al., 2015). This response is often mediated by HSP, particularly HSP70, which helps to prevent protein denaturation and aggregation under stress conditions (Mosser and Morimoto, 2004; Mayer and Bukau, 2005). In a study conducted in India, Bhat et al. (2016) focused on the HSP70 gene in Tharparkar cattle, aiming to identify polymorphisms and their potential impact on thermotolerance. They reported that HS can impair normal cellular functions, including material transport, DNA replication, transcription, and polypeptide synthesis. The study suggested that polymorphisms in the HSP70 gene could be a significant factor in determining heat tolerance in cattle, which could be beneficial for selecting thermotolerant cattle.

The induction of HSP70 is regulated by a transcription factor called HSF1, usually present in an inactive form in the cytoplasm, but upon HS, it translocates to the nucleus, where it binds to the HSE in the promoter region of the HSP70 gene, leading to the induction of

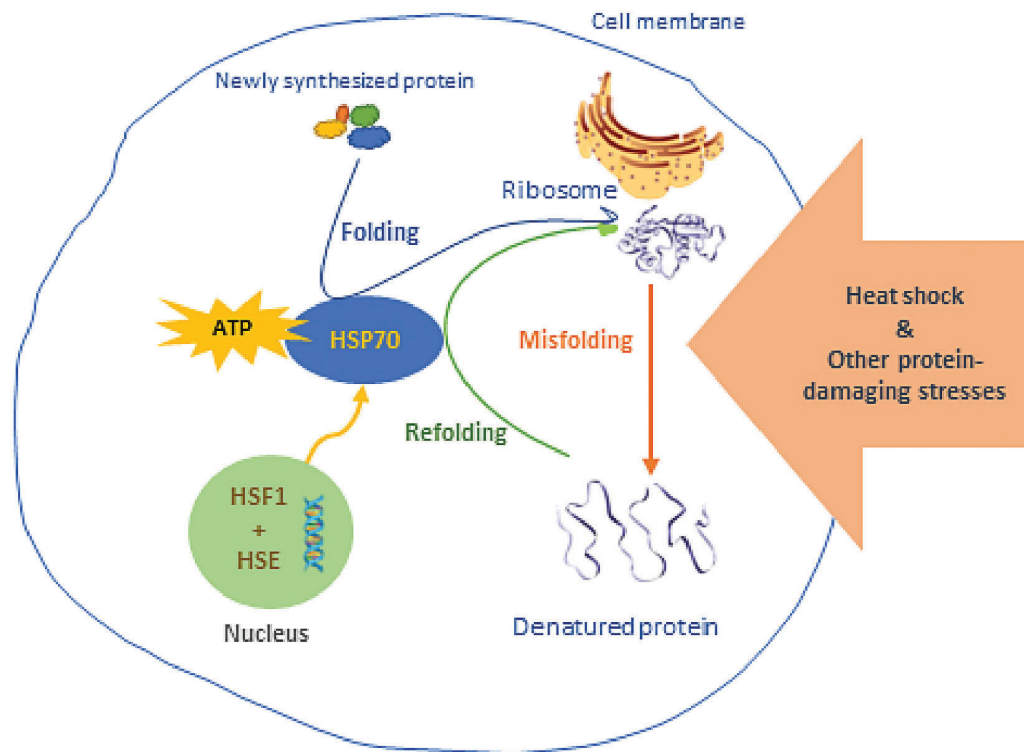


Figure 2. A diagrammatic representation of HSP70-assisted protein folding within an epithelial cell. HSP70 is required for the productive folding of newly synthesized proteins and their translocation across the intracellular membranes, for example, endoplasmic reticulum, mitochondria, and lysosomes. When exposed to heat shock and other protein-damaging stresses, proteins are prone to misfolding and aggregation. The presence of HSP70 limits protein misfolding and aggregation. HSP70 can rescue misfolded proteins through its refolding activity, ensuring that protein homeostasis is maintained within the cell.

HSP70 expression (Fujimoto and Nakai, 2010). According to Alberts et al. (2002), this protein attaches to newly forming peptide chains on ribosomes, safeguarding the hydrophobic surface that would otherwise be revealed to the solvent. This action averts abnormal folding or clumping until the entire peptide chain is created and appropriate folding can occur. However, once HSP70 is produced, it binds to denatured proteins within the cell to prevent them from aggregating (Figure 2). This binding occurs through the ATPase domain of HSP70, which binds to exposed hydrophobic residues on the denatured protein. HSP70 then uses the energy from ATP hydrolysis to facilitate the refolding of the denatured protein into its correct conformation (Mosser and Morimoto, 2004; Mayer and Bukau, 2005). HSP70 and related proteins have a strong ATP-binding capacity that accelerates polypeptide attachment and release. Although dimer and oligomer formation have been proposed, the monomeric protein is considered the functional chaperone (Mayer and Bukau, 2005).

The HSP70-assisted folding process involves substrate binding and release cycles, accelerated by ATP hydrolysis and regulated by co-chaperones such as HSP40, Hip

(Huntingtin interacting protein), Bag (B-cell leukemia/lymphoma-2-associated athanogene), and Hop (Hsp70-Hsp90 organizing protein; Mosser and Morimoto, 2004; Mayer and Bukau, 2005). HSP40, specifically human DnaJ homolog (Hdj)-1/Hdj-2, improves HSP70 chaperone activity by binding to its C-terminus, increasing ATPase activity, and stabilizing the substrate-bound form. Hop and carboxyl terminus of heat shock cognate 71 kDa-interacting protein (CHIP) are other co-chaperones that also bind to the C-terminus of HSP70, contributing to specificity in chaperone interactions. Co-chaperone involvement can be substrate-specific and essential for particular tasks, although their levels typically remain lower than those of HSP70 or HSP90, even under cellular stress (Mosser and Morimoto, 2004; Fujimoto and Nakai, 2010).

Furthermore, Wang et al. (2015) showed that triggering the production of HSP70 is vital for cellular thermotolerance and safeguarding cells against different types of stress, such as myocardial ischemia and oxidative stress. Cellular thermotolerance associated with HSP70 represents an important cellular response that helps to protect cells from damage and maintain cellular function

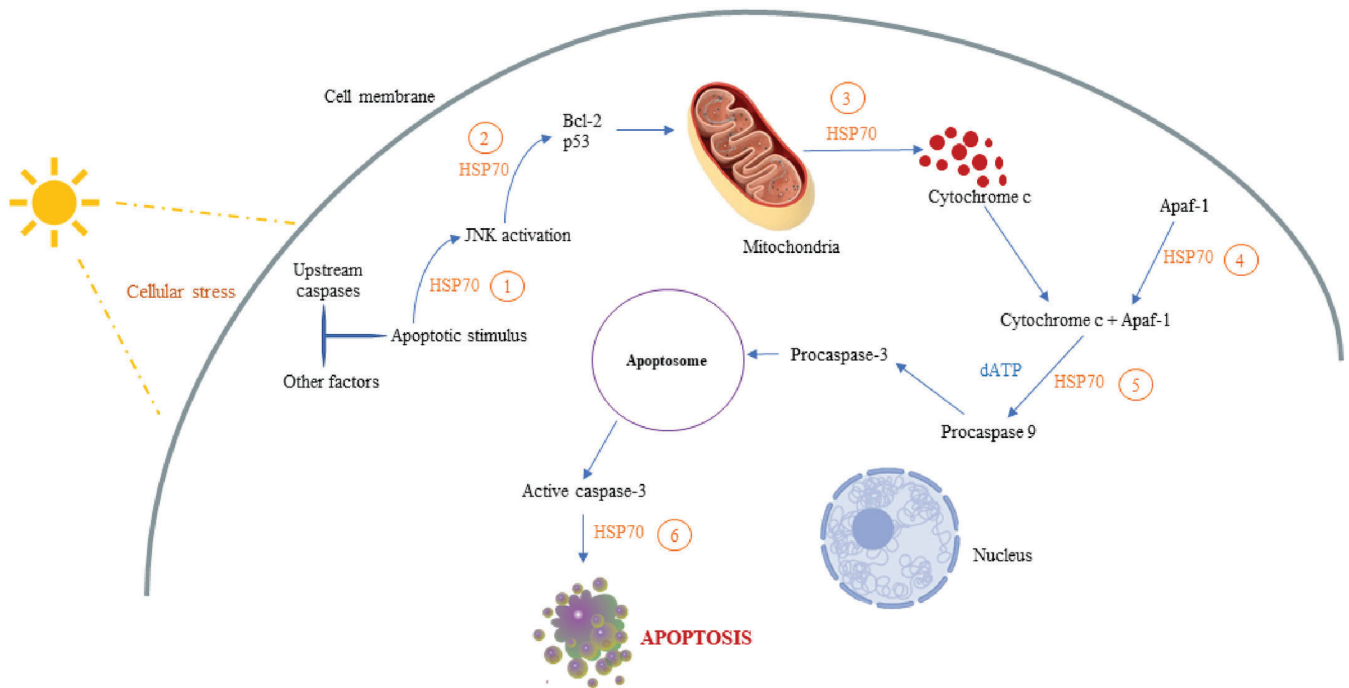


Figure 3. A diagrammatic representation of HSP70-mediated intervention in the apoptotic pathway within an epithelial cell. (1) After apoptotic stimulation, HSP70 immediately inhibits JNK activation. (2) HSP70 interacts with antiapoptotic Bcl-2 and p53 proteins to promote cell survival. (3) HSP70 blocks cytochrome c release from mitochondria. (4) HSP70 inhibits Apaf-1 oligomerization to suppress the caspase activity. (5) HSP70 prevents the recruitment of procaspase-9 to the apoptosome complex in presence of cytochrome c/dATP. (6) Finally, HSP70 interacts with active caspase-3 to stop the apoptosis. Expanded from Beere and Green (2001).

under stress conditions. The identification of HSP70 as a critical mediator of this response has significant implications for the development of therapeutic strategies aimed at mitigating the harmful effects of stress on cells.

Role of HSP70 in Cellular Apoptosis

In addition to its involvement in various cellular processes such as protein folding, transport, and degradation, HSP70 also plays a role in the regulation of apoptosis, which is the process of programmed cell death (Beere et al., 2000). Apoptosis (Figure 3) is critical for maintaining tissue homeostasis and removing damaged or abnormal cells. Dysregulation of apoptosis can lead to many diseases, including cancer (Muchowski and Wacker, 2005). Apoptosis commonly involves the release of mitochondrial proteins such as cytochrome c and apoptosis-inducing factor from the intermembrane space into the cytosol of stressed cells, where cytochrome c interacts with apoptotic protease activation factor-1 (Apaf-1) to form the apoptosome complex, in turn activating caspase-9 and initiating a caspase cascade that leads to apoptosis (Schmitt et al., 2006). HSP70 has been shown to regulate apoptosis by interacting with a variety of proteins involved in the apoptotic pathway, such as

caspases, B-cell lymphoma 2 (Bcl-2) family proteins, and tumor suppressor protein (p53; Beere et al., 2000). Moreover, HSP can modify various signaling cascades, such as JNK, NF- κ B, and AKT, which regulate important components in the apoptotic pathway. Thus, HSP can affect multiple steps in the pathway and help cells survive after being exposed to harmful stimuli (Beere, 2004).

Furthermore, HSP70 can prevent apoptosis by inhibiting the activation of caspases, which are proteases that cleave many cellular proteins, leading to cell destruction, which can also inhibit the release of cytochrome c from the mitochondria, which is an essential step in the apoptotic pathway (Beere and Green, 2001). Beere et al. (2000) found that HSP70 requires an active C-terminal peptide binding domain to inhibit caspase activity, and in vitro studies showed that HSP70 can inhibit cytochrome c-mediated procaspase-9 processing (Mosser and Morimoto, 2004). HSP70 can also directly interact with Apaf-1 to suppress caspase activity and prevent the recruitment of procaspase-9 to the apoptosome complex (Beere and Green, 2001; Schmitt et al., 2006). However, Beere et al. (2000) observed that HSP70 promotes the self-association of Apaf-1 in the presence of cytochrome c/dATP, which prevents the association of procaspase-9 and procaspase-3 with the partially assembled Apaf-1

complex. HSP70 can also interrupt the apoptotic pathway by inhibiting JNK activation, blocking cytochrome c release, binding to cytochrome c to prevent apoptosome formation, suppressing Apaf-1 oligomerization, and inhibiting procaspase enrolment (Beere and Green, 2001). Additionally, HSP70 can interact with and stabilize antiapoptotic Bcl-2 family proteins, preventing them from degrading and promoting cell survival. Although the precise mechanism of its antiapoptotic effects is still unclear, HSP70 has been shown to protect cells from apoptotic stimuli such as UV radiation, DNA damage, certain chemotherapeutic agents, and serum extraction (Hassan et al., 2019).

HSP70 plays a complex role in regulating apoptosis depending on the context (Beere, 2004). Further studies are needed to fully understand the mechanisms by which HSP70 regulates apoptosis and its potential as a therapeutic target for diseases involving dysregulated apoptosis.

Immunoregulatory Role of HSP70

HSP70 has been shown to have critical immunoregulatory roles in both innate and adaptive immune responses. One of the key roles of HSP70 in the immune system is its ability to act as a danger signal, or danger-associated molecular pattern (**DAMP**), that alerts the immune system to the presence of damaged or stressed cells (Yokota and Fujii, 2010). HSP70 can be detected by pattern recognition receptors on the surface of immune cells, activating innate immune responses such as proinflammatory cytokines and chemokines production and promote the recruitment of immune cells to the site of injury or infection (Asea et al., 2000; Yokota and Fujii, 2010). Studies have shown that extracellular HSP70 can activate toll-like receptor 4 (TLR4) and toll-like receptor 2 (TLR2) on immune cells, producing proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), IL-6, and IL-1 β . These proinflammatory cytokines can recruit immune cells such as neutrophils and monocytes to the site of injury or infection and promote tissue repair (Asea et al., 2000). On the other hand, (Martine et al., 2019) explored the regulatory function of HSP70 in NOD-leucine rich repeat and pyrin containing protein 3 (NLRP3) inflammasome activation and found that deficient HSP70 led to the exacerbation of NLRP3-mediated peritonitis in mice, whereas increased expression of HSP70 reduced caspase-1 activation and IL-1 β production, indicating the potential of treating inflammatory disorders through HSP70 induction or thermal stress.

HSP70 also plays a role in the adaptive immune response by acting as a chaperone for antigenic peptides. It can bind to and stabilize antigenic peptides, promoting

their uptake by antigen-presenting cells (**APC**) such as dendritic cells. This enhances the presentation of these antigens to T cells, leading to the activation and proliferation of antigen-specific T cells (Tsan and Gao, 2009). According to Kim et al. (2013), HSP70 can enhance the antigen-presenting function of dendritic cells by promoting the cross-presentation of exogenous antigens. HSP70 can also promote the presentation of endogenous antigens by stabilizing and delivering them to the major histocompatibility complex class I molecules on the surface of APC (Murshid et al., 2012). Additionally, HSP70 has been shown to enhance the immunogenicity of tumor antigens, leading to increased tumor-specific T-cell responses (Feng et al., 2003).

HSP70 has been found to play a role not only in innate and adaptive immune responses but also in the development of regulatory T cells, which are essential in suppressing immune responses and preventing autoimmune diseases. HSP70 can help in the growth and differentiation of regulatory T cells by triggering the expression of forkhead box protein P3 (Foxp3), a transcription factor critical to their development and function (Brenu et al., 2013). Moreover, HSP70 can stimulate the expression of indoleamine 2,3-dioxygenase, an enzyme that suppresses T-cell activation and promotes regulatory T-cell development (Munn and Mellor, 2007).

The multifaceted role of HSP70 in the immune system, including its ability to act as a DAMP, regulate immune responses, and induce the development of regulatory T cells, highlights its importance and potential as a target for immunotherapy.

HSP70 as a Biomarker for HS

There has been significant interest in developing reliable biomarkers for HS detection. One such biomarker is HSP70, which has been previously studied and proposed as an indicator of HS in various livestock species, including dairy cattle, pigs, and poultry (Gaughan et al., 2013; Hyder et al., 2017). Several authors have reported the detection of HSP70 in bovine plasma, serum, saliva, and milk, employing different types of ELISA methods (Table 2).

Although elevated blood and salivary HSP70 concentrations have been linked to HS in cattle (Haque et al., 2012; Gaughan et al., 2013; Lamy et al., 2017), the sampling procedures for these methods are invasive, which can induce stress in the animals (Lamy et al., 2017; Pathirana and Garcia, 2022). Noninvasive methods for HS detection in dairy cows are therefore needed. Pathirana and Garcia (2022) addressed this need by developing a competitive ELISA test specifically designed to detect HSP70 in foremilk samples from Holstein-Friesian cows under different weather conditions and milk yields. The

Table 2. Summary of detection methods of HSP70 in blood, saliva, tissue, or milk applying the ELISA method

Reference	Species or breed	Sample type	Detection method	HSP70 (ng/mL) detection range
Kumar et al. (2018)	Lactating Haryana and Sahiwal cows	Blood plasma	Commercial ELISA kit	20.0–120.0
Kumar et al. (2020)	Lactating Haryana cows	Blood plasma	Commercial ELISA kit	112.0–138.0
Haque et al. (2012)	Murrah buffaloes	Blood plasma	Modified ELISA assay	26.20–63.43
Gaughan et al. (2013)	Black Angus steers	Blood plasma	Modified ELISA assay	2.33–8.08
Lamy et al. (2017)	Holstein-Friesian cows	Saliva	Commercial ELISA kit	2.27–9.02
Pathirana and Garcia (2022)	Holstein-Friesian cows	Skim milk	Competitive ELISA	536.88–1,532.04
Bhanuprakash et al. (2016)	Sahiwal cattle	Peripheral blood-mononuclear cell (PBMC)	Indirect ELISA assay	0–3.50
Bhanuprakash et al. (2016)	Sahiwal and Holstein crossbreed cattle	PBMC	Indirect ELISA assay	0–2.55
Aggarwal et al. (2012)	Crossbred Karan Fries cows	Blood plasma	Modified ELISA assay	54.31–90.26

authors reported that milk samples showed higher levels of HSP70 during hot weather and in cows with both low and high yields, compared with milder weather conditions. These findings highlight the potential of detecting HSP70 in milk as a noninvasive method for identifying HS in dairy cows. Additionally, because cows are milked 1 to 3 times a day depending on the system, using milk as a diagnostic tool would be particularly advantageous. By demonstrating a correlation between HSP70 levels and environmental conditions, as well as production status, Pathirana and Garcia (2022) have laid the groundwork for utilizing milk-based assays as a practical tool for monitoring HS in dairy herds. This approach offers a promising avenue for enhancing HS management strategies and subsequently improving the welfare and productivity of dairy cattle.

In this regard, utilizing HSP70 as a biomarker for HS in livestock could offer several advantages. It enables direct and quantitative measurement of the cellular stress response with enhanced sensitivity and accuracy (Hassan et al., 2019). Monitoring HSP70 levels can also improve the assessment of individual animal susceptibility to HS as a supplementary method. Additionally, HSP70 measurement can be used in models with other physiological and behavioral variables to predict HS more accurately. This could be used to phenotype tolerant animals, applied for genetic selection, and used develop targeted management strategies.

However, challenges such as the time lag between HS onset and cellular expression of HSP70, the standardization of measurement protocols, the behavior of proteins under varying temperature and time intervals, the establishment of species-specific reference ranges, and the need to address interindividual variations must be overcome for widespread application in livestock management. Despite these challenges, the use of HSP70 as a biomarker represents a promising avenue for improving HS management strategies and enhancing the welfare and productivity of dairy cattle.

ALTERNATIVE INFLUENCES ON HSP70 EXPRESSION

The expression of HSP70 is also influenced by several factors other than HS as explored in this review (Figure 4). Understanding these factors is essential for unraveling the protein's diverse functions and therapeutic potential. The interconnected nature of these factors highlights the complexity of HSP70 regulation and its significance in cellular homeostasis. Environmental stressors, such as oxidative stress, heavy metals, radiation, toxins, infectious agents (including viruses, bacteria, and fungi), and exercise trigger the expression of HSP70 as a protective response against cellular damage, which involves specific signaling pathways and transcription factors such as HSF (Voellmy, 2004). Livestock species and breeds also exhibit variations in heat tolerance, which are influenced by differences in heat-regulating mechanisms and HSP concentrations (Gaughan et al., 2013). Genetic factors, particularly variations in the HSP70 gene promoter and regulatory regions, contribute to diverse HSP70 expression among dairy cows. Polymorphisms affecting transcription factor binding affinity can also alter the expression levels (Kampinga and Craig, 2010).

Age-related decline in HSP70 expression increases susceptibility to stress and diseases, though the underlying mechanisms require further investigation (Calderwood et al., 2009). Additionally, various hormones (such as cortisol), proinflammatory cytokines (such as IL-1 and TNF- α), and certain drugs (including proteasome inhibitors) can influence HSP70 expression through distinct mechanisms (Mosser and Morimoto, 2004). Alternatively, basal expression levels and the capacity to upregulate HSP70 in response to stress can differ based on the cellular and tissue context, and it is crucial to understand these factors for elucidating the differential regulation of HSP70 expression (Kampinga and Craig, 2010). Furthermore, nutritional factors significantly influence HSP70 expression and heat tolerance in dairy cows, with anti-

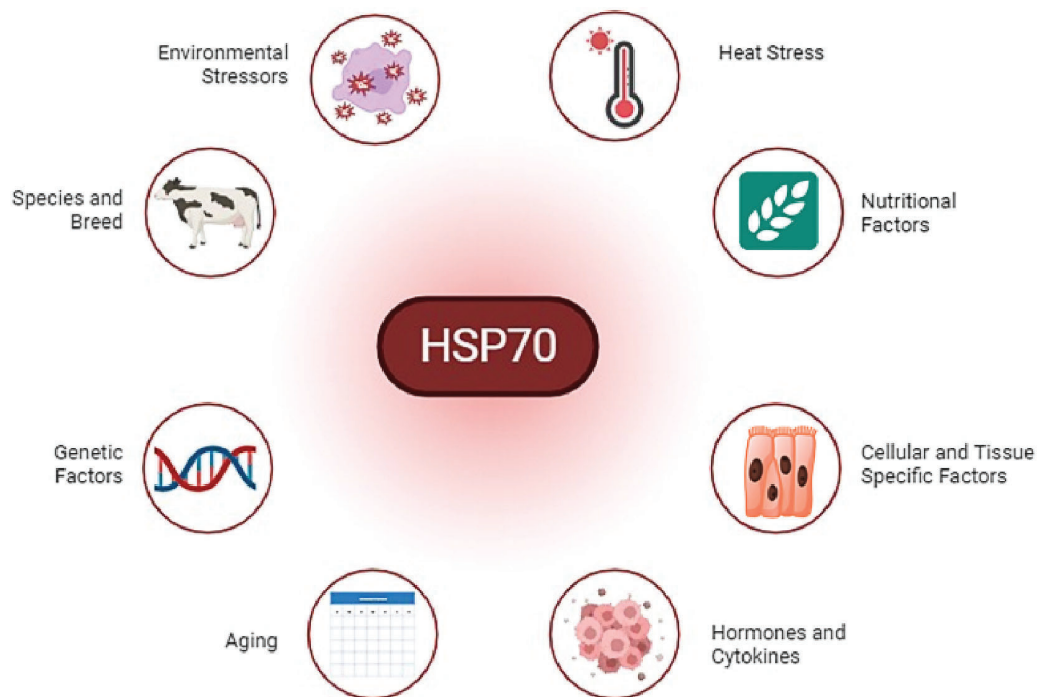


Figure 4. Factors influencing the expression of HSP70.

oxidants and minerals enhancing its expression, whereas specific nutrients (such as polyphenols, flavonoids, n-3 fatty acids) and caloric stress also induce HSP70 expression, underscoring the diverse influences on this crucial cellular protein (Samavat et al., 2018).

In general, HSP70 expression appears to be intricately influenced by several factors in addition to HS. This complex network of factors affecting HSP70 highlights the crucial role of HSP70 in maintaining cellular balance, making the protein a potential indicator of cell stress. However, its direct or specific application as an indicator of HS is yet to be confirmed. Further exploration of these factors holds the potential to enhance our comprehension of HSP70 expression and its diverse applications.

CONCLUSION AND FUTURE OPPORTUNITIES

This review discussed the critical role of HSP70 as a molecular chaperone in promoting cellular thermotolerance in dairy cows under HS. HSP70 can improve proper protein folding, prevents the aggregation of denatured proteins, and contributes to the cellular adaptive response to HS. Understanding these intricate mechanisms can help to develop targeted interventions and management strategies to improve the resilience of dairy cows to high temperatures. Using HSP70 as a biomarker for HS in dairy cows could offer several benefits, including directly measuring the cellular stress response and

enabling HS detection and its resistance. Monitoring HSP70 levels can also help to assess individual animal susceptibility to HS as a supplementary approach or be integrated into predictive models and guide targeted management strategies. However, additional studies are needed to fully explore these potential avenues.

Future research should focus on understanding the relationship between extracellular HSP70 expression and HS and on developing noninvasive techniques for detecting HSP70 under specific climatic conditions. This includes considering the time elapsed between HS and cellular expression, standardizing measurement protocols, assessing protein behavior across varying temperatures and time intervals, and exploring the specificity of detection methods, particularly the use of milk as a sampling medium. Because cows are milked 1 to 3 times daily, depending on the system, milk would be a particularly advantageous diagnostic tool. Addressing these challenges and advancing the understanding of HSP70 can enhance the sustainability and productivity of the dairy industry, along with ensuring animal wellbeing.

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Nonstandard abbreviations used: APC = antigen-presenting cell; BTSCC = bulk tank SCC; CBT = core body temperature; DAMP = danger-associated molecular pattern; HLI = heat load index; HS = heat stress; HSE = heat shock element; HSF = heat shock factor; HSP = heat shock protein; PBMC = peripheral blood-mononuclear cell; RH = relative humidity; RT = rectal temperature; T_{db} = dry-bulb temperature; T_{dp} = dew point temperature; THI = temperature-humidity index; TNZ = thermoneutral zone; T_{wb} = wet bulb temperature.

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CHAPTER 3

Comparison between in-house competitive and commercial ELISA for the detection of heat shock protein 70 in milk, blood, and saliva of dairy cows

A key gap identified in [Chapter 2](#) was the limited availability of analytical methods capable of reliably and reproducibly quantifying HSP70 in milk samples. [Chapter 3](#) compares an in-house competitive ELISA with a commercial assay across milk, blood, and saliva to evaluate agreement, precision, and practical performance. This work establishes the analytical foundation for subsequent investigations of HSP70 as a heat stress indicator in dairy cows.

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Comparison between in-house competitive and commercial enzyme-linked immunosorbent assays for the detection of heat shock protein 70 in milk, blood, and saliva of dairy cows

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ABSTRACT

Heat shock protein 70 (HSP70) has emerged as a potential physiological indicator of heat stress (HS), recognized for its substantial upregulation in response to increased temperatures and cellular stress. The accurate measurement of HSP70 is crucial for its application in research and on-farm monitoring. Commercial ELISA kits are widely available and exhibit high sensitivity; however, their limited working ranges require the dilution of high-concentration samples, which can potentially introduce variability. A recently developed in-house competitive ELISA provides a cost-effective alternative, previously validated for bovine HSP70 detection; however, its performance across various biological matrices has not been systematically evaluated against commercially available systems. A secondary objective was to determine whether HSP70 levels measured in less-invasive matrices such as milk and saliva, correspond to those obtained from blood. This study compared an in-house competitive ELISA to a commercial sandwich ELISA kit for detecting HSP70 in milk, blood, and saliva samples of dairy cows. Samples were analyzed with optimized methods, and assay performance was evaluated using descriptive statistics, Pearson correlations, and linear regression models. As expected for assays using different formats and calibration systems, the in-house ELISA indicated elevated absolute concentrations across all matrices; however, these differences were systematic and did not affect the strong agreement between methods. Blood samples had the lowest CV in both assays (2.76% in-house; 5.89% commercial), indicating greater repeatability than milk

(5.69% and 10.24%) and saliva (7.5% and 9.95%). The consistently lower CV for the in-house method across all fluids indicates that it is more precise and can lead to higher statistical power than commercial assays for a given sample size. Strong positive correlations were observed between assays across all matrices (milk $r = 0.91$; blood $r = 0.88$; saliva $r = 0.87$). Regression analysis validated these relationships, with R^2 values of 0.777, 0.833, and 0.752 for milk, blood, and saliva, respectively. The findings indicate that the in-house ELISA closely aligns with the performance of the commercial kit. Despite the lower detectable minimum concentration of the commercial assay, the in-house assay provides a more economical alternative for evaluating physiologically high and moderate HSP70 levels; however, the commercial kit remains widely available for purchase. Among biological matrices, plasma exhibited the highest cross-assay correlation, whereas milk and saliva showed consistent relative trends, indicating their potential as noninvasive alternatives for HS monitoring in dairy cows.

Key words: biomarkers, heat stress, heat shock protein 70, noninvasive

INTRODUCTION

Heat stress (HS) is a major constraint to dairy production worldwide, negatively affecting the welfare, health, and productivity of dairy cows (Wankar et al., 2021; Thornton et al., 2022). Among the physiological indicators of HS, heat shock protein 70 (HSP70) has gained substantial attention as a potential biomarker due to its upregulation in response to elevated temperatures and cellular stress (Hyder et al., 2017). Heat shock protein 70 plays a key role in maintaining protein homeostasis by promoting proper protein folding, stabilizing newly synthesized proteins, and preventing the aggregation of

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

denatured or misfolded proteins, while also protecting cellular function by limiting the growth of damaged protein aggregates during HS (Mosser and Morimoto, 2004; Mayer and Bukau, 2005). In dairy cattle, circulating HSP70 is generally low under thermoneutral conditions but increases during HS, typically by about 1.5- to 3-fold depending on the assay and biological matrix (Lamy et al., 2017; Li et al., 2021; Pathirana and Garcia, 2022; Altyeb et al., 2025). Increases in HSP70 are commonly observed when cows are exposed to elevated environmental temperatures and when core body temperature exceeds approximately 38.6°C (Gaughan et al., 2013). Heat shock protein 70 can be detected both intracellularly and in extracellular fluids following stress exposure, making it a promising candidate for HS monitoring in dairy cows. However, HSP70 concentrations are also affected by factors such as genetics, age, lactation stage, disease or inflammation, metabolic and oxidative stress, and management-related stressors (Rakib et al., 2024).

Elevated HSP70 levels have been reported across several livestock species under HS, using various biological matrices (Gaughan et al., 2013; Lamy et al., 2017; Pathirana and Garcia, 2022). Although blood-based detection methods, including plasma and peripheral blood mononuclear cells, remain common (Haque et al., 2012; Bhanuprakash et al., 2016), there is increasing interest in noninvasive alternatives such as milk (Pathirana and Garcia, 2022; Rakib et al., 2025). These matrices extend practical advantages for animal welfare, ease of sampling, and on-farm monitoring, especially in lactating animals.

Accurate measurement of HSP70 is essential for its effective use in both research and field-based diagnostics. Although commercial ELISA kits are readily available and generally offer high sensitivity, their limited detection range, requirement for sample dilution, and higher cost may restrict their practical applicability. In contrast, in-house ELISA systems are increasingly used in biomedical and veterinary fields because of their broader working ranges, greater flexibility in assay optimization, and significantly lower costs while maintaining comparable analytical performance to commercial kits (AL-Saeedi et al., 2023; Smaraki et al., 2024). Recently, an in-house competitive ELISA system developed by Pathirana and Garcia (2022) demonstrated potential for detecting HSP70 in bovine milk samples, offering an affordable and customizable alternative. However, their evaluation was limited to milk, without testing in blood, which is commonly considered the gold standard for biomarker detection, or saliva, which is gaining interest as a noninvasive alternative for HS monitoring. To demonstrate the broader applicability of this in-house method, a comprehensive comparison with commercial ELISA kits is therefore needed across multiple sample types and performance parameters.

This study aims to evaluate the analytical performance and reproducibility of the in-house competitive ELISA developed by Pathirana and Garcia (2022) and further validated by Rakib et al. (2025), in comparison with a selected commercial ELISA kit for the detection of HSP70 in milk, blood, and saliva samples, to determine its suitability as a practical, reliable, and economical alternative for routine bovine HS monitoring, and to evaluate whether milk and saliva provide HSP70 concentrations comparable to blood as less invasive sample matrices.

MATERIALS AND METHODS

Study Site and Experimental Animals

The experiment was conducted at Corstorphine Dairy Farm (University of Sydney, Camden, New South Wales, Australia) to compare the performance of the in-house and commercial ELISA systems. A total of 22 clinically healthy, third-parity Holstein Friesian cows (298.5 ± 26.7 DIM) were selected through health screening to exclude animals with lameness, mastitis, or other clinical problems that could influence HSP70 measurements. Cows were grazed on pasture year-round and given an average of 26 kg of DM/cow per day, comprising 8 kg of pelleted concentrate provided during milking, in addition to a mixture of forages including silage, dried distillers grains, fescue or prairie grass mix, and seasonal pasture species, such as ryegrass (*Lolium multiflorum* L.) in winter and spring and Kikuyu (*Cenchrus clandestinus*) in summer and autumn.

Sample Collection and Handling

Sampling was conducted on a single day in April 2024. On that day, cows were exposed to a moderate thermal load, with an average temperature-humidity index of 71.9 (25.8°C air temperature and 51% relative humidity). Heat shock protein 70 levels observed under these conditions consequently reflect baseline to moderately enhanced concentrations rather than peak summer responses. In the study region, April corresponds to the early autumn transition, when cows are no longer subjected to continuous summer heat and any previous acclimatization to prolonged high temperatures begins to decline (West, 2003). Therefore, the animals sampled during that period are anticipated to have relatively stable physiological responses, with HSP70 indicating a transient moderate heat load rather than a fully acclimatized HS condition.

Milk, blood, and saliva samples were collected from each animal during the afternoon milking period (1300–1500 h). The afternoon milking was selected to align with the daily maximum in ambient temperature and thermal load, ensuring uniform sampling under the most chal-

lenging thermal conditions. On the same day, 2 replicate samples from each fluid were processed, and HSP70 was measured in the laboratory. To decrease technical variability, replicates were averaged before statistical analysis.

Milk samples (~250 mL) were obtained from all 4 quarters of the udder of each cow during the mid-milking phase using rubber-sealed snap-cap containers using Waikato Milking Systems equipment (Waikato Milking Systems, Hamilton, New Zealand). Samples were immediately placed on ice and transported to the laboratory, and subsequently centrifuged at $1,000 \times g$ for 10 min at 4°C to isolate the skim milk fraction, following the protocol of Pathirana and Garcia (2022).

Blood samples (10 mL) were obtained via coccygeal vein using lithium heparin-coated vacutainer tubes (BD Vacutainer, BD-Plymouth, UK), as described by Gaughan et al. (2013). Immediately after collection, tubes were stored in ice boxes (2°C–4°C) and transported to the laboratory within 30 min. Samples were centrifuged at $1,328 \times g$ for 10 min at 2°C to isolate plasma, which was aliquoted into 5-mL portions for subsequent analysis.

Saliva samples were collected using sterile Salivette cotton swabs (Sarstedt, GmbH, Germany), placed into the cheek pouches of cows for approximately 30 s to absorb fluid. After collection, the swabs were stored in an ice container (2°C–4°C) and transported to the laboratory within 30 min. Saliva was extracted by centrifugation at $1,000 \times g$ at 4°C for 5 min, and the supernatant was separated into 5-mL aliquots for ELISA testing (Lamy et al., 2017).

In addition to samples collected for HSP70 determination, milk composition, including fat, protein, SNF, and lactose percentages, was determined using an ultrasonic milk analyzer (Master Eco, Milkotester, Bulgaria). Daily milk yield (L/d) was recorded using WB Ezi-Test meters (Tru-Test, New Zealand) during both morning and afternoon sessions.

HSP70 Measurement Using In-House Competitive ELISA

Concentrations of HSP70 in milk, blood, and saliva samples were determined in duplicate using the in-house competitive ELISA system as previously described by Pathirana and Garcia (2022). Key steps of the 3-d method are summarized below.

A polystyrene microtiter plate (Nunc Immuno Polysorp, Rochester, NY) was coated with human recombinant HSP70 solution (**HrHSP70**; ADI-NSP-555, Enzo Life Sciences, Farmingdale, NY) using a 1:1,000 dilution of HrHSP70 in working solution (0.05 M sodium bicarbonate, pH 9.7), with a final volume of 100 µL/well and incubated for overnight at 4°C. Blank wells were only coated with the buffer.

A series of standards was prepared by diluting HrHSP70 solution with a starting concentration of 8,000 ng/mL in assay buffer (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, 2% [wt/vol] bovine serum albumin, 0.02% [vol/vol] ProClin 950) and serially diluted (2-fold) to a final concentration of 62.5 ng/mL. Then, 100 µL of each sample or standard was mixed with 100 µL of monoclonal anti-human HSP70 antibody (1:800 dilution in assay buffer; ADI-SPA-810, Enzo Life Sciences, Farmingdale, NY) in 1.5-mL microcentrifuge tubes, preparing concentrations ranging from 4,000 to 31.25 ng/mL. All prepared standard or sample and antibody mixtures were incubated overnight at 4°C before the next step.

The next day, the coated plates were washed 3 times with 300 µL of washing buffer (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, 0.05% [vol/vol] Tween 20, 0.02% [vol/vol] ProClin 950), and nonspecific binding sites were blocked using 150 µL of assay buffer for 2 h at room temperature. Following this, 100 µL of each sample-antibody mixture or standard-antibody mixture was added in duplicate to the wells. Additional control wells included maximum binding wells containing a 1:1 mixture of anti-HSP70 antibody and assay buffer, as well as nonspecific binding wells that received assay buffer only. The plate was then incubated overnight at 4°C.

On the third day, the wells were washed 3-times, before adding 100 µL of secondary antibody (goat anti-mouse IgG conjugated with horseradish peroxidase; BML-SA204, Enzo Life Sciences, Farmingdale, NY; 1:200 dilution in assay buffer) to each well. After a 2-h incubation at room temperature, the wells were washed again and treated with 100 µL of a substrate solution containing 3,3',5,5'-tetramethylbenzidine (**TMB**; Sigma, Saint Louis, MO). After 30 min incubation at room temperature, the reaction was stopped using 50 µL of 1 N hydrochloric acid. Optical density was measured at 450 nm using an ELISA plate reader (MR9600, Accuris, NJ).

Standard curves were generated for each assay using Microplate Manager 6 software (Bio-Rad, Hercules, CA), and HSP70 concentrations were calculated accordingly. Additionally, the intra- and interassay CV were calculated as 7.20% and 15.83%, respectively, based on triplicate measurements ($n = 3$). The assay demonstrated a minimum detection limit of 31.25 ng/mL and a quantifiable working range of 31.25 to 2,000 ng/mL.

HSP70 Measurement Using Commercial ELISA

Heat shock protein 70 concentrations in milk, blood, and saliva samples were also measured using a commercially available sandwich ELISA kit (ab133060; Abcam, Cambridge, UK), following the manufacturer's protocol

Table 1. Descriptive statistics of HSP70 concentrations measured using in-house and commercial ELISA systems across 3 sample types

Variable	Mean	SD	Minimum	Maximum	CV%
In-house ELISA					
Milk HSP70 concentration (ng/mL)	390.53	22.21	356.53	424.06	5.69
Blood HSP70 concentration (ng/mL)	430.15	11.88	410.06	449.98	2.76
Saliva HSP70 concentration (ng/mL)	268.89	20.17	240.19	302.03	7.50
Commercial ELISA kit					
Milk HSP70 concentration (ng/mL)	31.51	3.23	26.83	37.83	10.24
Blood HSP70 concentration (ng/mL)	40.96	2.41	36.9	45.22	5.89
Saliva HSP70 concentration (ng/mL)	19.47	1.94	16.14	24.20	9.95

with minor adaptations for bovine biological matrices. All reagents and samples were brought to room temperature before use. Standards were prepared by serially diluting recombinant HSP70 protein, creating a standard curve ranging from 0.78 to 50 ng/mL. A 96-well plate precoated with a monoclonal anti-HSP70 antibody was used. A total of 100 μ L of each standard or sample was added to the wells in duplicate and incubated for 2 h at room temperature. Following incubation, wells were washed 4 times with $1\times$ wash buffer, after which 100 μ L of HSP70 detection antibody was added. The plate was sealed and incubated for an additional hour at room temperature with gentle agitation. After a second wash step, 100 μ L of horseradish peroxidase-conjugated anti-IgG antibody was added to each well and incubated for 1 h. Plates were then washed again, and 100 μ L of TMB substrate was added. After a 30-min incubation at room temperature on the plate shaker, the reaction was stopped with 100 μ L of Stop Solution (1 *N* hydrochloric acid), and absorbance was measured at 450 nm using a microplate reader (MR9600, Accuris). Samples exceeding the standard curve range were appropriately diluted in assay buffer and reassayed. The assay had a minimum detection limit of 0.2 ng/mL, whereas accurate quantification was possible within the standard curve range of 0.78 to 50 ng/mL. The assay demonstrated intra- and interassay CV of less than 5% and 13%, respectively.

Statistical Analysis

All statistical analyses were conducted in R statistical software (version 4.5.0). For each sample type (milk, blood, and saliva) descriptive statistics including the mean, standard deviation, minimum, maximum, and CV were calculated for HSP70 concentrations measured by both ELISA methods. Pearson correlation coefficients and associated *P*-values were computed to evaluate the linear relationships between HSP70 concentrations measured by the in-house and commercial ELISA methods. Correlation matrices were visualized using the *corrplot* package. To further explore agreement between the 2 methods, simple linear regression models were applied

separately for each matrix. Regression outputs included the equation, R^2 values, and corresponding significance levels. Scatter plots with fitted regression lines and statistical annotations were generated using the *ggplot2* package. Statistical significance was set at $P < 0.05$ for throughout the analyses.

RESULTS

Descriptive Statistics of HSP70 Concentrations

Table 1 presents the descriptive statistics of HSP70 concentrations (ng/mL) measured using in-house and commercial ELISA systems across milk, blood, and saliva samples. The in-house ELISA measured higher mean concentrations in all matrices (milk: 390.53 ng/mL; blood: 430.15 ng/mL; saliva: 268.89 ng/mL) compared with the commercial kit (milk: 31.51 ng/mL; blood: 40.96 ng/mL; saliva: 19.47 ng/mL). Coefficients of variation were lowest in blood samples for both the in-house (2.76%) and commercial (5.89%) assays, indicating higher consistency in plasma-based measurement. The in-house ELISA also showed relatively low variability in milk samples (CV = 5.69%), whereas the commercial kit showed higher variability in both milk (10.24%) and saliva (9.95%).

Correlation Between ELISA Systems

Among all matrices, milk samples exhibited the highest correlation ($r = 0.91$, $P < 0.001$), followed by blood ($r = 0.88$, $P < 0.001$) and saliva ($r = 0.87$, $P < 0.001$; Figure 1). These strong positive correlations reflect a high level of association between the 2 assays in quantifying HSP70 concentration across 3 different biological fluids collected from dairy cows.

Linear Regression Analysis Between ELISA Systems

Simple linear regression models were used to assess the relationship between HSP70 concentrations obtained from the in-house and commercial ELISA systems for

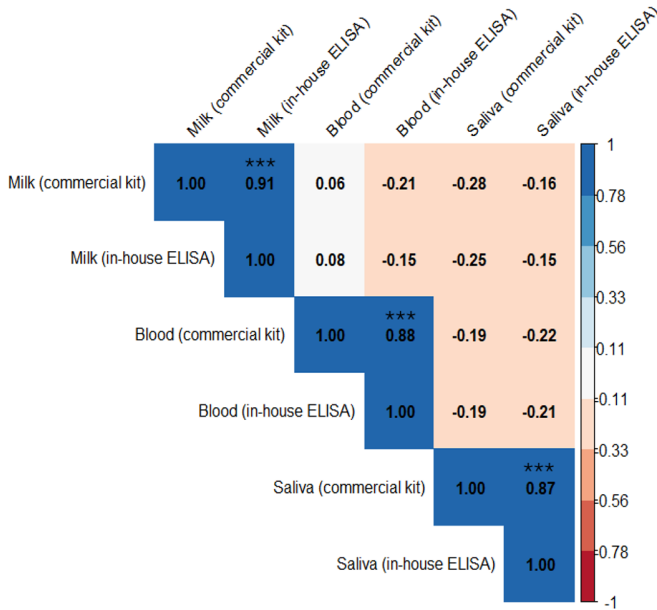


Figure 1. Pearson correlation matrix showing the relationships between HSP70 concentrations measured by in-house and commercial ELISA systems across milk, blood, and saliva samples. Correlation coefficients (r) are displayed within each cell. Positive correlations are shown in blue and negative correlations in red, with color intensity indicating the strength of the association. Asterisks indicate levels of statistical significance (*** $P < 0.001$).

each sample type (Figure 2A–C). All models showed strong linear associations with high significance ($P < 0.0001$). Among the sample types, blood samples exhibited the strongest linear fit with an R^2 of 0.833, followed by milk ($R^2 = 0.777$) and saliva ($R^2 = 0.752$). These findings indicate a strong linear association between the 2 assays across all biological matrices.

DISCUSSION

This study compared an in-house competitive ELISA with a commercial ELISA kit for detecting HSP70 levels in milk, blood, and saliva samples from dairy cows. Both assays exhibited robust analytical performance; however, notable differences were observed in their concentration ranges, variability, and the reliability with which they performed across different sample types. The key outcome of this comparison was the strong agreement between the 2 assays in their relative measurement of HSP70 across sample types.

Strong positive correlations were observed between the 2 ELISA systems across all matrices, with milk exhibiting the highest correlation coefficient ($r = 0.91$), followed by blood ($r = 0.88$) and saliva ($r = 0.87$). These results suggested that, although absolute concentrations differed between ELISA techniques, both assays produced similar relative levels of HSP70 across animals. The consistently lower CV values observed for the in-house ELISA indicate

that it provides more precise measurements, which requires fewer samples to achieve the same statistical power compared with the commercial kit. This higher analytical precision is beneficial when analyzing extensive field datasets or when sample availability is limited. However, the variation in absolute quantification implies that assay-specific calibration would be required before determining universal diagnostic thresholds for assessing HS. In general, the in-house assay captures the same biological trends as the commercial kit, supporting its potential use as a practical and lower-cost alternative for monitoring HSP70 responses in dairy cows. Similar findings have been reported in other ELISA comparison studies (AL-Saedi et al., 2023; Gobena et al., 2025). Linear regression analyses further confirmed the strong association between both ELISA systems, with R^2 values above 0.75 for all sample types. Blood samples showed the best linear fit ($R^2 = 0.833$), which may reflect the lower matrix interference compared with milk or saliva. This indicates that plasma not only shows consistency within a single assay but also shows stronger agreement when results are compared across different assays. Previous studies have similarly reported that blood-based biomarkers show closer agreement across different ELISA systems (Fry et al., 2008; Toaleb et al., 2025). Consistent with these observations, the present findings suggest that blood is the most reliable matrix for cross-assay comparisons. Furthermore, the in-house ELISA exhibited significant potential for field applications due to its adaptability across sample matrices and ease of assay optimization.

The in-house ELISA showed higher average HSP70 concentrations in all sample types compared with the commercial kit. Differences among them are mostly due to variations in assay design and standardization methods. In this study, the commercial ELISA kit exhibited not only a lower minimum but also a relatively limited detection range (0.78–50 ng/mL), making it necessary to dilute samples that exceeded the kit's upper detection limit before analysis. These additional dilution steps may introduce variability and increase the risk of underestimating the real value of the concentration (Verch et al., 2016). In contrast, the in-house assay showed a higher detectable minimum concentration but a broader detection range (31.25–2,000 ng/mL); no previous dilutions of the samples were required (Pathirana and Garcia, 2022). However, competitive tests are also more susceptible to influence from biological fluid components when compared with commercial sandwich ELISA assays, which could lead to higher apparent values (Njemini et al., 2005). Previous comparative ELISA studies have reported that, even when aiming for the same protein, assay format, antibody specificity, and standard curve preparation can introduce systematic variation in observed concentrations (Fry et al., 2008; Toaleb et al., 2025).

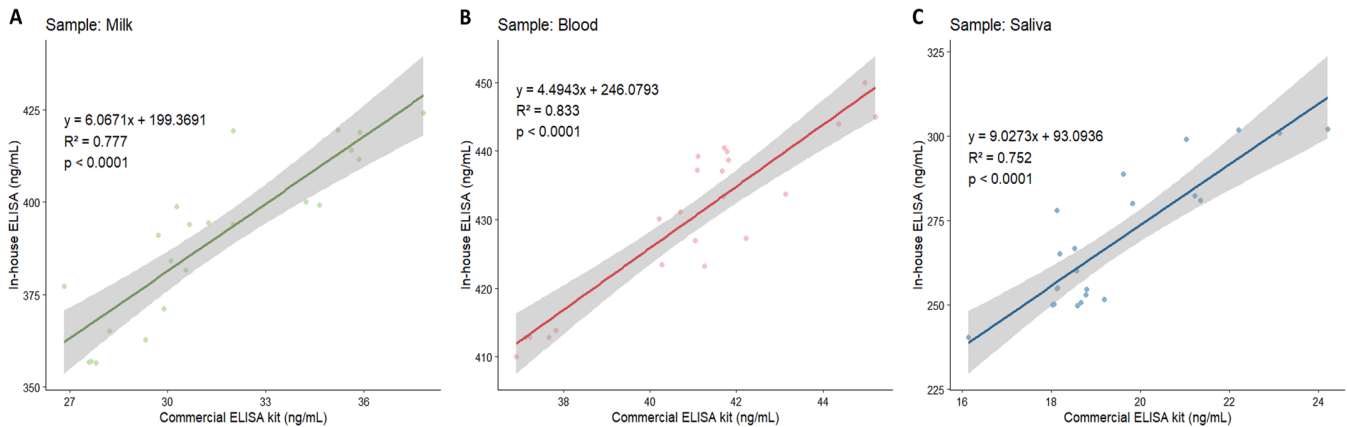


Figure 2. Simple linear regression plots showing the relationship between HSP70 concentrations measured by the in-house and commercial ELISA systems for (A) milk, (B) blood, and (C) saliva samples. Each panel shows the regression line with 95% CI (gray shading), along with the regression equation, coefficient of determination (R^2), and P -value.

Among matrices, the lowest CV% were observed in blood samples for both ELISA systems, indicating greater repeatability in plasma-based HSP70 measurements. This is probably because plasma has a more stable composition and less enzymatic activity than milk and saliva, where protein degradation or interference with antibody-antigen binding can lead to less consistent measurements (Mojsym et al., 2022). Saliva showed the highest variability, with CV values of 7.5% for the in-house assay and 9.95% for the commercial kit, which may reflect the influence of oral enzymes and other interfering compounds (Soo-Quee Koh and Choon-Huat Koh, 2007). The in-house assay also showed lower CV% in milk (5.69%) compared with the commercial kit (10.24%), indicating improved reliability without requiring dilution, which is advantageous for practical use in the field. The in-house ELISA showed consistently lower CV% across all sample types: 2.76% for blood, 5.69% for milk, and 7.50% for saliva, compared with 5.89%, 10.24%, and 9.95%, respectively, indicating that the in-house ELISA was more precise and repeatable than the commercial kit. This overall pattern among fluids highlights the robustness of the in-house method (Njemini et al., 2005), demonstrating its consistent performance despite variations in sample composition. This observation is also consistent with previous reports that additional dilution steps can introduce error and reduce precision in ELISA (Verch et al., 2016).

In addition to analytical performance, it is essential to evaluate the economic and practical aspects of the 2 assays. The in-house ELISA is considerably more economical, costing approximately US\$1–US\$1.5 per sample compared with US\$7.5–US\$8.5 per sample for the commercial kit, making it suitable for extensive or routine applications. However, this lowers expenses involves an extended processing time, as the in-house

technique takes around 3 d to complete, in contrast to the 4 to 6 h needed for the commercial test. The variations in cost and time, along with the labor required, will probably affect which option is better in practice. These cost-time trade-offs are not limited to HSP70 detection; similar trends are evident in contemporary diagnostic assay development, where in-house ELISA remain prevalent despite their additional manual processing requirements, primarily because of their significant cost advantages. An in-house SARS-CoV-2 serology ELISA was specifically developed as a cost-efficient substitute for commercial anti-S1 kits (AL-Saedi et al., 2023), and a similar emphasis on affordability and scalability has been reported in extensive serological screening of cattle for lumpy skin disease virus (Smaraki et al., 2024). A comprehensive assessment that considers both consumable expenses and labor costs would clarify the comparative feasibility of each method across different research and farm conditions.

To the best of our knowledge, this is the first study to directly evaluate commercial and in-house ELISA systems for the detection of HSP70 in milk, blood, and saliva of dairy cows. It provides valuable information on how well the test performs in both invasive and noninvasive sample types. A key limitation of this study is the use of a commercially available multispecies HSP70 ELISA that was not specifically validated for bovine samples. Although similar multispecies assays have been applied in bovine research and HSP70 is highly conserved across mammalian species, the lack of species-specific validation may influence the absolute quantification of HSP70 across the different biological matrices assessed. Moreover, the study included a limited number of animals, which may have constrained the broader applicability of the results. Further research should incorporate bovine-validated HSP70 assays, evaluate other commercially

available ELISA kits, and include larger sample sizes to enhance cross-matrix comparisons and improve assay reliability and usefulness across diverse cattle populations.

CONCLUSIONS

This study demonstrates that the in-house competitive ELISA provides a reliable and cost-effective alternative to a commercial ELISA for detecting HSP70 in dairy cows. The in-house assay avoided substantial dilution, showed strong agreement with the commercial kit, and provided greater reproducibility in blood and milk. Blood was the most consistent matrix, but milk and saliva also reflected useful noninvasive options for HS monitoring.

NOTES

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Nonstandard abbreviations used: HrHSP70 = human recombinant HSP70 solution; HS = heat stress; HSP70 = heat shock protein 70; TMB = 3,3',5,5'-tetramethylbenzidine.

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CHAPTER 4

Skimming and storage factors affect the detection of heat shock protein 70 in raw bovine milk

Once the analytical methodology for reliably measuring HSP70 in animal fluids was established (Chapter 3), appropriate sample handling became essential to ensure accurate HSP70 measurements in milk. Chapter 4 investigates the effects of milk processing and storage conditions on HSP70 detection, with particular emphasis on skimming, temperature, and preservation methods. By identifying factors influencing HSP70 stability, this chapter provides practical guidance on sample handling and the quantification of HSP70 in milk for subsequent investigations.

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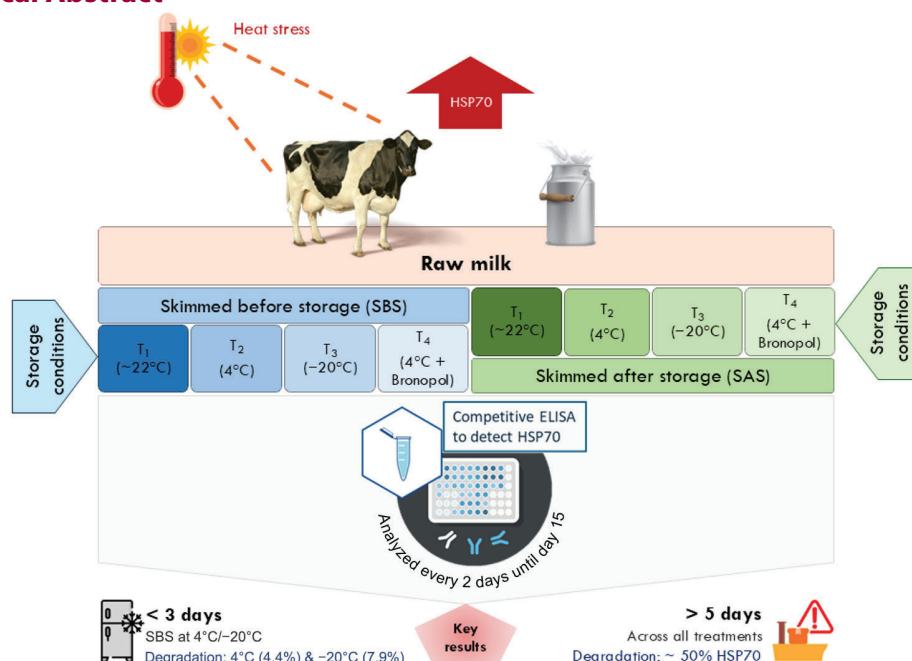
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Skimming and storage factors affect the detection of heat shock protein 70 in raw bovine milk

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Graphical Abstract



Summary

This study evaluated the impact of milk sample handling on the detection of heat shock protein 70 (HSP70) in bovine milk by using a competitive enzyme-linked immunosorbent assay (ELISA) system. Samples were processed as skimmed before storage (SBS) or skimmed after storage (SAS) and stored under various conditions: room temperature (~22°C; T₁), refrigeration (4°C; T₂), freezing (-20°C; T₃), and refrigeration with preservative (4°C + bronopol; T₄). Samples were analyzed every 2 days over 15 days to assess HSP70 stability. Results showed that SBS samples stored at 4°C or -20°C for up to 3 days preserved HSP70 levels with minimal degradation (4.4% and 7.9%, respectively), whereas SAS samples showed significantly higher degradation (44.2%–53.9%). Room temperature storage led to the greatest degradation, and bronopol use did not consistently maintain HSP70 levels. These findings highlight the importance of optimizing milk storage to preserve HSP70, a potential biomarker for cellular and heat stress in dairy cows.

Highlights

- Skimming milk before storage reduces HSP70 degradation compared with after storage.
- Skim milk stored at 4°C or -20°C preserves HSP70 levels effectively for up to 3 days.
- Storage beyond 5 days causes ~50% HSP70 degradation across all treatments.



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Skimming and storage factors affect the detection of heat shock protein 70 in raw bovine milk

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Abstract: Heat shock proteins (HSP) play a vital role in maintaining cellular function under elevated temperatures. Among these, heat shock protein 70 (HSP70) has recently been detected in bovine milk using competitive ELISA, highlighting its potential as a noninvasive marker of cellular stress and a tool for detecting heat stress (HS) in dairy cows. However, optimal management and storage conditions of milk samples for detection of HSP70 are yet to be determined. This study aimed to evaluate how different management and storage practices affect detection of HSP70 in milk samples. Samples were skimmed by centrifugation either before storage (SBS) or after storage (SAS), and then stored under different temperatures: (a) room temperature (T₁, ~22°C), (b) refrigerated (T₂, 4°C), (c) frozen (T₃, -20°C), and (d) refrigerated at 4°C with a preservative (bronopol; T₄). Samples were analyzed every 2 d until d 15 (d 1, d 3, ..., d 15) using a competitive ELISA system (sensitivity range 31.25–2,000 ng/mL) to detect HSP70 levels. Result showed that SBS samples had the smallest reduction in HSP70 levels on d 3 when stored at T₂ or T₃, with decreases of 4.4% and 7.9%, respectively. In contrast, SAS samples exhibited greater reductions (44.2% at T₂ and 53.9% at T₃). Samples stored at room temperature (T₁) showed the greatest degradation, especially in SAS samples, whereas the use of a preservative (T₄) did not consistently maintain HSP70 levels in either SBS or SAS samples. The HSP70 detection showed lower variability during early storage (d 1–3) across all treatments, with SBS samples demonstrating greater stability than SAS; however, variability significantly increased by d 9 in both sample types. An apparent increase in HSP70 levels was observed from d 11 to 15 in both SBS and SAS, suggesting the influence of protein stability and interactions affecting ELISA reactivity, where further research is required. Overall, samples stored as skim milk until d 3 at 4°C or -20°C preserved HSP70 levels more effectively than whole milk, with room temperature storage being the least favorable. This pioneering study explored how sample storage and management affect HSP70 detection, providing practical guidelines for farmers and processors to preserve milk sample integrity for assessment of HSP70 as indicator of cellular stress and, potentially, HS.

Heat stress (HS) in lactating dairy cows not only reduces feed intake, milk yield, and reproductive performance, but also affects milk quality and composition, which has direct implications for dairy processing and product consistency (Becker et al., 2020; Rakib et al., 2020). Managing HS has become more complex due to the increasing number of high-producing animals, which are metabolically more active (Polisky and Von Keyserlingk, 2017), and the ongoing impact of climate change, with more frequent and severe heat events (IPCC, 2021).

Several methods for detecting HS in dairy cows have been explored, ranging from physiological and behavioral measurements to advanced applications of remote sensing and machine learning technologies (Rakib et al., 2024). Identifying practical biomarkers for HS is crucial for improving herd management and minimizing production losses. Among potential biomarkers, HSP70 is of particular interest due to its critical role in cellular stress response in dairy cattle and other livestock species, including pigs and poultry (Gaughan et al., 2013). HSP70 helps protect cells under stress by facilitating proper protein folding and preventing the aggregation of denatured proteins. By binding to misfolded proteins, it helps

maintain protein homeostasis and limits the accumulation of cellular damage during HS (Mosser and Morimoto, 2004; Mayer and Bukau, 2005). Additionally, HSP70 is involved in signaling and regulatory pathways that enhance the cellular adaptive response. Its expression is upregulated through a complex network involving heat shock factors (HSF), element-binding proteins, and co-chaperones (Voellmy, 2004). Given its role in maintaining cellular balance and its release both within and outside cells in response to stress, HSP70 shows strong potential as an indicator of tissue-level stress (Rakib et al., 2024). However, HSP70 is not specific to HS alone; its expression can also be induced by other physiological stressors, including inflammation and disease conditions such as mastitis (Hyder et al., 2017). Understanding these influences is essential for interpreting HSP70 levels accurately and for exploring its broader diagnostic potential. Nevertheless, HSP70 potentially remains as a valuable supplementary tool for identifying heat stress or resilience in individual animals, supporting genetic selection, and informing herd management strategies (Rakib et al., 2024).

Although increases in HSP70 levels in blood and saliva have been associated with HS in cattle (Haque et al., 2012; Lamy et al.,

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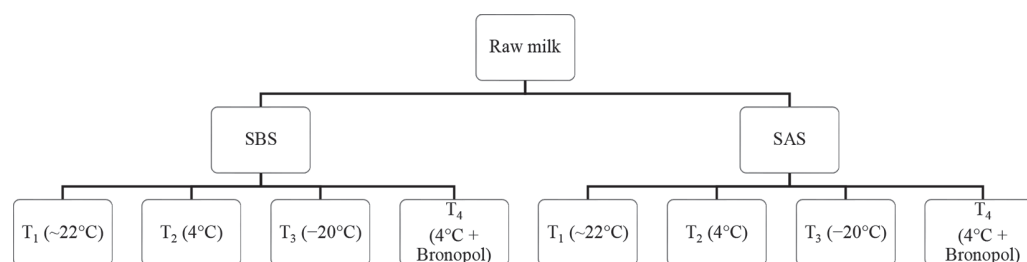


Figure 1. Experimental design of storage conditions to detect HSP70 in raw bovine milk. SBS = skimmed before storage; SAS = skimmed after storage; T₁ = room temperature (~-22°C); T₂ = refrigerated (4°C); T₃ = frozen (-20°C); T₄ = adding bronopol preservative and refrigerated (4°C).

2017), the invasiveness of sample collection can itself induce stress the animals and the process is notably labor-intensive (Lamy et al., 2017; Pathirana and Garcia, 2022), making routine application impractical for farmers. Pathirana and Garcia (2022) developed a competitive ELISA test for detecting HSP70 in milk samples, presenting promising avenues for further exploration. However, sample management factors such as storage conditions before analysis could affect the accuracy and limit the potential application of the method, yet these factors have not been quantified. For instance, if immediate analysis were required after collection, the method would be impractical on farms. Therefore, the objective of this research was to evaluate the effect of different milk management and storage practices on the detection of HSP70 in dairy cows using a competitive ELISA system.

The study was conducted at Corstorphine Dairy Farm, The University of Sydney, Camden, NSW, Australia, between August and September 2023. Milk samples were collected from 20 Holstein Friesian cows in their third lactation, with an average of 114 DIM. All cows grazed on annual ryegrass-based pasture and showed no signs of health issues such as mastitis or lameness, minimizing potential confounding effects on HSP70 levels. Milk sampling was performed on the first day of the experiment during a single, scheduled afternoon milking session between 1300 and 1400 h, with samples collected from all 4 quarters of each cow. According to per-minute environmental data from the Australian Bureau of Meteorology, ambient temperature during this sampling period ranged from 20.4°C to 22.6°C, and relative humidity ranged from 34% to 39%. These consistent environmental conditions minimized the potential influence of acute thermal stress on HSP70 levels across animals on the sampling day. Moreover, the ~-22°C ambient temperature also aligned with the designated room temperature condition used in this experiment, which was selected to reflect typical sample handling environments encountered in laboratories or farm-side settings, particularly in moderate climates.

Approximately 250 mL of milk per cow was collected into rubber snap-seal sample bottles. Milk yield (L/d) was recorded using WB Ezi-Test milk meters (Tru-Test, New Zealand). Milk composition, including fat, SNF, protein, and lactose, was analyzed using a MASTER ECO ultrasonic milk analyzer (Milko-tester Ltd., Bulgaria) following the manufacturer's instructions. Each milk sample was then divided into eight 15-mL subsamples (Figure 1). Subsamples were handled as follows: skimmed before storage (SBS): fat was removed via centrifugation (1,000 × g for 10 min at 4°C) immediately after collection; and skimmed after storage (SAS): fat was removed via centrifugation (1,000 × g for

10 min at 4°C) after the samples had been stored under specified conditions.

Subsequently, samples were subjected to different storage conditions: (a) room temperature (T₁, ~-22°C), (b) refrigerated (T₂, 4°C), (c) frozen (T₃, -20°C), and (d) refrigerated at 4°C (T₄) with added preservative (bronopol). Each subsample (15 mL) was stored in a Falcon tube placed inside a labeled plastic bag. Samples were analyzed every 2 d until d 15 (d 1, d 3, ..., d 15). Before ELISA analysis, frozen samples were thawed completely at room temperature, ensuring consistency in the measurement process.

The concentration of HSP70 in milk samples, analyzed in duplicate, was measured using a competitive ELISA system, exactly as previously described by Pathirana and Garcia (2022). In brief, 96-well polystyrene microtiter plates (Nunc Immuno Polysorp, Rochester, NY) were coated with recombinant human HSP70 (ADI-NSP-555, Enzo Life Sciences, Farmingdale, NY) and incubated overnight at 4°C. Samples and standards were pre-incubated with a monoclonal anti-human HSP70 antibody (ADI-SPA-810, Enzo Life Sciences) overnight at 4°C and then added to the blocked wells. After incubation and washing, a goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP, BML-SA204, Enzo Life Sciences) was applied. The color reaction was obtained using 3,3',5,5'-tetramethylbenzidine (TMB, Sigma, St. Louis, MO) substrate, and the reaction was stopped with 1 N hydrochloric acid. Absorbance was measured at 450 nm using an ELISA plate reader (MR9600, Accuris, NJ). The intra- and inter-assay coefficients of variation were determined to be 8.1% (n = 3) and 16.9% (n = 3), respectively. The minimum detection limit of the assay was 31.25 ng/mL, and the detection was reliable in the range from 31.25 to 2,000 ng/mL.

Data processing, statistical analysis, and visualization were performed using R (version 4.3.2; <https://www.r-project.org/>) statistical software. Descriptive statistics, including the mean, SD, coefficient of variation %, and the range (minimum and maximum values), were calculated for HSP70 and key variables such as milk yield, fat, SNF, protein, and lactose (Table 1). Pearson correlation coefficients and corresponding *P*-values were calculated to investigate the relationships between HSP70 concentration (ng/mL), milk yield (L/d), and milk composition (fat, SNF, protein, and lactose). A linear mixed model (*lme4* package) was used to compare the effects of sample handling methods (SBS and SAS), storage conditions, storage time, and their interactions on HSP70 levels. Animal ID was included as a random effect to account for multiple observations per sample. Pairwise comparisons of estimated marginal means were conducted within each combination

Table 1. Descriptive statistics of the variables from the dataset at d 1

Variable	Mean	SD	CV (%)	Minimum	Maximum
HSP70 concentration (ng/mL)	210.08	17.37	8.27	177.08	248.44
Milk yield (L/d)	32.75	10.63	32.47	12.50	51.25
Milk composition (%)					
Fat	3.99	1.51	37.94	1.30	6.00
SNF	8.87	0.48	5.37	7.90	9.90
Protein	3.22	0.17	5.17	2.90	3.60
Lactose	4.84	0.26	5.35	4.30	5.40

of sample handling method, storage condition, and storage time using the *emmeans* and *multcomp* packages, with Sidak adjustment applied for multiple testing. Model residuals were assessed for normality and heteroscedasticity, and extreme outliers were removed before final analysis. Moreover, Levene's test was performed to evaluate homogeneity of variances across sample handling methods $\times$ storage condition $\times$ storage time combinations. In addition, generalized least squares (GLS) modeling was applied to compare models assuming constant residual variance with those allowing group-specific variances using the *varIdent* function from the *nlme* package. Model comparison was conducted using likelihood ratio tests. All tests were performed at a significance level of $P < 0.05$ with 95% CI reported where applicable.

As shown in Table 1, HSP70 levels averaged 210.08 ng/mL, with a moderate variability in d 1 (CV = 8.27%). Milk yield had a high variability (CV = 32.47%), whereas milk composition variables showed a range of variation, with fat percentage having the highest CV (37.94%), whereas SNF, protein, and lactose percentages exhibited more consistency, with CV around 5%.

Additionally, the Pearson correlation coefficients between HSP70 concentration (ng/mL), milk yield (L/d), and different milk composition parameters (fat, SNF, protein, and lactose) were calculated in d 1. HSP70 exhibited a negative correlation with milk yield (-0.41), suggesting that higher HSP70 levels are associated with reduced milk production. However, no significant correlations were observed between HSP70 concentration and milk composition (fat, SNF, protein, lactose). These results indicate that although HSP70 concentration is linked to milk yield, it is not directly correlated with milk composition.

The main effects of sample handling methods, storage conditions, and storage time, together with their 2-way and 3-way interactions, were all statistically significant ($P < 0.0001$). Given the significant interactions observed, pairwise comparisons were interpreted within each day $\times$ handling method $\times$ storage condition combination. On d 3, SBS samples showed the least reduction in HSP70 levels, with decreases of 4.4% for T_2 (4°C), 7.9% for T_3 (-20°C), 21.3% for T_1 (room temperature, $\sim 22^\circ\text{C}$), and 57.9% for T_4 (4°C with preservative), compared with d 1 (210.08 ng/mL; Figure 2). In contrast, SAS samples on d 3 exhibited larger reductions, with 44.2% for T_2 , 49.7% for T_4 , 53.9% for T_3 , and 56.6% for T_1 , indicating that skim milk stored at T_2 (4°C) was most effective in preserving HSP70 levels until d 3, followed by frozen storage at T_3 (-20°C). Samples stored at room temperature (T_1) showed a higher degradation, particularly for SAS, whereas the use of a preservative (T_4) did not consistently maintain HSP70 levels in either SBS or SAS samples.

After d 3, a decrease in HSP70 levels was observed in both SBS and SAS samples, with further reductions from d 3 to 9 across

all storage conditions. However, after d 5, HSP70 was no longer detected in T_1 samples (room temperature, $\sim 22^\circ\text{C}$) due to sample spoilage. The HSP70 levels in SBS samples remained relatively higher than in SAS samples until d 3, after which both handling methods showed a similar trend, suggesting that centrifugation may help to preserve protein stability in the early stages of storage. From d 11 to 15, an increase in measured HSP70 levels was observed in both handling methods (data not shown), indicating the influence of protein stability and interactions affecting ELISA reactivity, warranting further research.

The CV of HSP70 ranged from 8.27% to 40.95% across samples, with lower variability observed in the earlier days (d 1–3) for most treatments, indicating more consistent HSP70 detection during this period. In SAS samples, variability ranged from 10.3% to 12.7% on d 3 across different treatments but increased significantly to 36.43% by d 9. The SBS samples were generally more stable in shorter treatments, with the lowest CV of 8.26% observed on d 1; however, variability increased to a range of 30.55% to 32.52% by d 9 for different treatments.

Similarly, Levene's test revealed significant heterogeneity in HSP70 variability across all combinations of sample handling method, storage condition, and storage time ($F = 7.99$, $P < 0.001$), as well as for each factor individually (all $P < 0.001$). The GLS modeling further confirmed that residual variance differed significantly across these groups: the model allowing group-specific variances to fit substantially better than the constant-variance model (likelihood ratio = 806.1, $P < 0.001$), which suggest that both sample handling methods and treatment duration significantly influence the stability and detectability of HSP70.

Previous research on the effects of sample handling, storage conditions, and time on ELISA results is limited but provides valuable insights. Charlier et al. (2005) demonstrated that storing whole milk samples for up to 4 d at 4°C did not significantly affect the detection of gastrointestinal nematode (*Ostertagia ostertagi*) infections in adult cows using an indirect ELISA, compared with skim milk samples. Additionally, bronopol, a common preservative used in milk sample collection, has generally been reported to have no significant impact on ELISA performance in certain assays (Sweeney et al., 1994; Sanchez et al., 2002). For example, Sanchez et al. (2002) found high repeatability between preserved and non-preserved samples stored for up to 42 d at 4°C using an *O. ostertagi* ELISA, and da Silva et al. (2017) similarly reported stable results in bronopol-treated samples analyzed for pregnancy-associated glycoproteins. However, in this study, bronopol-preserved samples consistently showed lower HSP70 concentrations compared with samples stored at 4°C without preservative. A potential explanation for this is that bronopol generates reactive oxygen species and oxidizes thiol groups to disulfides. As HSP70 contains cys-

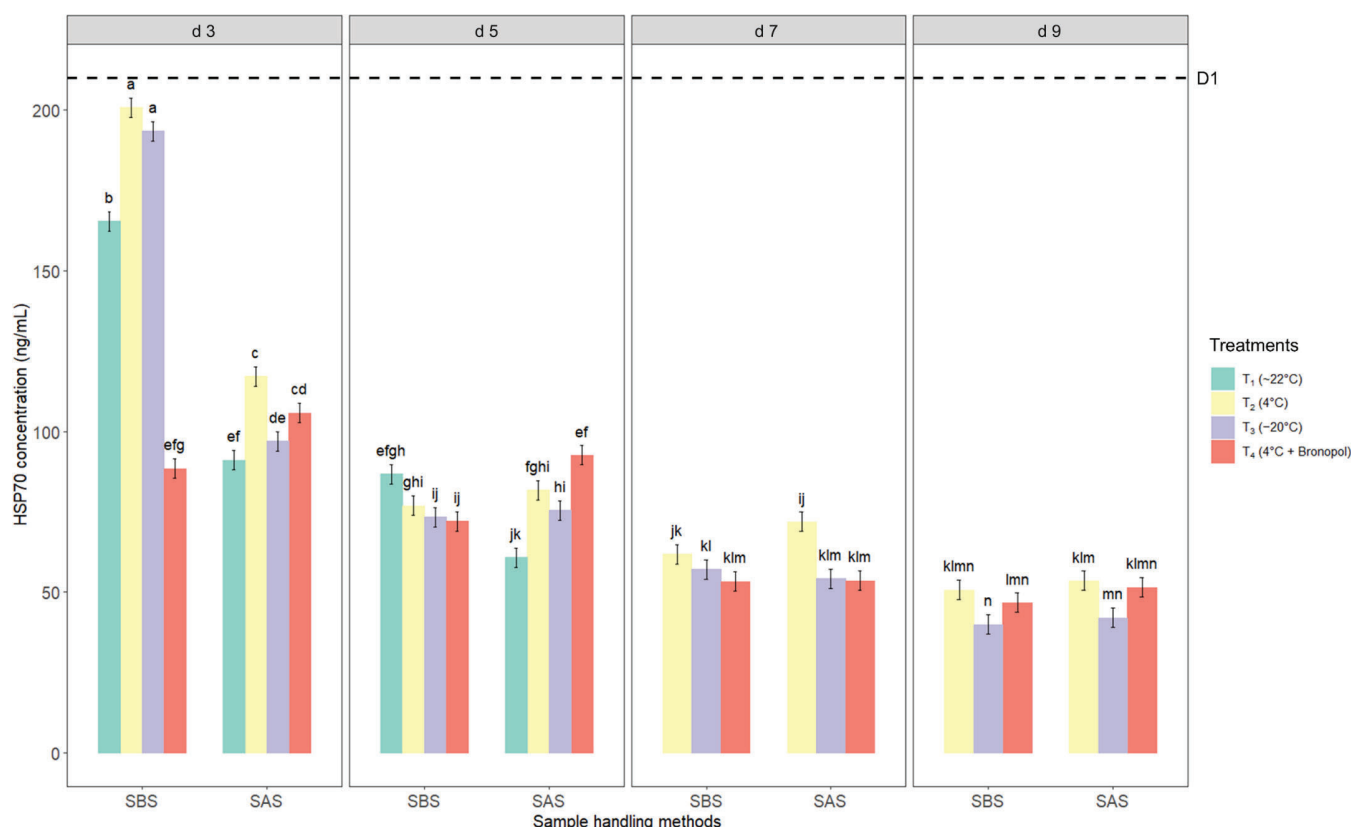


Figure 2. Effect of sample handling methods, storage conditions, and storage time on HSP70 concentration (ng/mL) in skim milk before and after storage. Vertical lines represent the SEM. Bars with different letters indicate significant differences ($P < 0.05$) based on Sidak-adjusted pairwise comparisons within each combination of sample handling method, storage condition, and storage time. The dashed line represents expression of HSP70 at d 1 without storage effect.

teine residues with reactive thiol groups, bronopol may alter its structure, potentially affecting antibody recognition and detection in ELISA (Shepherd et al., 1988; Zhang et al., 2022). This aligns with findings from other ELISA-based studies suggesting that bronopol can interfere with specific assay components. Thomas et al. (2016) observed altered ELISA readings for C-reactive protein in bronopol-treated milk, whereas Wilson et al. (2012) reported reduced detection of anti-bovine viral diarrhea antibodies in bronopol-preserved milk stored at room temperature. Klintevall et al. (1991) further demonstrated that excessively high concentrations of bronopol led to reduced ELISA absorbance values, likely due to interference with colorimetric detection rather than analyte degradation. These studies support the interpretation that bronopol may interact with the ELISA system possibly affecting antibody binding or enzymatic activity resulting in lower apparent concentrations. This highlights the need for further validation of preservative compatibility in analyte-specific ELISA and cautions against assuming uniform preservative behavior across assay types.

From d 11 to 15, both SBS and SAS samples exhibited an unexpected rise in HSP70 levels, suggesting that prolonged storage might influence protein stability and interactions. One possible explanation is the degradation of proteins, which could release HSP70 previously bound to other molecules, thereby increasing

its detectable levels. Another possibility is the denaturation of proteins, which could lead to cross-reactivity with the test assay, as hypothesized by Wynands et al. (2017). These authors evaluated the effect of storage temperature and the time from milk sample collection to laboratory analysis on pregnancy-associated glycoproteins levels using ELISA for pregnancy diagnosis in dairy cows. While such increases may not significantly affect routine diagnostic tests for producers, they could have implications for research-focused sample storage and the interpretation of results.

To the best of our knowledge, this is the first study to investigate the effects of sample storage and skimming on HSP70 detection in bovine milk, providing important insights into ways for minimizing its degradation. Our findings reveal that storing skim milk refrigerated at 4°C or frozen at -20°C until d 3 maximizes protein stability and minimizes protein degradation allowing detection of HSP70 with a competitive ELISA system. In contrast, room temperature storage decreased stability of HSP70 leading to protein degradation process, especially in SAS samples. The centrifugation process in SBS samples appeared to enhance protein stability, minimizing HSP70 degradation process. In contrast, bronopol-preserved samples showed lower HSP70 levels compared with those stored at 4°C without preservative, suggesting a potential interaction between the preservative and HSP70, though further research is

needed to confirm this. These results provide practical guidelines for farmers and processors to preserve milk sample integrity for assessment of HSP70 as indicator of cellular and heat stress.

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Notes

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The study was conducted in accordance with ethical guidelines and received approval from the Animal Ethics Authority at The University of Sydney, Australia (Project Identifier no.: 2022/AE002230).

The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: GLS = generalized least squares; HS = heat stress; HSF = heat shock factors; HSP = heat shock proteins; SAS = skimmed after storage; SBS = skimmed before storage; T₁ = room temperature (~22°C); T₂ = refrigerated (4°C); T₃ = frozen (–20°C); T₄ = adding bronopol preservative and refrigerated (4°C).

CHAPTER 5

Detecting heat shock protein 70 in milk, blood, and saliva of dairy cows exposed to different seasonal conditions

Establishing analytical reliability (Chapter 3) and appropriate sample handling procedures (Chapter 4) enabled investigation of the biological variability of HSP70 across milk, blood, and saliva under different seasonal conditions. Chapter 5 reports on associations between HSP70 concentrations and environmental heat load to determine whether HSP70 reflects seasonal changes in heat stress. These findings provide the first integrated assessment of matrix-specific responses prior to the temporal analyses presented in subsequent chapters.

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Detecting heat shock protein 70 in milk, blood, and saliva of dairy cows exposed to different seasonal conditions

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ABSTRACT

Heat stress (HS) negatively affects the health, reproduction, and milk production of dairy cows, indicating a major challenge to animal welfare and farm profitability. Although environmental and physiological indicators are commonly used to monitor HS, cellular biomarkers such as heat shock protein 70 (HSP70) offer a promising approach for detecting HS at the molecular level. This research aimed to evaluate and compare HSP70 levels in the milk, blood, and saliva of lactating dairy cows across seasons, assess their environmental responsiveness using the temperature-humidity index (THI) as the standard environmental indicator of HS under temperate Australian conditions, and investigate the potential of milk HSP70 as a noninvasive biomarker of HS at the cellular level. Twenty multiparous Holstein Friesian cows were monitored across 4 seasons (summer, autumn, winter, and spring) over 1 yr, and HSP70 concentrations were measured using a competitive ELISA system. Weather data, including THI, as well as milk yield and composition traits, were also recorded. Results showed significant seasonal variation in HSP70 levels, with the highest concentrations observed in summer across all sample types. Blood exhibited the highest (416.45 ng/mL) HSP70 concentrations overall, followed by saliva (304.89 ng/mL) and milk (279.02 ng/mL). Milk HSP70 showed the highest variability (CV = 53.76%), indicating that additional factors beyond HS may influence its expression, whereas blood and saliva appeared to be more consistent. The associations between HSP70 concentrations in biological fluids were initially evalu-

ated using Pearson correlation analysis. To account for repeated measurements in cows and identify key predictors, linear mixed-effects models with cow identification as a random effect were then fitted. Moderate positive correlations were found between the concentration of milk HSP70 and both blood ($r = 0.58$) and saliva HSP70 ($r = 0.53$), indicating that milk HSP70 may reflect systemic stress responses in dairy cows. Multi-variable linear mixed models identified average THI as a significant predictor of HSP70 concentrations across fluids. In the milk HSP70 model, DIM was also a significant predictor, and milk yield was not. Overall, this study provides preliminary evidence that milk HSP70 may have potential as a noninvasive indicator of HS in dairy cows. These findings also contribute to a better understanding of HS physiology and may inform future approaches for herd-level monitoring and management under climate change.

Key words: biomarkers, heat stress, HSP70, noninvasive

INTRODUCTION

Heat stress (HS) poses a major challenge in dairy farming, reducing milk production, impairing reproduction, and compromising animal welfare (West, 2003; Sammad et al., 2020). Effective monitoring and mitigation of HS are crucial for maintaining dairy cow productivity and farm sustainability. A range of methods, including environmental indicators and physiological, behavioral, and molecular biomarkers, have been developed to detect and monitor HS in dairy cows (Rakib et al., 2024). Among these, visual observation remains the simplest and most widely used method on farms, including signs such as increased respiration rate, panting, drooling, and reduced activity (Shu et al., 2021). Declines in milk yield are also used as indirect evidence of HS; however, they reflect

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

cumulative rather than real-time stress responses (Tao et al., 2020).

The temperature-humidity index (THI) is the most widely used measure to quantify environmental HS in dairy cattle and remains the industry standard across both experimental and on-farm monitoring systems (Polsky and Von Keyserlingk, 2017; Islam et al., 2020). Even though it has some limitations, such as not accounting for individual-cow variability or environmental factors like wind speed and solar radiation (Dunshea et al., 2013), THI remains the standard approach for assessing environmental HS because of its extensive validation, simplicity, and historical consistency across research. To improve upon these aspects, composite indexes such as the Grazing Heat Load Index have been developed (Bryant et al., 2023) and recently updated for New Zealand dairy systems (Hitchman et al., 2025).

At the individual-cow level, rectal temperature is considered the gold standard for measuring core body temperature; however, this measurement can be labor-intensive, and influenced by defecation, handling stress, and measurement frequency (Islam et al., 2021). Sensor technologies such as boluses, ear tags, and neck collars offer near real-time monitoring of temperature and behavior, although their cost may restrict widespread adoption, especially in smaller herds (Islam et al., 2020). In addition, even though accelerometer-based systems that monitor lying, standing, and rumination behaviors are among the few that have been externally validated, several other commercial sensors, including cameras, milk sensors, boluses, and load cells, remain unvalidated or provide inconsistent performance across various farm conditions, with only 14% of them going through external validation (Stygar et al., 2021). Another technology, infrared thermography, has also emerged as a noninvasive tool to detect changes in surface temperature across different body regions including the eye, tail, muzzle, vulva, and udder; however, its accuracy can be influenced by environmental factors such as wind speed, humidity, and solar radiation (Church et al., 2014).

Indicators of HS at the cellular level have received less attention. Heat shock proteins (HSP), particularly HSP70, have emerged as promising biomarkers because of their role in cellular function under elevated temperatures (Hyder et al., 2017). Elevated HSP70 levels have been reported under HS in various livestock species, including beef cattle (Gaughan et al., 2013), dairy cows (Lamy et al., 2017; Pathirana and Garcia, 2022), pigs (Khazzaka et al., 2006), and poultry (Xia et al., 2013) using different sample types. Although many studies focus on HSP70 detection in blood, either through plasma (Haque et al., 2012) or peripheral blood-mononuclear cells (Bhanuprakash et al., 2016), others have explored salivary (Lamy et al., 2017; Rajamanickam et al., 2025) and milk-based detec-

tion methods (Pathirana and Garcia, 2022; Rakib et al., 2025). Mojsym et al. (2022) compared protein patterns in plasma and saliva of pregnant cows and found that saliva partially reflects the systemic protein profile of plasma, which suggests that blood may be a more sensitive matrix for detecting systemic physiological changes. However, no previous research has concurrently compared HSP70 concentrations in milk, blood, and saliva across different environmental conditions.

Although milk sampling provides a noninvasive, stress-free alternative for HS monitoring, existing research has been limited to short-term assessments, without considering seasonal variation or comparing HSP70 expression across multiple biological fluids (Pathirana and Garcia, 2022). This also highlights the need for broader studies that evaluate the HSP70 levels in milk alongside blood and saliva across different seasons, while also accounting for environmental conditions and production traits. The integration of HSP70 measurements with other physiological and behavioral variables could enhance predictive models, leading to more accurate HS detection. Our null hypothesis was that HSP70 concentrations would be similar across blood, milk, and saliva and that all 3 fluids would exhibit seasonal variation in response to environmental conditions. Therefore, the objective of this study was to evaluate and compare the concentrations of HSP70 in milk, blood, and saliva of lactating dairy cows across 4 seasons, assess their environmental responsiveness using THI as the standard environmental indicator of HS, and investigate the potential of milk HSP70 as a noninvasive biomarker of HS at the cellular level.

MATERIALS AND METHODS

Study Location and Experimental Animals

The study was conducted at Corstorphine Dairy Farm, The University of Sydney, Camden, New South Wales, Australia, over one year, during winter and spring of 2023, followed by summer and autumn of 2024. Twenty multiparous Holstein Friesian cows in their third parity were selected for the study (Figure 1). The majority of cows were maintained across all 4 seasons; however, due to unavoidable events, one cow was replaced in spring following death, and 9 cows were replaced in autumn after being dried off. Individual-cow variability, including animal replacement, was accounted for in the statistical analyses. The average DIM at the time of seasonal sampling was 116.5 ± 33.9 d in winter, 174.8 ± 33.0 d in spring, 263.0 ± 29.9 d in summer, and 303.9 ± 33.1 d in autumn ($n = 20$ per season; $\pm$ SD).

Multiparous cows were selected to reduce variability associated with parity-related changes in milk production, metabolic status, and stress response. All cows were

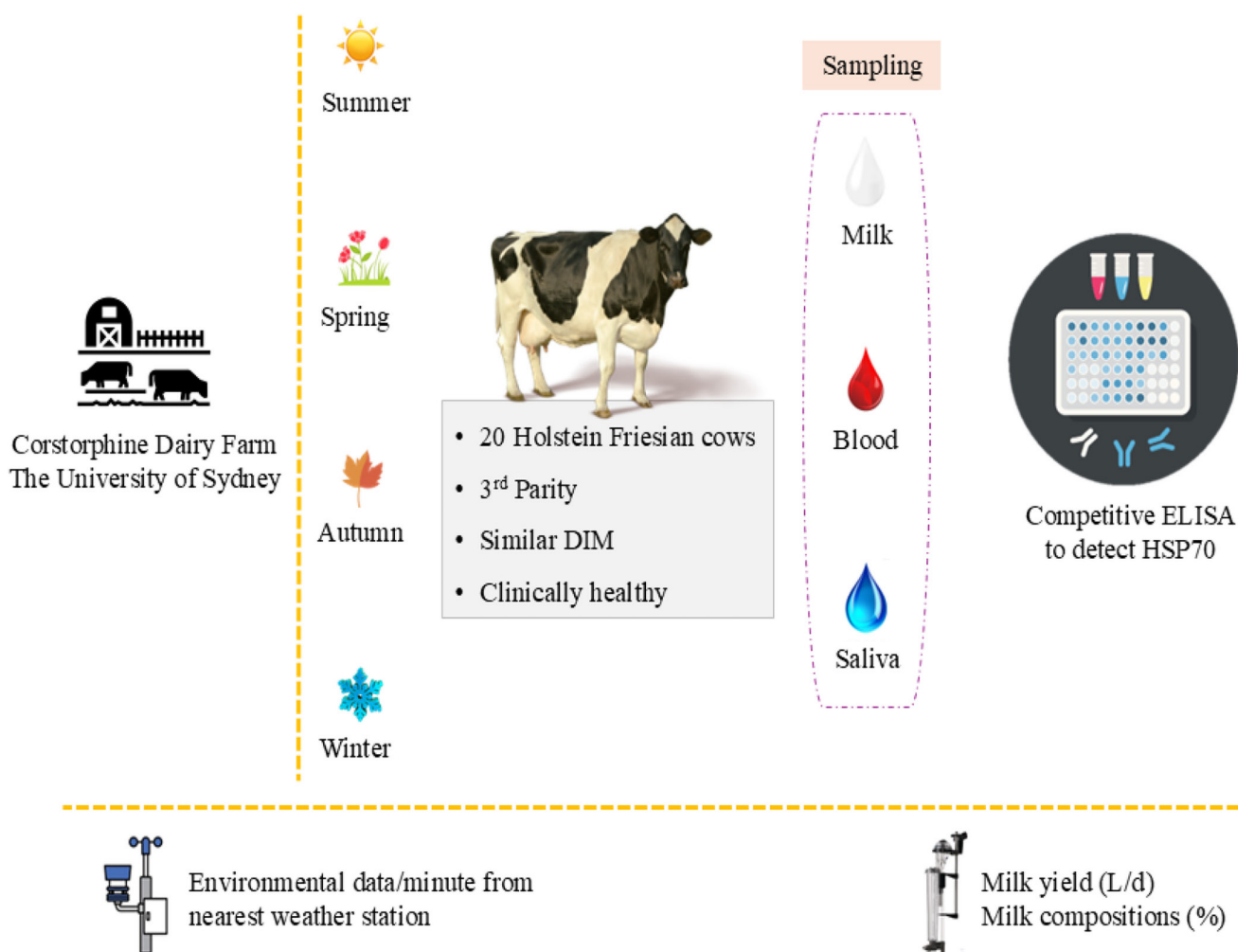


Figure 1. Experimental design for detecting HSP70 in milk, blood, and saliva of dairy cows under different seasons.

clinically healthy and a free from lameness, mastitis, or other health issues to avoid confounding effects on HSP70 levels. Cow health was assessed daily by trained farm staff during milking and herd checks, with formal herd health evaluations conducted fortnightly.

Cows were maintained on pasture throughout the year and were provided an estimated total feed allowance of ~26 kg of DM per head per day, including ~8 kg of pelleted concentrate offered during milking, along with mixed forages consisting of silage, fescue or prairie grass mix, and seasonal pasture species (ryegrass in winter and spring, and Kikuyu in summer and autumn) according to farm feeding records and the nutritionist's formulation. The concentrate composition varied slightly between batches, but generally comprised high-energy grains, canola meal, minerals, vitamins, and additives. Feed composition and pasture quality were assessed monthly, and diets were adjusted as necessary by the farm nutritionist to meet the nutritional requirements of lactating dairy cows and maintain consistency across seasons. Feeding management was uniform for all cows,

and nutrition was not treated as an experimental variable in this study. Cows had year-round access to pasture with natural tree shelter and were not housed.

The study was conducted in accordance with ethical guidelines and received approval from the Animal Ethics Authority at The University of Sydney, Australia (Project Identifier No: 2022/AE002230).

Environmental Data Collection

Ambient temperature and relative humidity were recorded every minute throughout the study period from the Camden Airport Automatic Weather Station, located 3.17 km from the farm (BOM, 2024), a licensed and quality-controlled dataset. These variables were used to calculate the THI using the equation of NRC (1971), which is commonly applied in Australian pasture-based dairy systems (Islam et al., 2020). Moreover, Dikmen and Hansen (2009) reported a moderate correlation ($r = 0.51$) between the (NRC, 1971) based THI and rectal temperature under subtropical conditions:

$$\text{THI} = (1.8 \times T + 32) - [(0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T - 26.8)],$$

where THI = temperature-humidity index, T = ambient temperature (°C), and RH = relative humidity (%).

For each sampling day, THI was averaged from 1-min weather data recorded on that day, and these THI values were used for all statistical analyses, including seasonal comparisons and correlation analyses with HSP70 concentrations and production traits. Based on this method, average THI values during sampling were highest in summer (82.49), followed by autumn (71.90), spring (70.23), and winter (67.51).

Sample Collection and Processing

Milk, blood, and saliva samples were collected from 20 multiparous cows during the afternoon milking (1300–1500 h) once per season (summer, autumn, winter, and spring), providing 4 structured sampling points across the year. Cows were milked twice daily, and sampling was standardized to the afternoon milking to ensure consistency across seasons and to correspond with peak daily heat load. Sampling dates were selected to represent the midpoint of each season, based on prevailing THI conditions. Milk composition (fat, protein, SNF, and lactose) was analyzed for comparative purposes using milk from the same afternoon sampling by an ultrasonic milk analyzer (Master Eco, Milkotester, Bulgaria). For each sample type, 2 replicate samples were processed and analyzed for HSP70 on the same day in the laboratory. To reduce technical variability, replications were averaged before statistical analysis.

Approximately 250 mL of milk per cow was collected into sterile snap-seal bottles during routine afternoon milking in a herringbone parlor using Waikato Milking Systems equipment (Waikato Milking Systems, Hamilton, New Zealand). Samples were collected via the in-line sampling port to ensure representation from all 4 quarters of the udder. Cows were milked twice daily in the parlor and grazed on pasture between milking sessions. Samples were immediately placed on ice and transported to the laboratory for analysis. Milk was centrifuged at $1,000 \times g$ for 10 min at 4°C to separate the skim milk fraction (Pathirana and Garcia, 2022). Milk yield (L/d) was recorded for each cow using WB Ezi-Test milk meters (Tru-Test, New Zealand) during both morning and afternoon milking sessions on each seasonal sampling day.

Blood samples (10 mL) were collected from the coccygeal vein immediately after milk collection during the afternoon milking, according to Gaughan et al. (2013)

using lithium heparin-coated Vacutainers (BD Vacutainer, BD-Plymouth, UK). Immediately after collection, the samples were placed in an ice box (2°C–4°C) and transported to the laboratory within 30 min. Blood samples were centrifuged at $1,328 \times g$ for 10 min at 2°C to obtain blood plasma (Gaughan et al., 2013), which was divided into 5-mL aliquots for analysis.

Saliva samples were collected using Salivette cotton rolls (Sarstedt, GmbH, Germany), which were held in the cheek pouch for ~30 s to absorb saliva. Immediately after collection, the samples were placed in an ice box (2°C–4°C) and transported to the laboratory within 30 min. Saliva samples were centrifuged at $1,000 \times g$ at 4°C for 5 min to obtain the fresh saliva supernatant, which was then divided into 5-mL aliquots for ELISA assay (Lamy et al., 2017).

HSP70 Measurement

The HSP70 concentrations in milk, blood, and saliva samples were assessed in duplicates with a competitive ELISA system as previously described by Pathirana and Garcia (2022). Briefly, 96-well polystyrene microtiter plates (Nunc Immuno Polysorp, Rochester, NY) were coated with recombinant human HSP70 (ADI-NSP-555, Enzo Life Sciences, Farmingdale, NY) and incubated overnight at 4°C. Before assay, samples and standards were pre-incubated with a monoclonal anti-human HSP70 antibody (ADI-SPA-810, Enzo Life Sciences) at 4°C overnight. The antibody-sample mixtures were then transferred to the blocked wells. Following incubation and washing steps, a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (BML-SA204, Enzo Life Sciences) was added. The color reaction was developed using 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, MO) and stopped with 1 N hydrochloric acid. Absorbance was measured at 450 nm using an ELISA plate reader (MR9600, Accuris, NJ). The intra- and interassay CV were 8.21% and 14.27%, respectively ($n = 3$ each). The assay demonstrated a detection limit of 31.25 ng/mL, with reliable quantification within the range of 31.25 to 2,000 ng/mL.

Because milk yield can fluctuate seasonally and potentially dilute biomarker concentrations, HSP70 values in milk (ng/mL) were normalized to daily milk production. This adjustment allowed estimation of the total HSP70 yield per cow (ng/d) according to the following formula:

$$\text{Milk HSP70 yield (ng/d)} = \text{milk HSP70 concentration (ng/mL)} \times \text{milk yield (L/d)} \times 1,000.$$

Table 1. Descriptive statistics for HSP70 concentrations in milk, blood, and saliva, and milk production traits across all seasons

Variable	Mean	SD	CV%	Minimum	Maximum
Milk HSP70 concentration (ng/mL)	279.02	150.01	53.76	76.45	546.93
Blood HSP70 concentration (ng/mL)	416.45	143.14	34.37	183.17	1,156.44
Saliva HSP70 concentration (ng/mL)	304.89	98.65	32.35	142.51	650.65
Milk HSP70 yield (ng/d)	5.89×10^6	3.24×10^6	55.03	1.07×10^6	13.6×10^6
Milk yield (L/d)	23.64	9.68	40.94	3.00	51.25
Fat (%)	4.11	1.75	42.51	1.10	9.60
SNF (%)	8.77	0.42	4.82	8.00	9.80
Protein (%)	3.20	0.14	4.45	2.95	3.55
Lactose (%)	4.80	0.23	4.85	4.35	5.35

Statistical Analysis

Data processing, statistical analysis, and visualization were conducted using R (version 4.5.0; <https://www.r-project.org>) statistical software. Descriptive statistics, including mean, SD, CV (%), and range (minimum–maximum), were computed for HSP70 concentrations in milk, blood, and saliva, as well as for milk HSP70 yield, milk yield, and key milk composition parameters (fat, SNF, protein, and lactose; Table 1). To assess associations between HSP70 concentrations in milk, blood, and saliva, as well as with milk production traits, Pearson correlations and pairwise linear mixed-effects models (*lme4* and *lmerTest* packages) were used. For each pairwise model, HSP70 concentration in one fluid was treated as the dependent variable and HSP70 from another fluid as the explanatory variable, with cow identification (**ID**) included as a random effect to account for repeated measures. Model performance was summarized using both marginal and conditional R^2 (using the *MuMIn* package).

In addition, univariable linear mixed models were used to evaluate the associations between HSP70 concentrations in each fluid and a set of production, physiological, and environmental variables, with cow ID as a random effect. Subsequently, multivariable linear mixed-effects models were developed to identify factors jointly associated with HSP70 concentrations in each fluid. Variables with P -values <0.20 in the univariable analysis were considered for inclusion in the initial multivariable model. Pairwise correlations were used to assess multicollinearity among candidate predictors, and variables with correlation coefficients >0.7 were not included together in the same model. Final models were obtained using manual backward elimination, retaining only variables with $P < 0.05$.

Finally, a separate mixed-effects model was fitted to assess the effects of season, sample type (milk, blood, and saliva), and their interaction on HSP70 concentrations, also including cow ID as a random effect. An ANOVA-like analysis was performed on the fitted model, and estimated marginal means (**EMM**) were compared using the *emmeans* and *multcomp* packages, with Sidak adjustment

to control for multiple testing. For each model, the fixed-effect estimate, SE, 95% CI, P -value, and both marginal and conditional R^2 (using *MuMIn* package) were reported. Data were visualized using bar plots (*ggplot2* package) with SE bars and statistical group annotations based on the adjusted comparisons.

RESULTS

Descriptive Statistics for HSP70 and Milk Parameters

Among the HSP70 measures, milk HSP70 concentration ranged from 76.45 ng/mL to 546.93 ng/mL and showed the greatest variability (CV = 53.76%), followed by the HSP70 concentrations in blood (34.37%) and saliva (32.35%) samples (Table 1). Milk HSP70 yield (ng/d) also exhibited high variability (CV = 55.03%). However, milk yield (L/d) and milk fat also exhibited considerable variation (CV = 40.94% and 42.51%, respectively), whereas SNF (%), milk protein (%) and lactose (%) had lower variability (CV $< 5\%$).

Association Between HSP70 and Production Traits

Milk HSP70 concentration was negatively correlated with milk yield ($r = -0.49$, $P < 0.01$) and showed weaker negative correlations with milk components including SNF%, protein%, and lactose% ($r = -0.05$ to -0.09 ; Figure 2). However, this association between milk HSP70 concentration and milk yield was not significant ($P = 0.39$) after adjusting DIM in the multivariable linear mixed-effects model. Blood and saliva HSP70 concentrations also exhibited negative correlations with production traits, although these associations were generally weaker (Figure 2). However, moderate positive correlations were observed among the 3 HSP70 sources, particularly between milk and blood ($r = 0.58$, $P < 0.001$), and milk and saliva ($r = 0.53$, $P < 0.001$), indicating a consistent pattern of HSP70 expression across sample types. In addition, the linear mixed model showed that milk HSP70 concentrations were significantly associated with both blood ($P < 0.001$; marginal and conditional $R^2 = 32.94\%$)

Table 2. Linear mixed model estimates of associations between HSP70 concentrations (ng/mL) in milk, blood, and saliva samples (n = 80)¹

Model	Estimate	SE	CI lower	CI upper	P-value	R_m^2	R_c^2
Milk HSP70 ~blood HSP70	0.60	0.10	0.41	0.79	<0.001	32.94%	32.94%
Milk HSP70 ~saliva HSP70	0.80	0.15	0.51	1.09	<0.001	27.31%	27.31%
Blood HSP70 ~saliva HSP70	0.72	0.14	0.46	0.98	<0.001	23.17%	40.17%

¹y ~x indicates that variable y is the response variable and x is the explanatory variable in the mixed model. Estimate = regression coefficient; CI lower = lower bound of the 95% CI; CI upper = upper bound of the 95% CI. R_m^2 = marginal R-squared (variance explained by fixed effects); R_c^2 = conditional R-squared (variance explained by both fixed and random effects).

and saliva HSP70 ($P < 0.001$; marginal and conditional $R^2 = 27.31\%$), accounting for repeated measures within cows (Table 2). A strong positive association was also observed between blood and saliva HSP70 ($P < 0.001$; marginal $R^2 = 23.17\%$; conditional $R^2 = 40.17\%$).

Factors Associated with HSP70 Concentrations

Univariable linear mixed models were conducted to examine the individual associations between HSP70 con-

centrations in milk, blood, and saliva and various production, physiological, and environmental factors (Table 3). Milk yield (L/d) showed a strong negative association with milk HSP70 concentrations ($P < 0.001$), explaining 23.92% of the variance. Although the associations between milk yield and HSP70 concentrations in blood and saliva were not significant, the direction of the effect remained consistent across all sample types.

Days in milk were significantly associated with HSP70 concentrations across all sample types, with marginal R^2

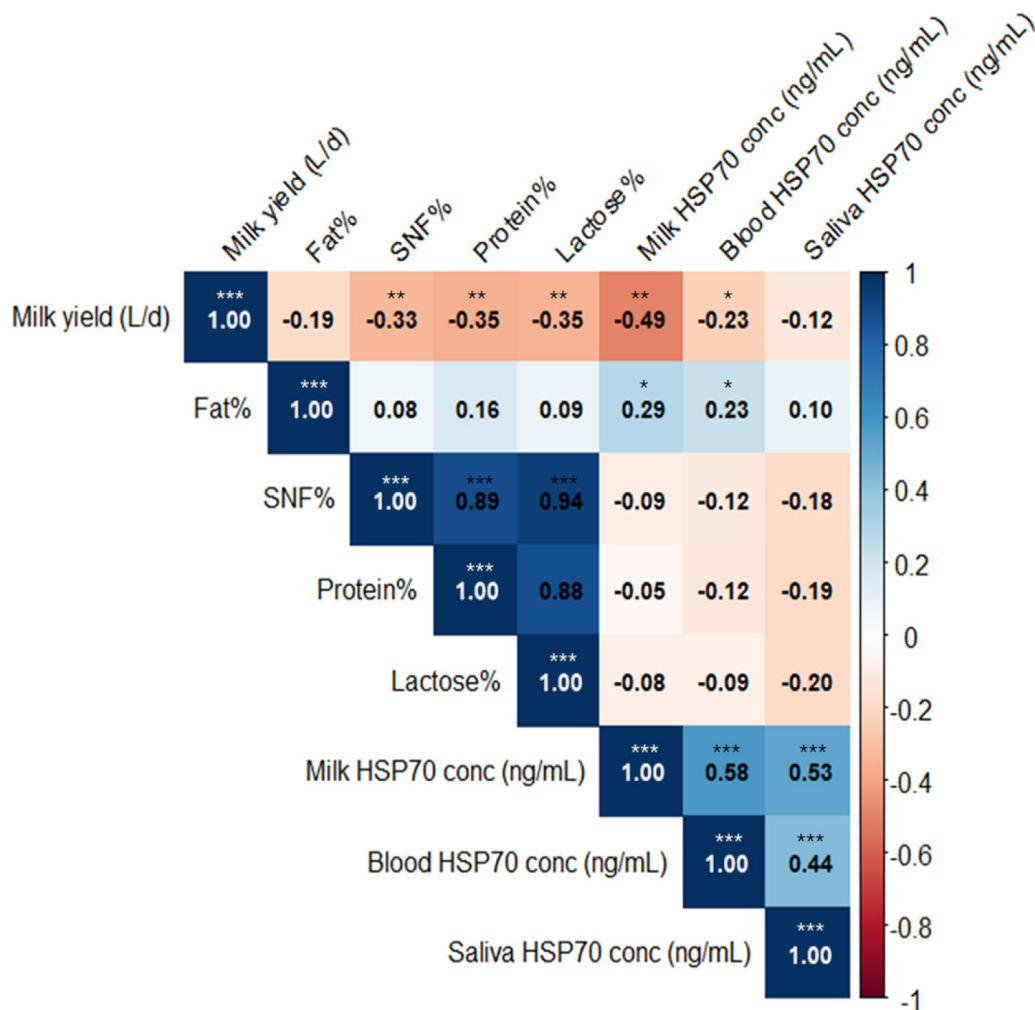


Figure 2. Pearson correlation matrix showing relationships among HSP70 concentrations (milk, blood, and saliva) and milk production traits. Positive correlations are shown in blue and negative correlations in red, with color intensity indicating the strength of the association. Asterisks indicate significant correlations (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). conc = concentration.

Table 3. Effects of production, physiological, and environmental variables on milk, blood, and saliva HSP70 concentrations based on univariable linear mixed-effects modeling¹

Variable	Estimate	SE	CI lower	CI upper	P-value	R_m^2	R_c^2
Milk HSP70 (ng/mL)							
Milk yield (L/d)	-7.62	1.53	-10.61	-4.62	<0.001	23.92%	23.92%
Fat (%)	24.50	9.32	6.24	42.75	0.01	8.04%	8.04%
SNF (%)	-32.61	39.99	-110.94	45.73	0.42	0.83%	0.83%
Protein (%)	-48.64	119.30	-282.33	185.04	0.69	0.21%	0.21%
Lactose (%)	-54.60	72.82	-197.23	88.03	0.46	0.71%	0.71%
DIM	1.482	0.11	1.23	1.70	<0.001	68.47%	74.56%
Average THI	21.56	1.70	18.24	24.88	<0.001	67.21%	67.21%
Blood HSP70 (ng/mL)							
Milk yield (L/d)	-3.77	1.64	-7.08	-0.39	0.02	6.34%	13.08%
Fat (%)	19.34	9.18	1.17	37.58	0.04	5.48%	8.74%
SNF (%)	-40.26	38.32	-115.21	34.78	0.30	1.39%	3.31%
Protein (%)	-126.67	113.79	-349.14	96.05	0.27	1.56%	3.40%
Lactose (%)	-53.90	69.86	-190.37	82.57	0.44	0.75%	2.27%
DIM	0.65	0.16	0.31	0.97	<0.001	15.33%	26.40%
Average THI	15.44	1.96	11.58	19.30	<0.001	37.82%	52.12%
Saliva HSP70 (ng/mL)							
Milk yield (L/d)	-1.23	1.15	-3.48	1.01	0.28	1.45%	1.45%
Fat (%)	5.57	6.36	-6.89	18.04	0.38	1.00%	1.00%
SNF (%)	-42.03	25.98	-92.92	8.86	0.11	3.21%	3.21%
Protein (%)	-133.9	77.06	-284.84	17.04	0.09	3.68%	3.68%
Lactose (%)	-84.75	47.09	-176.99	7.48	0.08	3.94%	3.94%
DIM	0.39	0.12	0.16	0.62	<0.01	12.03%	12.03%
Average THI	13.21	1.26	10.75	15.68	<0.001	58.30%	58.30%

¹CI lower = lower bound of the 95% CI; CI upper = upper bound of the 95% CI; R_m^2 = marginal R-squared (variance explained by fixed effects); R_c^2 = conditional R-squared (variance explained by both fixed and random effects).

values of 68.47% (milk), 15.33% (blood), and 12.03% (saliva). Among environmental variables, average THI explained the largest proportion of variance in HSP70 concentrations ($P < 0.001$ for all) for milk (conditional $R^2 = 67.21\%$), blood (52.12%), and saliva (58.30%) samples. Other variables, including ambient temperature, RH, and solar radiation were also significantly associated with HSP70 concentrations but explained less variation. Because these environmental variables are seasonally regulated, the results align with observed seasonal trends in HSP70 expression.

Multivariable linear mixed-effects models were then fitted to identify key predictors associated with HSP70 concentrations across fluids (Table 4). For milk HSP70, both average THI and DIM were significant predictors ($P < 0.001$), and although milk yield was included in the model it was not significant ($P = 0.39$). Together, these variables explained a large proportion of the variance (marginal $R^2 = 82.58\%$; conditional $R^2 = 95.70\%$). Days in milk was included as a covariate to account for differences in lactation stage, thereby standardizing HSP70 concentrations across cows at different stages of lactation. Blood HSP70 concentrations were significantly associated with average THI ($P < 0.001$), while for saliva HSP70, both milk yield ($P < 0.05$) and average THI ($P < 0.001$) were retained in the final model. These models confirmed the strong influence of

environmental conditions, particularly THI, across all biological fluids.

Seasonal Variation of HSP70

The HSP70 concentrations peaked in summer and generally declined toward winter across all biological fluids, with some variation in spring (Figure 3), although these seasonal trends do not account for DIM. Blood exhibited the highest HSP70 levels across all seasons ($P < 0.05$), with mean concentrations of 559.40 ng/mL in summer, followed by 425.54 ng/mL in autumn, 337.15 ng/mL in winter, and 343.72 ng/mL in spring. In contrast, milk and saliva showed significantly lower concentrations ($P < 0.05$). Milk HSP70 levels declined from 450.06 ng/mL in summer to 389.67 ng/mL in autumn, 95.14 ng/mL in winter, and then increased to 181.22 ng/mL in spring, remaining consistently lower than blood levels. Saliva followed a similar seasonal pattern to blood, peaking in summer (441.78 ng/mL) and showing comparable levels across autumn (269.77 ng/mL), winter (273.36 ng/mL), and spring (234.66 ng/mL), with no significant differences among the cooler seasons.

Moreover, to account for seasonal variation in milk volume and minimize the influence of dilution, milk HSP70 yield per cow (ng/d) was calculated to provide a clearer estimation of actual HSP70 secretion across

Table 4. Linear mixed model results showing factors associated with different HSP70 measurements¹

Factor	Estimate	SE	CI lower	CI upper	P-value	R_m^2	R_c^2
Milk HSP70 (ng/mL)							
(Intercept)	-912.54	61.10	-1,031.21	794.23	<0.001	82.58%	95.70%
Average THI	13.25	0.85	11.58	14.87	<0.001		
Milk yield (L/d)	0.66	0.75	-0.80	2.12	0.39		
DIM	1.04	0.08	0.88	1.20	<0.001		
Blood HSP70 (ng/mL)							
(Intercept)	-710.68	143.61	-993.54	-427.06	<0.001	37.82%	52.12%
Average THI	15.44	1.96	11.58	19.30	<0.001		
Saliva HSP70 (ng/mL)							
(Intercept)	-776.69	101.49	-974.24	-572.72	<0.001	60.33%	62.86%
Milk yield (L/d)	1.79	0.78	0.17	3.41	<0.05		
Average THI	14.23	1.28	11.69	16.71	<0.001		

¹CI lower = lower bound of the 95% CI; CI upper = upper bound of the 95% CI; R_m^2 = marginal R-squared (variance explained by fixed effects); R_c^2 = conditional R-squared (variance explained by both fixed and random effects).

seasons. Milk HSP70 yield increased steadily from winter (3.28×10^6 ng/d) to summer (9.23×10^6 ng/d), with intermediate values in autumn (7.21×10^6 ng/d) and spring (4.30×10^6 ng/d). Although HSP70 concentrations (ng/mL) differed significantly across all 4 seasons, the yield-based comparison showed a more gradual pattern, with no significant difference observed between winter and spring.

DISCUSSION

This study provides novel insights into the seasonal HSP70 variation in dairy cows by simultaneously evaluating 3 biological fluids (milk, blood, and saliva from the same animal) under varying seasonal conditions. Our model-based results (EMM, not adjusted for DIM) revealed that HSP70 levels were higher during summer and decreased during cooler seasons across all sample types, aligning with the reduction in HS. This pattern of HSP70 expression reflects a physiological adaptation to environmental heat load (Dangi et al., 2012; Manjari et al., 2015). Average THI values during sampling were highest in summer (82.49), followed by autumn (71.90), spring (70.23), and winter (67.51), indicating a clear seasonal pattern in HS exposure. Zimbelman et al. (2009) reported that milk yield in dairy cows begins to decline when THI exceeds 68, with potential losses of up to 2.2 kg/d. This threshold is approximately equivalent to the environmental conditions of 22°C at 45% RH or 24°C at 30% RH, showing that even moderate temperature and humidity levels can trigger early HS and reduce productivity. These findings also highlight that cows in this study were exposed to HS during a significant portion of the year and it was also reflected in the physiological responses observed across all sample types. Blood consistently exhibited the highest HSP70 levels across all seasons ($P < 0.05$) when compared with saliva and

milk samples, as previously reported in cattle, buffalo, and goats (Gaughan et al., 2013; Banerjee et al., 2014; Chen et al., 2025). The rise in HSP70 concentrations during summer likely reflects the animals' physiological response to higher HS, while the gradual decrease across the cooler seasons reflects reduced cellular stress under milder environmental conditions. Given the observed associations with THI, an essential next step would be to determine how many more or less cases of HS may be identified if HSP70 could be used alongside or instead of conventional indicators.

Salivary HSP70 followed a similar seasonal pattern to blood, peaking in summer (441.78 ng/mL) and showing comparable concentrations across autumn (269.77 ng/mL), spring (234.66 ng/mL), and winter (273.36 ng/mL), with no significant differences among the cooler seasons. These findings are consistent with those of Lamy et al. (2017), who first confirmed the presence of HSP70 in bovine saliva and observed significantly higher levels during summer in high-producing cows. They also reported that salivary HSP70 did not correlate with rectal temperature, suggesting that its expression may be influenced by factors beyond acute thermal load, such as metabolic status, energy balance, or local salivary gland regulation. The relatively stable concentrations observed in cooler seasons may reflect a threshold level of salivary HSP70, highlighting the need for further research to clarify its diagnostic value.

Milk HSP70 concentrations were consistently lower in absolute terms but demonstrated a significant ($P < 0.05$) seasonal decline from 450.06 ng/mL in summer to 389.67 ng/mL in autumn, 181.22 ng/mL in spring, and 95.14 ng/mL in winter. This distinct pattern reinforces its potential as a noninvasive indicator of HS. Alhussien and Dang (2018) also reported higher relative expression of milk HSP70 during hot-humid seasons compared with winter and hot-dry seasons while investigating neutrophil func-

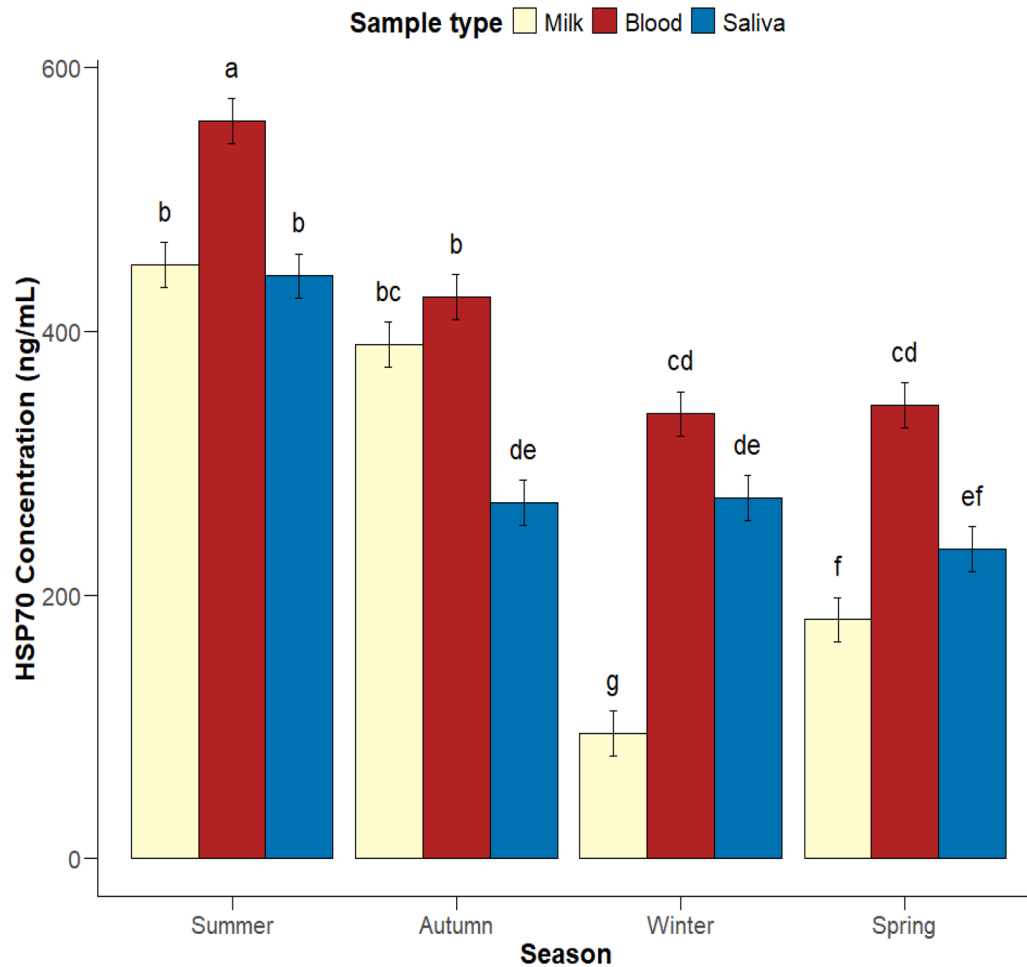


Figure 3. Effect of seasonal variation on HSP70 concentrations (ng/mL) in milk, blood, and saliva samples. Bars represent EMM ($\pm$ SEM) from a mixed-effects model with cow ID as a random effect (EMM not adjusted for DIM). Different lowercase letters (a–g) indicate significant differences between groups based on Sidak-adjusted pairwise comparisons ($P < 0.05$).

tionality in 3 Indian native cattle breeds. However, in the present study, milk HSP70 showed the highest variability among the sample types ($CV = 53.76\%$), with blood and saliva showing lower variability ($CV = 34.37\%$ and 32.35% , respectively). The calculated milk HSP70 yield (ng/d) also showed high variability ($CV = 55.03\%$), which suggests that milk may have lower sensitivity or reliability in detecting physiological changes compared with blood or saliva. The higher variability in milk could be attributed to dilution effects related to fluctuations in milk volume (Boland et al., 2013) or to local secretion dynamics within the mammary gland (Collier et al., 2008; Hu et al., 2016).

There were moderate positive correlations between milk and blood ($r = 0.58$, $P < 0.001$, marginal and conditional $R^2 = 32.94\%$), and between milk and saliva ($r = 0.53$, $P < 0.001$, marginal and conditional $R^2 = 27.31\%$), even though all samples were taken at the same time, further supports this relationship. These findings highlight the need to better understand the origin and biological transport of HSP70 into noninvasively collected fluids

like milk, as well as any lag time (potentially present) between peaks of HSP70 in the different fluids. Calderwood et al. (2007) reported that HSP70 can be actively released into extracellular fluids through physiological secretion mechanisms. Furthermore, studies have shown that bovine mammary epithelial cells not only express HSP70 but also significantly upregulate its gene expression and protein synthesis in response to HS, indicating that the mammary gland is a responsive source of HSP70 under stress conditions (Collier et al., 2008; Hu et al., 2016). The presence of HSP70 in milk has been confirmed in rats using ELISA-based detection methods (Liedel et al., 2011), and more recently in cow milk by Pathirana and Garcia (2022) and Rakib et al. (2025), highlighting the feasibility of milk as a noninvasive marker for monitoring HS in dairy cows.

The negative correlation between milk HSP70 concentration and milk yield ($r = -0.49$) initially suggested that elevated cellular stress may be associated with reduced lactational performance, consistent with previous findings for salivary HSP70 (Rajamanickam et al., 2025).

However, after accounting for lactation stage by including DIM as a covariate in the multivariable model, this association was no longer significant. This suggests that the relationship may be driven by physiological changes across the course of lactation or by a dilution effect, rather than a direct effect of stress on milk production. It is also possible that HSP70 reflects a delayed physiological response, with any resulting effect on milk yield occurring over a longer timeframe and requiring further study. In contrast, the weaker and inconsistent negative correlations between milk HSP70 and nutritional components such as SNF, protein, and lactose suggest that milk composition may also be affected under prolonged or severe HS conditions (Rakib et al., 2025). However, the relatively weaker correlations between blood and saliva HSP70 levels and milk production traits may be due to the different origins of these fluids. Milk is produced directly by the mammary gland, that is, where milk synthesis and secretion occur (Cai et al., 2018). In contrast, blood represents a systemic fluid reflecting whole-body stress responses (Edwards and Hoareau, 2021), while saliva is a locally secreted fluid (Soo-Quee Koh and Choon-Huat Koh, 2007), less directly connected to lactation. Given these differences, a lag effect in HSP70 detection in milk is highly likely, where elevated HSP70 concentrations may reflect cumulative cellular stress that eventually contributes to a decline in milk yield. Further investigation using larger and longitudinal datasets is necessary to confirm this relationship and to better understand both the immediate and delayed physiological responses to HS in dairy cows.

The strong association between DIM and HSP70 concentrations, particularly in milk (marginal $R^2 = 68.47\%$), may reflect both physiological changes during lactation and accumulated environmental exposure due to the design of this study. Yániz et al. (2009) reported increased HSP70 levels in bovine blood as gestation progressed, suggesting that the expression of HSP70 is modulated by physiological stage. Moreover, HSP70 synthesis can be triggered by a range of internal stressors beyond thermal load, including hypoxia, nutritional factors, aging, oxidative stress, and acidosis (Kregel, 2002; Rakib et al., 2024), which may become more pronounced as lactation progresses, potentially contributing to elevated HSP70 expression. Because the same cows were repeatedly sampled across seasons, except for one replacement in spring due to death and 9 in autumn due to drying off, DIM and season may be partially confounded in this design. However, the use of cows with similar lactation stages allowed for meaningful within-animal comparisons over time. Seasonal changes in milk HSP70 concentration may also be influenced by differences in milk volume. By calculating HSP70 yield (ng/d), we adjusted this dilution effect and observed a more conservative seasonal

pattern, particularly between winter and spring. This suggests that part of the variation in raw concentrations could reflect fluctuations in milk production rather than true shifts in HSP70 synthesis.

Further supporting this, multivariable linear mixed-effects models identified both DIM and average THI as significant predictors of milk HSP70, but milk yield was not significant after adjusting for lactation stage. For saliva HSP70, both milk yield and THI remained in the final model, and blood HSP70 was associated only with THI. These results show that environmental predictors (THI) consistently influenced HSP70 levels across all fluids, while other physiological factors varied depending on the sample type. Future research using separate groups of cows at standardized lactation stages across seasons could help separate these overlapping effects and better isolate the specific contribution of HS to HSP70 expression.

This study used the THI and seasonal variation to represent environmental HS, instead of using direct animal-based physiological indicators such as rectal temperature, respiration rate, core body temperature, or BW, which may also influence thermoregulatory capacity and metabolic heat load. Despite this limitation, the THI remains the industry standard and the most extensively validated environmental benchmark for evaluating HS in dairy cattle (Polsky and Von Keyserlingk, 2017), showing moderate-to-strong correlations with physiological indicators, including rectal temperature and milk yield (Bohmanova et al., 2007; Dikmen and Hansen, 2009; Ekine-Dzivenu et al., 2020). Gaughan et al. (2013) further demonstrated that plasma HSP70 concentrations in beef cattle significantly increased under prolonged heat exposure and exhibited a moderate correlation with core body temperature changes above 38.6°C , supporting the physiological significance of HSP70 as a marker for HS. In addition, HSP70 measurement in this study was performed using ELISA as a laboratory-based research tool for biomarker validation, rather than as a method intended for direct on-farm implementation.

Moreover, the use of weather station data, although practical, may not fully capture the actual climatic conditions experienced by cows within farm environments. On-site environmental sensors and continuous physiological monitoring (e.g., rectal temperature, infrared thermography, and sensor-based core body temperature) would improve the precision of future assessments. Furthermore, incorporating time-series sampling during acute heat events (e.g., heatwaves) and increasing sample size will be crucial to characterizing both immediate and delayed HSP70 responses, as well as for evaluating the sensitivity and specificity of HSP70 in milk, blood, and saliva relative to established HS thresholds. Collectively, these refinements in experimental design and real-time

monitoring will strengthen the physiological validation of HSP70 and enhance the robustness of future HS investigations.

Overall, this study provides valuable insights into how HSP70 levels in milk, blood, and saliva change with the seasons and different stages of lactation in dairy cows. These findings provide a strong basis for future research into the use of HSP70 as a tool for HS monitoring and for identifying heat-tolerant cows in dairy herd management.

CONCLUSIONS

This study provides the first comprehensive comparison of HSP70 concentrations in milk, blood, and saliva of dairy cows under different seasonal conditions. The findings confirm that HSP70 levels are highest during summer and are strongly influenced by THI under temperate Australian conditions, highlighting their utility as a biomarker of HS. Among the fluids evaluated, blood exhibited the highest HSP70 levels, and milk, despite lower concentrations and greater CV%, showed parallel seasonal trends and significant correlations with both production traits and other types of fluids. These results highlight the potential of milk HSP70 as a prospective noninvasive marker for HS monitoring in dairy cows. Further research is needed to incorporate animal-based physiological markers to validate these findings under large-scale and commercial conditions and to explore their role in the selection of heat-tolerant animals.

NOTES

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Nonstandard abbreviations used: conc = concentration; EMM = estimated marginal means; HS = heat stress; HSP = heat shock proteins; ID = identification; THI = temperature-humidity index.

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CHAPTER 6

Comparative evaluation of environmental, physiological, and milk HSP70 indicators for detecting heat stress in dairy cows

With seasonal variation in HSP70 quantified in [Chapter 5](#), [Chapter 6](#) undertakes a comparative evaluation of environmental and physiological indicators of heat stress alongside milk HSP70. Environmental indicators (e.g., temperature-humidity index), rectal temperature, infrared thermography, and sensor-derived reticulorumen temperature are evaluated in relation to milk HSP70 to examine their relative sensitivity, temporal lag, and practical usefulness. This chapter explains how these indicators differ in their responsiveness and defines the role of milk HSP70 within a multi-level heat stress detection framework.

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Comparative evaluation of environmental, physiological, and milk HSP70 indicators for detecting heat stress in dairy cows

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Abstract

Heat stress (HS) poses a major challenge to dairy productivity and cow welfare, yet available detection methods differ significantly in their sensitivity and responsiveness to environmental heat load. This study compared environmental, physiological indicators, including rectal temperature (RT), infrared thermography (IRT), and sensor-derived reticulorumen temperature (RRT), along with milk-based heat shock protein 70 (HSP70) indicators to evaluate their sensitivity, temporal lag, and usefulness, and to assess the potential of milk HSP70 as a non-invasive HS biomarker. Data were collected from 25 third-parity lactating Holstein Friesian cows (211.8 ± 21.3 DIM) managed in a pasture-based system over seven consecutive weeks in the Australian summer of 2024. Rectal temperature, IRT, milk HSP70, milk yield, and composition were measured twice weekly; RRT was recorded every 10 min via rumen boluses; and environmental variables, including temperature-humidity index (THI), were recorded every minute from the nearest weather station. Associations among indicators were examined using time-lagged correlation analyses and linear mixed models. Results revealed that RT ($r = 0.58$, $P < 0.001$; $R^2_c = 41.34\%$) and IRT ($r = 0.64$, $P < 0.001$; $R^2_c = 43.51\%$) showed the maximum associations with THI in real time. Sensor-derived RRT exhibited its highest association with THI at 0-8 h time-lag ($r = 0.42-0.43$, $P < 0.001$; $R^2_c = 29.23-30.52\%$). Milk HSP70 concentrations showed a significantly delayed association with THI, with the maximum

association at a 35 h time-lag ($r = 0.30$; $P < 0.001$; $R^2_c = 9.14\%$). Milk yield (L/d) declined in response to THI, with the maximum correlation at a 48 h lag ($r = -0.25$, $P < 0.001$; $R^2_c = 34.38\%$). RRT correlated with RT ($r = 0.74$ at 9 h, $P < 0.001$; $R^2_c = 55.67\%$) and IRT ($r = 0.62$ at 9 h, $P < 0.001$; $R^2_c = 42.71\%$), but weakly with milk HSP70 ($r = 0.26$ at 40 h, $P < 0.001$; $R^2_c = 7.17\%$). An initial milk HSP70 threshold of ~ 460 ng/mL, with relatively high sensitivity (0.833) but low specificity (0.435), was also identified. Overall, RT and IRT showed sensitivity to short-term changes in environmental heat load, while RRT reflected slower internal heat accumulation, and milk HSP70 was associated with delayed cellular stress responses. In this context, milk HSP70 shows potential as a supplementary biomarker that captures delayed HS responses and might provide a future avenue for monitoring broader physiological stress within integrated HS detection frameworks.

Keywords: Infrared thermography, non-invasive, rectal temperature, reticulorumen temperature, temperature-humidity index

Introduction

Heat stress (HS) is one of the most significant challenges in modern dairy farming, reducing milk production (Chen et al., 2024), affecting fertility (Liu et al., 2019), and compromising the health and well-being (Rakib et al., 2020) of dairy cows. The economic impact of HS on the dairy sector is substantial; it has been estimated to cost $\sim$ USD 8,500 per year for a 100-cow herd in Australia, although the overall losses are challenging to quantify due to its interacting effects on feed intake, milk production and quality, as well as reproductive performance (Lees, 2018). This challenge is more significant in pasture-based systems, which dominate Australian dairy production, as cows are directly exposed to variable environmental conditions (Chang-Fung-Martel et al., 2017).

Effective monitoring of HS is crucial for ensuring animal productivity, welfare, and the long-term sustainability of dairy farms. Timely detection enables the implementation of appropriate management strategies to mitigate its impact (Sejian et al., 2022). Providing shade, activating sprinklers or cooling systems, adjusting feeding schedules, or modifying grazing practices can help reduce the impact of HS and associated production losses (Dahl, 2018).

A variety of techniques have been developed for detecting HS in dairy cows, including environmental parameters, and animal-based indicators such as physiological and behavioural metrics (Becker et al., 2021; Rakib et al., 2024). Visual observation is considered the most straightforward and commonly used technique on farms (Shu et al., 2021), which includes looking for signs of increased respiratory rate, panting, drooling, and reduced activity (Tresoldi et al., 2018). Declines in milk production are commonly observed during HS; however, while milk yield is affected by various nutritional, physiological, and management factors, it reflects the accumulated impact of prior heat load rather than an immediate indicator of HS (Tao et al., 2020). The temperature-humidity index (THI) is the most widely used tool for evaluating HS; however, it lacks consideration of individual cow variability and excludes parameters including wind speed and solar radiation (Dunshea et al., 2013). New indices such as the grazing heat load index (GHLI), which incorporates air temperature, solar radiation, and wind speed, have been developed (Hitchman et al., 2025), however, these indicators are still indirect and unable to reflect the physiological state of individual cows (Rakib et al., 2024).

Rectal temperature (RT) measurement remains the standard method for evaluating core body temperature (CBT) of individual cows, but RT is an invasive, time-consuming, and potentially stressful procedure (Islam et al., 2021). Therefore, its usefulness on farms is limited, particularly as herd size increases, despite its reliability in research settings. Progress in sensor technologies, including ruminal boluses, ear tags, and accelerometers, allows continuous monitoring of CBT and behaviour; however, widespread adoption remains constrained by cost, the need for an individual device for each cow, and ongoing validation requirements (Islam et al., 2020; Stygar et al., 2021). Non-invasive imaging techniques, such as infrared thermography (IRT), have also been used to assess surface temperatures of different body regions, including the eye, muzzle, and udder (Daltro et al., 2017; Bang et al., 2022), however their accuracy can be impacted by environmental factors, (Church et al., 2014).

Few studies have focused exclusively on cellular biomarkers of HS. Heat shock proteins (HSPs), especially HSP70, play a crucial role in cellular stress and have been identified as potential indicators of HS (Hyder et al., 2017). Increased HSP70 levels have been reported in various livestock species across diverse biological matrices, including blood (Haque et al., 2012), saliva (Lamy et al., 2017), and milk (Pathirana and Garcia, 2022). In dairy cows, the detection of HSP70 in milk has opened the potential for a non-invasive biomarker that could

complement current HS detection methods, as milk sampling is a routine practice in the dairy system (Rakib et al., 2024).

Despite the availability of several detection techniques, no previous research has compared environmental, physiological, and biomarker indicators to detect HS at an individual cow level. We hypothesised that HS detection methods might vary in their sensitivity and response time. The direct physiological measures, including RT and IRT, respond rapidly to changes in heat load and are therefore useful for capturing short-term physiological responses (Idris et al., 2021). However, sensor-based reticulorumen temperature (RRT) measures can provide continuous real-time data with short biological delays (Bewley et al., 2008), while milk HSP70 biomarkers indicate delayed and cellular stress responses (Rakib et al., 2024). Based on this, the study aimed to compare environmental, physiological, and milk HSP70 indicators for detecting HS in dairy cows and assess the potential of milk HSP70 as a non-invasive biomarker for HS detection.

Materials and methods

Study location and experimental animals

The experiment was conducted at Corstorphine Dairy Farm, University of Sydney, Camden, New South Wales, Australia, during the summer (January-February) of 2024. Twenty-five multiparous Holstein Friesian cows, all in their third parity with 211.8 ± 21.3 DIM, were selected to ensure a biologically homogeneous population and minimize variation associated with parity and lactation stage. No signs of illness, including lameness or mastitis, were observed in any of the studied animals. Throughout the study, the herd was managed on pasture based on Kikuyu grass (*Cenchrus clandestinus*), and each cow received a supplement of approximately 8 kg of pelleted concentrate offered during milking (03:30 h and 13:00 h).

Sampling design and measurements

During each sampling, milk was obtained during the afternoon milking, subsequently followed by RT and IRT measurements. These variables were collected twice weekly (Mondays and Thursdays) throughout the 7-week study period using a fixed sampling schedule. In contrast, RRT was recorded continuously and reported at 10 min intervals using reticulorumen bolus sensors, whereas meteorological data were collected every minute at the nearest weather station

(Figure 1). All procedures were approved by The University of Sydney Animal Ethics Authority (Project Identifier No: 2022/AE002230).

Environmental data management

Ambient temperature and relative humidity were logged at one-minute intervals using data from the Camden Airport Automatic Weather Station (BOM, 2024), situated approximately 3.17 km from the study site, and providing accredited, quality-assured measurements. The temperature-humidity index (THI) was calculated using the following formula, which is widely used in dairy studies (NRC, 1971; Mader et al., 2006; Islam et al., 2020):

$$\text{THI} = (1.8 \times T + 32) - [(0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T - 26.8)]$$

where, THI = temperature-humidity index, T = ambient temperature (°C), and RH = relative humidity (%).

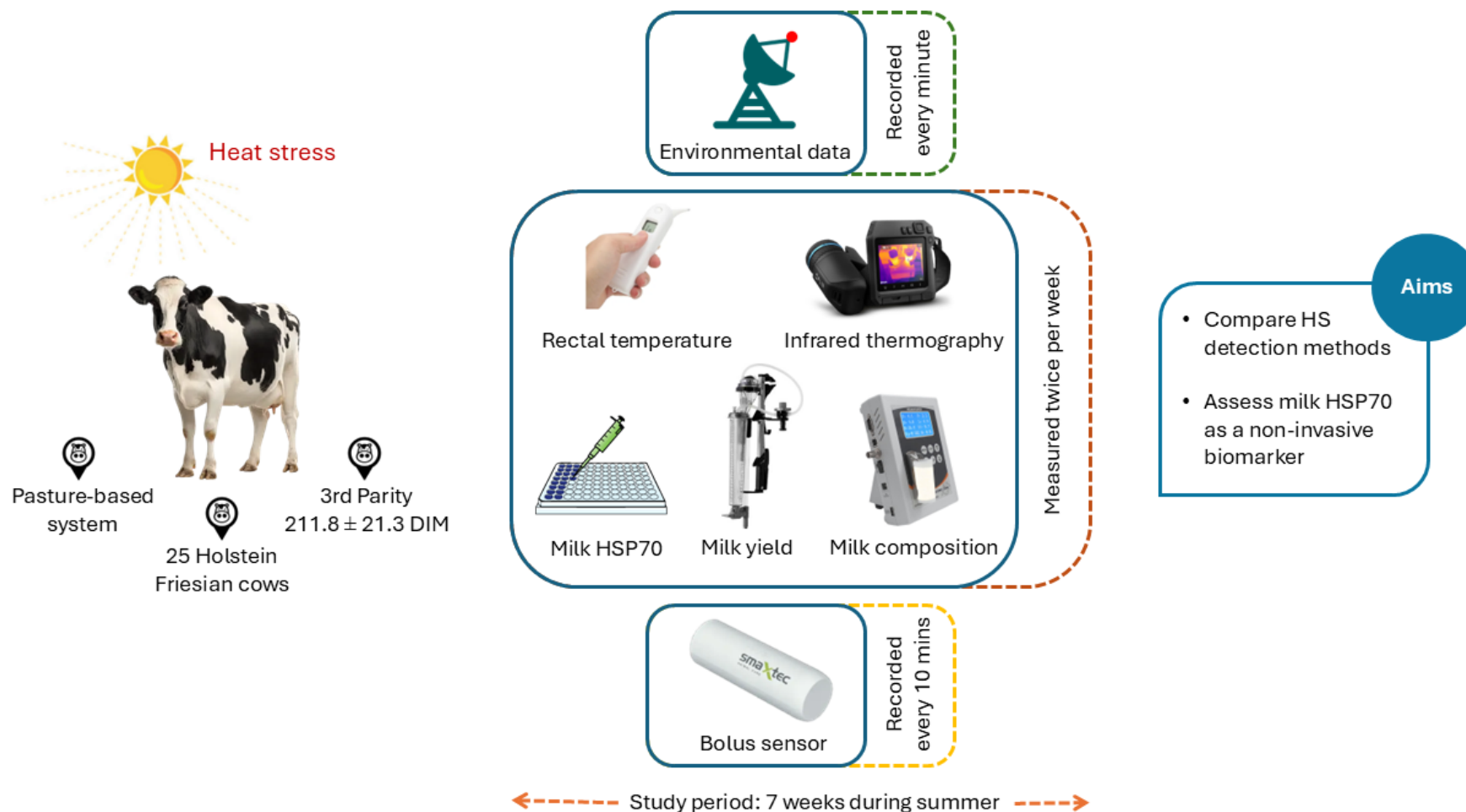


Figure 1. A schematic diagram of the experimental design for evaluating different heat stress detection techniques.

Physiological temperature measurement

a) Rectal temperature: Rectal temperature (°C) was measured immediately after milking using a calibrated digital veterinary thermometer (Livestock Veterinary Thermometer, Pssopp, China), inserted approximately 10 cm into the rectum until a stable reading was confirmed by an audible beep, in accordance with the manufacturer's guidelines (Figure 2). Two consecutive readings were recorded for each cow; the average of these readings was used for analysis.

b) Infrared thermography: Images were obtained immediately after RT measurement with an infrared camera (FLIR T420bx, FLIR Systems Inc., Sweden), while the cows were restrained in a shaded handling crush to minimise solar interference (Figure 2). The specifications of the FLIR T420bx were as follows: a resolution of 320×240 pixels, a thermal sensitivity of $<0.045^{\circ}\text{C}$, an accuracy of $\pm 2^{\circ}\text{C}$ or $\pm 2\%$ of reading, and a temperature measurement range of -20 to 350°C . All images were calibrated with an emissivity setting of 0.95, which reflects the capacity of skin to emit infrared radiation, and were captured from a distance of 1 m from the animal (Racewicz et al., 2018; Wang et al., 2021). Previous studies have assessed surface temperature from the lateral udder region of the cow (Daltro et al., 2017); however, in this study, infrared images were consistently obtained from the right fore-udder quarter to standardise the region of interest across cows and sampling days, and to reduce variability related to udder conformation and hind-limb interference. Images were analysed using FLIR software, and a 120×80 -pixel rectangle was placed over the udder area of the image to calculate the average, minimum, and maximum temperatures, ensuring that the recorded values excluded surrounding non-target surfaces.



Figure 2. A) Rectal temperature measurement using a digital veterinary thermometer. B) Infrared image captured with a FLIR T420bx camera. C) Infrared temperature extraction using FLIR software; within each outlined rectangle, the solid red triangle indicates the maximum temperature (hottest point) and the solid blue triangle indicates the minimum temperature (coolest point).

c) *Sensor-based reticulorumen temperature:* Continuous RRT was recorded using smaXtec bolus sensors (model MX-2042; smaXtec Animal Care GmbH, Graz, Austria). All cows had been equipped with these boluses as part of routine herd management, and data were collected according to the manufacturer's guidelines. This device records the raw RRT, which is then adjusted by the manufacturer's proprietary algorithm to account for drinking events. Data was transmitted through an antenna to a base station and stored in SmaXtec Messenger® software and recorded at 10-minute intervals.

Milk analysis

a) *Milk sampling and milk HSP70 measurement:* A sample of ~ 250 mL of milk per cow was collected during the afternoon milking session (13:00-15:00 h) on each sampling day (Mondays and Thursdays), aligning with the peak ambient temperature period of the day. The milk sample was transferred into sterile snap-seal bottles using the in-line sampling port to ensure representation from all four udder quarters. Samples were immediately stored in an icebox and transferred to the laboratory. Milk samples were centrifuged at $1,000 \times g$ for 10 min at 4 °C to obtain the skim milk (Rakib et al., 2025). Two replicate aliquots from each cow were analysed on the same day for HSP70 using an in-house competitive ELISA, as previously reported by Pathirana and Garcia (2022) and (Rakib et al., 2026b). In summary, 96-well polystyrene microtiter plates (Nunc Immuno Polysorp, Rochester, NY, USA) were coated with recombinant human HSP70 (ADI-NSP-555, Enzo Life Sciences, Farmingdale, NY, USA) and incubated overnight at 4°C. Before the experiment, samples and standards were pre-incubated overnight at 4°C with a monoclonal anti-human HSP70 antibody (ADI-SPA-810, Enzo Life Sciences). The antibody-sample combinations were subsequently transferred to the blocked wells. Following the incubation and washing procedures, an HRP-conjugated goat anti-mouse IgG antibody (BML-SA204, Enzo Life Sciences) was added. The colourimetric reaction was initiated with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, St. Louis, MO, USA) and immediately stopped with 1 N hydrochloric acid. Absorbance was measured at 450 nm with an ELISA plate reader (MR9600, Accuris, NJ, USA). The intra-assay and inter-assay coefficients of variation (CV) were 7.72% and 16.36%, respectively (n = 3 for each). The assay exhibited a detection limit of 31.25 ng/mL, with reproducible quantification ranging from 31.25 to 2000 ng/mL.

b) *Milk yield and composition:* Milk yield (L/day) was recorded during morning (03:30-05:30 h) and afternoon (13:00-15:00 h) milkings using WB Ezi-Test milk meters (Tru-Test, New

Zealand) from all quarters of the udder. Milk composition (fat, protein, SNF, and lactose in percentage) was determined using an ultrasonic milk analyzer (Master Eco, Milkotester, Bulgaria) during the sampling days.

Data processing and statistical analysis

All data processing and analysis were performed using R (version 4.5.0) statistical software. The sampling time was set at 16:00 h, serving as the reference point for aligning all variables. A temporal lag window extending up to 48 h prior to sample collection was defined to assess time-lagged correlations across environmental, physiological, and biomarker variables (Figure 3). Descriptive statistics, including mean, standard deviation (SD), coefficient of variation (CV), and range (maximum and minimum), were computed for RT, IRT, RRT, milk HSP70 concentration, milk yield, and milk composition traits. Pearson's correlation analysis was used to investigate pairwise associations across predefined lag grids for both THI and RRT, using continuous time-series data. In addition, RRT at the time of milk sampling (RRT_t0) was analysed to evaluate its concurrent association with THI during sampling. Correlation coefficients were calculated for each lag hour and visualised as heatmaps to characterise both the strength and the temporal structure of associations.

Linear mixed models were used with cow ID as a random effect to evaluate the interaction between each outcome variable (RT, IRT, RRT, milk HSP70, and milk yield) and either THI or RRT across different lag hours. Among the IRTs, only the average IRT was considered for modelling because it showed the strongest association with THI during the Pearson's correlation analysis, providing a more stable representation of surface temperature dynamics. Milk composition traits were excluded from this analysis because no significant correlations with acute HS indicators were observed. Both marginal and conditional R^2 , the Akaike information criterion (AIC), the Bayesian information criterion (BIC), the root mean square error (RMSE), and the mean absolute error (MAE) were used to assess the models.

In addition, receiver operating characteristic (ROC) analysis was performed to identify the diagnostic threshold for milk HSP70 in detecting HS at the individual cow level, with HS status defined by RRT at the selected time lag. RRT was used as the physiological reference indicator instead of RT because it provides continuous, cow-specific measures of internal thermal status, whereas RT was measured only twice weekly. Optimal cutoff points were determined using the

Youden index, and bootstrap resampling was used to estimate sensitivity and specificity with 95% confidence intervals.

Indicators	Time of Sampling								
	Time (h)			11:00	12:00	13:00	14:00	15:00	16:00
	Lag grid	-48		-5	-4	-3	-2	-1	0
THI		*	*	*	*	*	*	*	*
RT									*
IRT									*
RRT		*	*	*	*	*	*	*	*
RRT_t0									*
Milk HSP70									*
Milk yield									*
Milk compositions									

* = Data collected

Figure 3. A schematic diagram of a lag grid of 48h prior to sample collection for evaluating delayed correlations between environmental, physiological, milk HSP70, and milk production variables. THI = temperature-humidity index; RT = rectal temperature; IRT = infrared thermography; RRT = reticulorumen temperature; RRT_t0 = baseline reticulorumen temperature; HSP70 = heat shock protein 70.

Results

Descriptive statistics of study variables

A summary of the descriptive statistics for the environmental, physiological, HSP70 biomarker, and milk production variables monitored throughout the study period is presented in Table 1. The ambient temperature averaged 29.44 °C, and the relative humidity was 62.71%, resulting in a mean THI of 77.24, with minimum and maximum THI values of 67.66 and 86.42, respectively, during the study period. Physiological indicators, including RT and RRT, ranged from 37.93 °C to 40.75 °C and 38.37 °C to 41.51 °C, respectively; however, IRT had a broader range from 25.60 °C to 40.50 °C. The average HSP70 concentration in milk was 496.73 ng/mL, with a range of 280.06 to 770.69 ng/mL. Milk composition values averaged 4.21% fat, 3.18% protein, 8.73% SNF, and 4.78% lactose, with a mean daily milk yield of 20.90 L.

Table 1. Descriptive statistics of environmental, physiological, HSP70 biomarker, and milk production variables during the study period.

Variables	Mean	SD	CV%	Maximum	Minimum
Environmental variables					
Ambient temperature (°C)	29.44	4.59	15.58	38.90	20.20
RH (%)	62.71	15.35	24.47	99.00	37.00
THI	77.24	4.44	5.75	86.42	67.66
Physiological variables					
RT (°C)	39.39	0.56	1.43	40.75	37.93
Average IRT (°C)	35.63	1.37	3.86	38.70	31.70
Maximum IRT (°C)	38.10	1.00	2.61	40.50	35.60
Minimum IRT (°C)	31.65	2.24	7.08	36.50	25.60
RRT (°C)	39.46	0.53	1.33	41.51	38.37
HSP70 biomarker variable					
Milk HSP70 concentration					
(ng/mL)	496.73	100.89	20.31	770.69	280.06
Milk production variables					
Milk yield (L/day)	20.90	6.63	31.70	45.50	2.00
Fat (%)	4.21	1.27	30.15	10.25	1.20
Protein (%)	3.18	0.15	4.71	3.55	2.80
SNF (%)	8.73	0.47	5.38	9.70	4.40
Lactose (%)	4.78	0.25	5.30	5.80	4.00

SD = standard deviation; CV = coefficient of variation; RH = relative humidity; THI = temperature-humidity index; RT = rectal temperature; IRT = infrared thermography; RRT = reticulorumen temperature; HSP70 = heat shock protein 70; SNF = solids-not-fat.

Association between environmental, physiological, and biomarker variables

Figures 4(a) and 4(b) illustrate the time-lagged pairwise correlations among environmental, physiological, and HSP70 biomarker variables, along with milk yield and composition. The association between THI and both RT ($r = 0.58$) and average IRT ($r = 0.64$) was strongest at the time of measurement. In contrast, RRT_t0 showed its highest correlation slightly later, within 0-8 h after heat exposure ($r = 0.42$ - 0.43). A delayed association between THI and milk HSP70 was observed, with the maximum correlation occurring at a 35-h lag ($r = 0.30$). Milk yield (L/d) showed a negative correlation with THI, decreasing from a 25-h lag ($r = -0.20$) and reaching its highest decline at 48 h ($r = -0.25$). Among milk constituents, protein (%), lactose (%), and SNF (%) exhibited weak negative correlations ($r = -0.12$ to -0.18) with THI at an 11 h lag, although fat (%) showed a minimal and delayed interaction ($r = -0.09$ at 36 h lag).

At the same sampling point, RRT showed a moderate correlation with RT ($r = 0.44$); however, a relatively strong correlation ($r = 0.74$) was observed at a 9-h lag. Besides, RRT also exhibited moderate correlations with average IRT ($r = 0.41$ - 0.62) across 0-9 h and a weak positive association with milk HSP70 concentration ($r = 0.26$ at 40 h lag), while its relationship with milk yield was minimal ($r = -0.10$ at 47 h lag). However, milk constituents showed weak negative correlations ($r = -0.14$ to -0.22) with RRT at a 19 h lag.

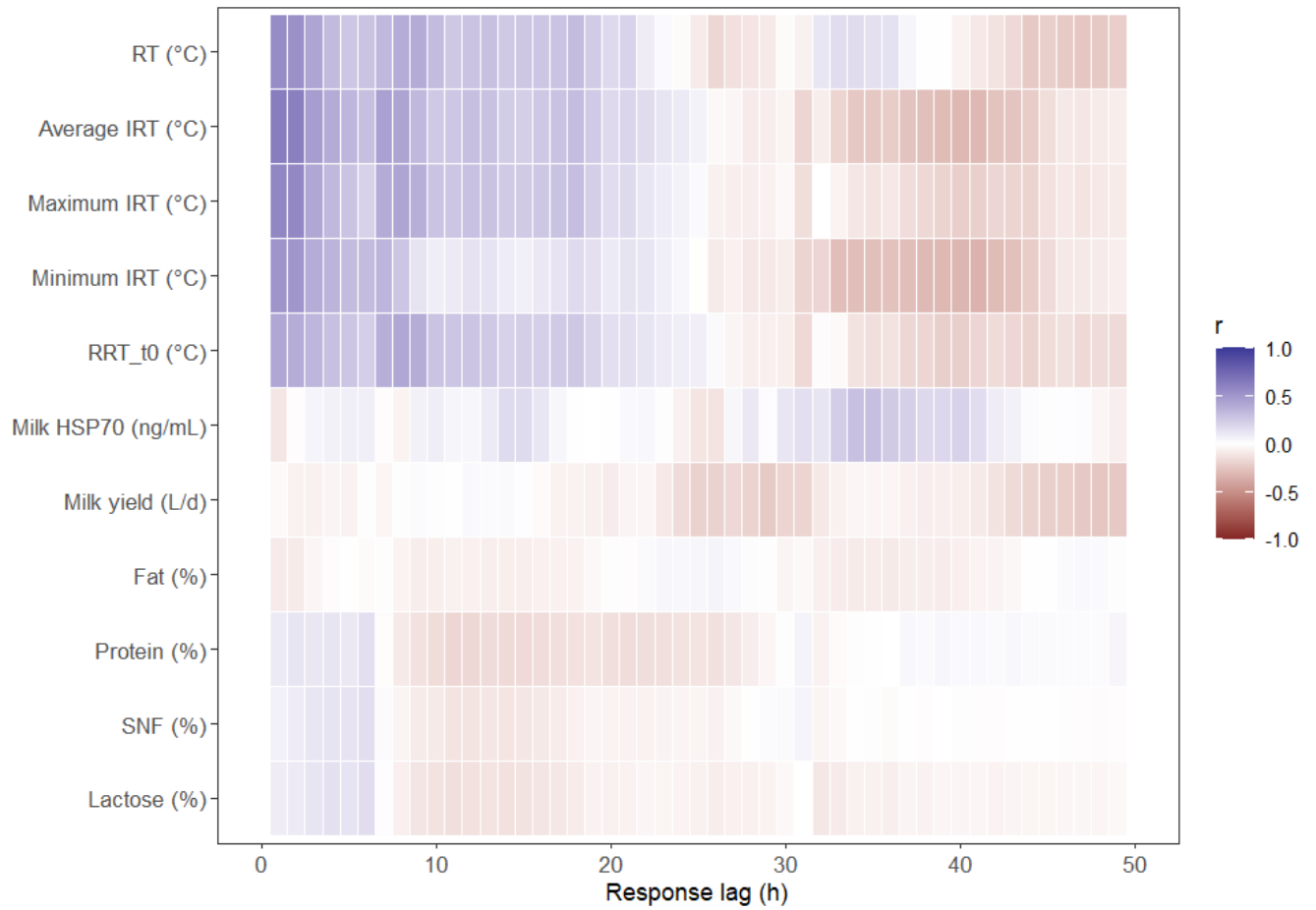


Figure 4(a). Pearson correlations between temperature-humidity index (THI) and physiological, HSP70 biomarker, and milk production variables across lag hours. Positive correlations are shown in dark blue and negative correlations in dark red, with colour intensity indicating the strength of the association. RT = rectal temperature; IRT = infrared thermography; RRT_t0 = baseline reticulorumen temperature; HSP70 = heat shock protein 70.

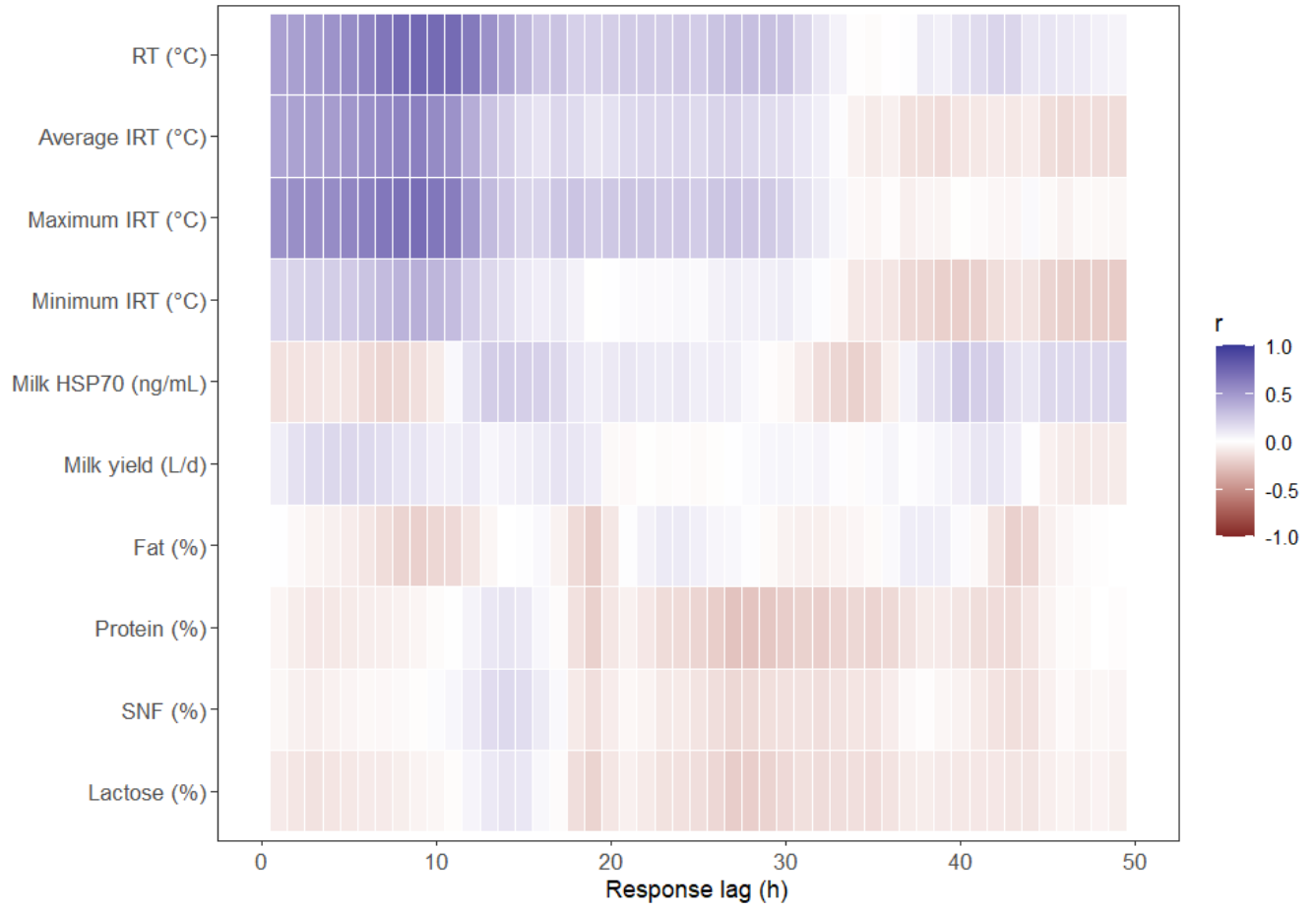


Figure 4(b). Pearson correlations between reticulorumen temperature (RRT) and physiological, HSP70 biomarker, and milk production variables across lag hours. Positive correlations are shown in dark blue and negative correlations in dark red, with colour intensity indicating the strength of the association. RT = rectal temperature; IRT = infrared thermography; HSP70 = heat shock protein 70.

Linear mixed model analysis

Following the pairwise correlations, linear mixed models were used to further assess interactions among environmental, physiological, and HSP70 biomarker variables across lag hours (Table 2).

The most significant lag correlations previously observed [Figures 4(a) and 4(b)] were aligned with the optimal models found in this analysis. RT and average IRT exhibited strong positive correlations with THI in real time ($P < 0.001$), with R^2_c values of 41.34% and 43.51%, respectively. In addition, RRT showed a positive correlation with THI, with similar model performance across 0 to 8 h lags ($P < 0.001$; $R^2_c = 29.23\text{-}30.52\%$). The milk HSP70 concentration showed a delayed response to THI, with the optimal model indicating a 35 h lag ($P < 0.001$; $R^2_c = 9.14\%$). In contrast, milk yield (L/d) showed a negative association with THI, with the maximum association observed at a 48 h lag ($P < 0.001$; $R^2_c = 34.38\%$).

When RRT was used as the predictor, RT showed the maximum correlation at a 9 h lag ($P < 0.001$; $R^2_c = 55.67\%$), followed by moderate associations with average IRT across 0 to 9 h lags ($P < 0.001$; $R^2_c = 18.28\text{-}42.71\%$). Weak positive correlations were observed between RRT and milk HSP70 ($P < 0.001$; $R^2_c = 7.17\%$ at a 40 h lag), whereas milk yield exhibited a negative correlation with RRT, with the best model at a 47 h lag ($P < 0.05$; $R^2_c = 29.40\%$).

Table 2. Linear mixed model results showing associations between environmental, physiological, and HSP70 biomarker variables at selected lag hours.

Outcome	Predictor (lag hours)	Estimate	SE	<i>p</i> -value	R^2_m (%)	R^2_c (%)	AIC	BIC	MAE	RMSE
RT (°C)	THI (0)	0.07	0.01	<0.001	33.75	41.34	429.80	445.16	0.37	0.46
Average IRT (°C)	THI (0)	0.22	0.02	<0.001	40.14	43.51	684.60	698.35	0.84	1.06
RRT (°C)	THI (0)	0.06	0.01	<0.001	18.54	30.52	522.43	537.70	0.41	0.54
RRT (°C)	THI (8)	0.08	0.01	<0.001	17.35	29.23	528.14	543.40	0.41	0.54
Milk HSP70 (ng/mL)	THI (35)	6.27	1.11	<0.001	9.14	9.14	3813.39	3828.44	76.96	96.00
Milk yield (L/day)	THI (48)	-0.35	0.06	<0.001	6.19	34.38	2228.50	2243.94	4.99	6.41
RT (°C)	RRT (0)	0.41	0.05	<0.001	18.59	20.44	496.50	511.71	0.40	0.51
RT (°C)	RRT (9)	0.57	0.03	<0.001	55.02	55.67	301.26	316.47	0.29	0.38
Average IRT (°C)	RRT (0)	0.95	0.14	<0.001	17.29	18.28	736.49	750.10	0.98	1.25
Average IRT (°C)	RRT (9)	1.28	0.11	<0.001	38.99	42.71	669.92	683.53	0.84	1.08
Milk HSP70 (ng/mL)	RRT (40)	54.37	11.71	<0.001	6.66	7.17	3661.39	3676.27	74.48	96.57
Milk yield (L/day)	RRT (47)	-1.55	0.64	<0.05	1.40	29.40	2164.12	2179.39	5.20	6.58

RT = rectal temperature; *THI* = temperature-humidity index; *IRT* = infrared thermography; *RRT* = reticulorumen temperature; *HSP70* = heat shock protein 70; *SE* = standard error; R^2_m = marginal *R*-squared (variance explained by fixed effects); R^2_c = conditional *R*-squared (variance explained by both fixed and random effects); *AIC* = Akaike information criterion; *BIC* = Bayesian information criterion; *MAE* = mean absolute error; *RMSE* = root mean square error. *MAE* and *RMSE* are expressed in the same units as the corresponding outcome variable.

Milk HSP70 threshold

To evaluate the ability of milk HSP70 concentrations to assess HS, a ROC analysis was performed, with HS defined by RRT with a 40-h lag, as this lag hour showed the highest association between milk HSP70 and RRT. The ROC analysis produced an area under the curve (AUC) of 0.613 (95% CI: 0.550-0.676), indicating fair predictive performance. The optimal diagnostic threshold, determined by the Youden index, was approximately 460 ng/mL. At this threshold, the sensitivity was 0.833, indicating that 83.3% of HS cases were accurately identified, while the specificity was 0.435, indicating that only 43.5% of non-HS samples were correctly identified. Sensitivity and specificity were assessed by bootstrap resampling, and 95% confidence intervals were calculated.

Discussion

This study investigated environmental and physiological indicators, including RT, IRT, and RRT, as well as milk-based HSP70 biomarkers to evaluate their sensitivity, temporal lag, and practical application for assessing HS in dairy cows. During the study period, cows were exposed to moderate to severe HS conditions, as indicated by a mean THI of 77.24 (range: 67.66-86.42), exceeding commonly accepted thresholds associated with HS in dairy cattle (Yan et al., 2021). Consistent with its widespread use in both experimental and commercial settings, THI was therefore used as the standard environmental indicator for this study to compare physiological and biomarker-based responses.

At the individual cow level, RT remains the reference physiological indicator of CBT, and its short-term responsiveness to heat load makes it a key benchmark for assessing other HS detection methods (Idris et al., 2021). This study found that the average RT was 39.39 °C, with a range of 37.93 to 40.75°C, indicating that the cows were subjected to mild to severe heat load under the observed THI conditions (Yan et al., 2021). The real-time correlation between RT and THI ($r = 0.58$; $P < 0.001$; $R^2_c = 41.34\%$) indicates a moderate but biologically meaningful relationship, which aligns with Kaufman et al. (2018), who reported weak to moderate associations between THI and RT. Moreover, Dikmen and Hansen (2009) found a similar correlation between RT and THI ($r = 0.51$) when THI was calculated using the NRC (1971) formula, the same THI formula used in the present study. The findings indicate that THI can measure a significant portion of the environmental heat load, but it can't explain all physiological changes observed in individual cows. These variations are caused by differences

in metabolic demand, behavior, and lactation status. On the other hand, RT provides a precise physiological measure of heat load at the individual cow level; however, it is not practical and therefore not used widely in commercial farms (Islam et al., 2020).

IRT measures surface temperature through the proportional emission of radiated heat, which offers a non-invasive tool with considerably greater automation prospects compared to RT (Macmillan et al., 2019). Its potential to assess large numbers of animals with minimal restraint also improves labour efficiency and supports rapid screening in commercial dairy systems (Idris et al., 2021). In the present study, average IRT varied between 31.70 and 38.70 °C (mean 35.63 °C) and exhibited a higher real-time association with THI ($r = 0.64$; $P < 0.001$; $R^2_c = 43.51\%$) compared to the RT correlation with THI ($r = 0.58$; $P < 0.001$; $R^2_c = 41.34\%$). In contrast, previous studies Bang et al. (2022) reported relatively weak to moderate relationships between IRT, panting score ($r = 0.23$ - 0.50 ; $P < 0.001$), and THI ($r = 0.14$ - 0.48 ; $P < 0.05$) across various body regions, including inner tail base, outer vulva, muzzle, ocular area, paralumbar fossa, fore and hind hoof, fore and rear udder, and armpit. The stronger associations observed in our study might reflect increased peripheral blood flow during heat exposure, which elevates skin temperature and can be rapidly detected by thermal imaging (Stewart et al., 2005).

IRT also showed significant correlations with physiological indicators of internal body temperature. Daltro et al. (2017) found a strong relationship between udder surface temperature and RT ($r = 0.74$), indicating that increases in surface temperature typically correspond to increases in internal body temperature. In our investigation, IRT also showed moderate correlations with RRT across 0-9 h lags ($r = 0.41$ - 0.62 ; $P < 0.001$; $R^2_c = 18.28$ - 42.71%), suggesting that variations at the body surface temperature are aligned with fluctuations in internal temperature. These delays in RRT are expected because the reticulorumen responds to temperature changes more slowly than peripheral tissues, due to its larger volume and lower heat transfer efficiency (Bewley et al., 2008). Although IRT is highly responsive to environmental conditions, its accuracy is affected by several climatic factors. Church et al. (2014) have shown that moderate wind speeds can lower eye-region mean IRT by 0.43 °C, while elevated wind speeds can reduce it by up to around 0.78 °C; whereas, direct sunlight can elevate IRT readings by approximately 0.56 °C.

Sensor-based rumen bolus provides a continuous internal measurement of thermal status and is less invasive than RT; however, its response dynamics vary due to the thermal buffering

capacity of the rumen. This study found that the average RRT was 39.46 °C (range 38.37-41.51 °C), which is consistent with findings reported in earlier research on dairy cows under HS (Liang et al., 2013). It showed moderate real-time associations with THI ($r = 0.42-0.43$), and model performance remained relatively stable during 0-8 h lags ($P < 0.001$; $R^2_c = 29.23-30.52\%$). Timsit et al. (2011) also reported delayed RRT responses in young bulls using bolus sensors to identify bovine respiratory disorders. Moreover, the delayed response aligns with previous research on RRT dynamics, which indicates that RRT typically require 20-120 min to return to baseline temperature following a drinking interval, due to the substantial fluid volume and thermal capacity of the reticulorumen environment (Rogers Simmons et al., 1965; Brod et al., 1982; Yamada et al., 2001).

In our study, the correlations observed between RRT and other HS indicators also indicate similar physiological associations. Although the real-time correlation between RRT and RT was moderate ($r = 0.44$), the association strengthened significantly at a 9 h lag ($r = 0.74$; $P < 0.001$; $R^2_c = 55.67\%$). This indicates that changes in RRT lag behind RT and gradually represent the accumulated internal HS. This aligns with previous research indicating moderate to strong correlations between RRT and RT ($r \approx 0.48-0.89$), with the level of correlation influenced by sampling intervals, the physiological condition of the animal, and management and environmental factors (Rose-Dye et al., 2011; Lees et al., 2019).

Individual variations in breed and production levels can also affect RRT characteristics. Liang et al. (2013) reported that Holstein cows exhibit greater increases in RRT at elevated ambient temperatures than crossbreds (Holstein $\times$ Jersey and multi-breed crosses), possibly due to a higher metabolic heat load. High-producing cows also exhibit significant increases in RRT as ambient temperature rises (Purwanto et al., 1990), highlighting their susceptibility to HS. Although breed effects were not evaluated in the present study, the observed RRT range, including values exceeding 41 °C, reflects substantial internal heat accumulation within the study population, consistent with studies indicating that high-producing *Bos taurus* cattle are more vulnerable to HS (West, 2003; Renaudeau et al., 2012). Overall, RRT is a valuable indicator of sustained heat load within the reticulorumen, complementing RT and IRT rather than reflecting immediate heat exposure.

Milk HSP70 has recently been reported as a non-invasive indicator of cellular stress in dairy cows (Pathirana and Garcia, 2022), while research in this field is still limited compared to

conventional HS detection methods. In the present study, milk HSP70 concentrations averaged 496.7 ng/mL (range 280.1-770.7 ng/mL), consistent with the lower end of values reported by Pathirana and Garcia (2022), who also observed elevated HSP70 levels in milk at higher temperatures. A key feature of our findings is the time-lagged correlation between milk HSP70 and environmental HS. While RT and IRT responded to short-term changes in THI, milk HSP70 showed its most significant correlation with THI at a 35 h lag ($r = 0.30$; $P < 0.001$; $R^2_c = 9.14\%$). A similar delay was observed between RRT and milk HSP70, with maximum correlation at 40 h lag ($r = 0.26$; $P < 0.001$; $R^2_c = 7.17\%$). These patterns are consistent with the biology of heat-shock responses. HSP70 induction triggered by cellular stress, followed by transcription, translation, and release into extracellular fluids (Mosser and Morimoto, 2004; Mayer and Bukau, 2005; Bagath et al., 2019). However, measurable increases in extracellular fluids might be delayed due to protein accumulation, secretion dynamics, and tissue-specific transfer mechanisms. These factors might be useful to explain the lag observed in milk HSP70 in the current study, but the precise mechanisms underlying this delay require further investigation. However, variability in milk HSP70 could arise from dilution effects associated with milk volume (Boland et al., 2013) and specialised secretion dynamics in the mammary gland (Collier et al., 2008; Hu et al., 2016). These findings collectively indicate that milk HSP70 does not reflect heat load at the time of sampling but rather reflects a delay in response to previous heat exposure.

Moreover, (Rakib et al., 2026a) identified significant seasonal variations in HSP70 levels in milk, blood, and saliva, with moderate correlations among these fluids, indicating that milk HSP70 partially reflects systemic stress physiology. They further demonstrated a strong analytical agreement between in-house and commercial ELISA methods ($r = 0.91$), supporting the reliability of milk HSP70 measurement (Rakib et al., 2026b). Previous research also reported elevated levels of blood and saliva HSP70 in response to thermal or environmental stress (Gaughan et al., 2013; Lamy et al., 2017), indicating the role of extracellular HSP70 as a general systemic stress indicator (Hyder et al., 2017).

Establishing a diagnostic threshold for any biomarker requires comprehensive validation across multiple herds, seasons, and controlled heat-challenge experiments. In the present study, HS was defined using RRT as a physiological indicator. The use of rumen temperature boluses to identify and predict HS in grazing dairy cows has previously been reported, with changes in RRT used as indicators of HS under pasture-based conditions (Woodward et al., 2024). Sensor-

derived RRT provides continuous, individual cow evaluations of internal temperature and closely correlates with CBT (Bewley et al., 2008; Lees et al., 2019). This exploratory investigation used ROC analysis based on RRT-defined HS at a 40-h lag, obtaining an estimated threshold of approximately 460 ng/mL (sensitivity 0.833; specificity 0.435). This is the initial effort to establish an operational milk HSP70 cut point for HS detection, which will require refinement in subsequent investigations.

In addition to biomarker responses, milk production parameters exhibited variations consistent with a delayed effect of HS. Milk yield had a negative correlation with THI, with the most significant decrease observed at a 48 h lag ($P < 0.001$; $R^2_c = 34.38\%$), and a similar delayed negative correlation with RRT at 47 h lag ($P < 0.05$; $R^2_c = 29.40\%$). These findings align with previous research demonstrating that milk yield can decrease after continuous or repeated heat load, often observed 1-3 days later rather than immediately (Wildridge et al., 2018). This delay is consistent with the time required for reduced feed intake and metabolic adaptations to be observed in production losses (Hagiya et al., 2019; Li et al., 2021a). In contrast, changes in milk composition were minimal, with the percentage of protein, lactose, and SNF showing only slight negative correlations with THI at an 11-h lag, while milk fat exhibited a minimal, delayed response, which suggests that yield was more responsive to cumulative HS than to overall compositional traits (Rakib et al., 2025).

This study provides an initial step toward understanding the temporal behavior and potential diagnostic value of milk HSP70; however, some limitations should be considered when interpreting the results. The relatively low proportion of variation explained by THI or RRT suggests that milk HSP70 is influenced by several biological factors beyond HS alone, including immune function, oxidative stress, lactation stage, and mammary physiology, while cellular origin within milk remains unclear. In this study, RRT was used as the primary internal temperature reference because it was continuously measured throughout the investigation. However, the maximum correlation between RT and RRT was observed at a 9 h lag and explained only about half of the variability, which indicates that RRT might not fully capture immediate changes in CBT. On the other hand, RT was only measured twice weekly, which limited its ability to serve as a continuous reference measure of HS. The RT and IRT showed moderate associations with THI; while they only explained around 40% of the variation. This underscores the biological variability and limitations of THI as a continuous HS indicator. In

future investigations, the use of physiologically defined HS indicators might allow for a more precise evaluation of the indicator performance.

Besides that, different sampling patterns across variables limit the interpretation of temporal correlations. Environmental parameters and RRT were continually monitored, while milk HSP70, RT, and IRT were measured at specific times of the sampling day. This discrepancy inhibits precise alignment between heat exposure and biological response, suggesting caution when interpreting lag-based correlations. Although the lagged association with THI suggests a delayed response, it cannot be demonstrated that milk HSP70 reflects cumulative HS; rather, it could represent accumulated cellular stress after prolonged heat exposure. In addition, the observed lag does not mean that the cow was constantly exposed to HS, as times of cooling, particularly overnight, might help ease some of the psychological stress. To determine whether milk HSP70 is an indicator of cumulative heat exposure, delayed activation, or both, further investigation will be needed that considers the integrated heat load over the previous few days and includes more frequent milk samples, including an evaluation of nighttime cooling.

Finally, the study was conducted on a single herd under a specific management system, which might limit the application of the results to other management systems or extreme THIs. Although the study focused on overall population responses, individual cow variability in milk HSP70 was not investigated in detail. Future research involving multiple herds, controlled heat-challenge conditions, and repeated measurements within individual animals would help clarify baseline variability and determine whether milk HSP70 can contribute to understanding individual differences in heat responses or resilience, rather than serving as a supplementary HS detection indicator.

Conclusion

This study demonstrated that environmental variables, physiological indicators (RT, IRT, and RRT), and milk HSP70 reflect distinct biological responses to HS in dairy cows. Despite the substantial biological variability, RT and IRT showed moderate, short-term correlations with THI, indicating sensitivity to variations in environmental heat load. In contrast, RRT indicated a relatively slower rate of internal heat accumulation, providing valuable insights into prolonged heat exposure. Milk HSP70 exhibited a significantly delayed response, consistent with cellular stress mechanisms, highlighting its potential as a supplementary

indicator of HS in dairy cows. The different response times of these indicators suggest a multi-tiered monitoring approach, in which physiological measures inform short-term thermal responses, while delayed milk HSP70 responses might reflect accumulated stress and recovery processes. Collectively, our findings underscore the opportunity to develop more comprehensive HS assessment frameworks that integrate real-time physiological indicators with molecular biomarkers to assist with management strategies for HS in dairy cows

Notes

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Ethical Statement

The study was conducted in accordance with ethical guidelines and received approval from the Animal Ethics Authority at The University of Sydney, Australia (Project Identifier No: 2022/AE002230).

Conflict of Interest

The authors have not stated any conflicts of interest.

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CHAPTER 7

Daily variation in milk HSP70 in dairy cows during a heat wave

Chapter 7 investigates the daily variation in milk HSP70 during a heat wave, extending the temporal framework established in Chapter 6. Daily HSP70 dynamics are analysed in relation to temperature-humidity index and sensor-derived reticulorumen temperature across five consecutive days, providing field-based insight into cellular stress responses during prolonged heat exposure.

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Daily variation in milk HSP70 in dairy cows during a heat wave

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Abstract

Heat waves (HWs) are increasing in frequency and severity in many regions under climate change, posing challenges to the welfare and productivity of dairy cows. Physiological and environmental indicators are widely used to assess heat stress (HS) in dairy cows; however, the short-term temporal dynamics and lagged responses of molecular stress biomarkers during HW events remain poorly characterised. This study aimed to evaluate daily variation in milk HSP70 during a HW and to assess its association with the environmental temperature-humidity index (THI) and sensor-derived reticulorumen temperature (RRT). The research was carried out over five consecutive days of HW in a pasture-based dairy farm in Camden, New South Wales, Australia, investigating 20 clinically healthy, third-parity Holstein Friesian cows. Daily milk samples were obtained during afternoon milking for HSP70 assessment using competitive ELISA. Environmental data were recorded at 1-min intervals to calculate THI, while RRT was continuously monitored at 10-min intervals using rumen bolus sensors. Associations between THI, RRT, and milk HSP70 were analysed using Pearson correlations and univariable linear mixed models across lag windows of up to 50 h prior to sample collection. Diagnostic efficacy was evaluated using receiver operating characteristic (ROC) analysis. The results revealed

delayed associations between milk HSP70 and both THI and RRT. Associations between milk HSP70 and THI were evident within the delayed (~48 h) window after heat exposure, with peak correlations occurring between 40 and 44 h ($r = 0.67-0.69$; $P < 0.001$; $R^2_c = 72.38-75.66\%$). Similarly, associations with RRT were observed within a longer delayed (~48 h) window after heat exposure, with peak correlations occurring between 48 and 50 h ($r = 0.41-0.51$; $P < 0.001$; $R^2_c = 40.92-51.71\%$). In contrast, RRT responded rapidly to environmental heat load, showing the strongest associations with THI occurring within the immediate (0-6 h) window ($r = 0.45-0.53$; $P < 0.001$; $R^2_c = 48.15-57.21\%$). An exploratory cut-point for milk HSP70 of approximately 550 ng/mL was identified, with a sensitivity of 0.782 and specificity of 0.909 for RRT-defined HS. Overall, the findings indicate that milk HSP70 exhibits a distinct temporal delay in response to HW exposure, consistent with cumulative cellular stress, and may offer supplementary insights into commonly used environmental and physiological indicators for evaluating prolonged HS in dairy cows in pasture-based settings.

Key words: heat stress; temperature-humidity index; reticulorumen temperature; biomarker, non-invasive

Introduction

Heat waves (HWs) are typically defined as periods of persistently high temperatures lasting a minimum of three consecutive days, during which daily maximum and minimum temperatures exceed location-specific climatological thresholds (McCarthy et al., 2019; Trancoso et al., 2020; BOM, 2024), resulting in sustained heat load that challenges thermoregulation in dairy cows. The severity of HWs is often intensified by increased humidity and solar radiation, which contribute to the environmental heat load (Vitali et al., 2015). In many regions of Australia, HWs have become more intense, frequent, and longer in duration over the past seven decades, with a notable acceleration in recent years, indicating an escalation in the intensity of thermal extremes at the national level (Trancoso et al., 2020). These changes are consistent with broader global warming trends, as average global temperatures have increased by approximately 1.0 °C since the 1800s and are projected to exceed 1.5 °C above pre-industrial levels as early as 2030 (IPCC, 2021). In southern Australia, the average duration of high temperature events has increased from 2 to 4 consecutive days between 1960 and 2008 (Nidumolu et al., 2014), posing increasing challenges for the livestock industry (Beniston et al., 2007; Polsky and Von Keyserlingk, 2017). Managing heat stress (HS) in modern dairy herds has become more

difficult due to selective breeding for improved productivity, which has increased metabolic activity and, consequently, susceptibility to HS (Polsky and Von Keyserlingk, 2017). Dairy cows are sensitive to climatic conditions, particularly ambient temperature and humidity, due to their relatively low thermoneutral zone, which limits their ability to dissipate excess heat and makes them more susceptible to prolonged heat load (West, 2003). Prolonged or intense HS can result in significant declines in feed intake, milk production, and reproductive performance (Becker et al., 2020; Rakib et al., 2020), as well as increased mortality rates (Vitali et al., 2009).

The temperature-humidity index (THI) is widely used to describe environmental heat load; however, it does not account for individual variation in physiological HS or modifying factors such as solar radiation and wind speed (Dunshea et al., 2013). Continuous monitoring of reticulorumen temperature (RRT) via rumen bolus sensors provides a reliable proxy for core body temperature (CBT) and offers improved insight into the thermal status of the animal HS under field conditions (Lees et al., 2019). Despite these advances, both approaches primarily reflect environmental exposure and whole-animal responses, providing limited insight into underlying cellular stress processes.

In this context, molecular biomarkers have gained interest as potential indicators of cellular stress responses in dairy cattle. Heat shock protein 70 (HSP70) is a highly conserved molecular chaperone involved in cellular stress responses, including heat, metabolic, and immune-related stress, where it plays a key role in maintaining protein homeostasis and supporting cell survival (Rakib et al., 2024). It has been detected in several biological fluids of livestock species, including blood, saliva, and milk (Lamy et al., 2017; Kumar et al., 2020; Pathirana and Garcia, 2022). The measurement of HSP70 in milk is convenient as it allows non-invasive sampling during routine milking and may serve as an objective indicator of physiological stress responses in dairy cows, including those associated with heat load (Rakib et al., 2025).

However, most studies on milk HSP70 in dairy cows have used single-time-point sampling (Rajamanickam et al., 2025; Rakib et al., 2026a), providing a limited understanding of its short-term variability during HW events. Thus, it remains unclear how milk HSP70 changes daily during the entire period of HW, and how these changes correlate with both external (THI) and internal (RRT) indicators of HS. Understanding these short-term patterns is crucial for evaluating whether milk HSP70 could serve as a biomarker of HS in dairy cows under HW

conditions. We hypothesised that milk HSP70 concentration would increase during the rising and peak phases of HW, in association with elevated THI and RRT, and subsequently decline as environmental heat load (THI) decreased. This study aimed to measure daily variations in milk HSP70 during a HW, evaluate its relationship with THI and RRT, and explore its potential for classifying HS using an RRT-based reference.

Materials and methods

Study design and animal management

The study was conducted in December 2023 at Corstorphine Dairy Farm, The University of Sydney, Camden, New South Wales, Australia (Figure 1). Intensive monitoring was initiated based on short-term forecasts from the Australian Bureau of Meteorology (BoM), which indicated a high probability of consecutive days with elevated temperature and humidity conditions consistent with HW development. The severity and duration of the HW were determined post hoc using observed meteorological data. Heatwave classification followed the BOM climatological percentile-based threshold approach, which defines a HW as a period of at least three consecutive days during which both daily maximum and minimum temperatures exceed location-specific climatological thresholds derived from historical temperature records (McCarthy et al., 2019; Trancoso et al., 2020; BOM, 2024). Meteorological data described above were used to calculate daily maximum and minimum temperatures for the study period, and each day was evaluated against these thresholds. During the identified event, daily maximum temperatures reached up to 42.10 °C, and THI values exceeded 84 (Table 1), indicating substantial environmental heat load. Based on these criteria, five consecutive days met the HW definition and were therefore classified as the HW period (HW-1 to HW-5), which was used for subsequent analyses.

Twenty clinically healthy, third-parity Holstein Friesian cows (201.8 ± 26.9 DIM) were selected following a health assessment to exclude cows with lameness, mastitis, or other disorders that could affect HSP70 concentrations. Information on age, body weight, and pregnancy status was not recorded; however, all cows were managed under the same herd conditions in a pasture-based system throughout the year, with a feeding target of approximately 26 kg DM/cow/day. A typical ration included approximately 8 kg pelleted concentrate offered during milking (03:30 and 13:00 h), 1.5 kg silage, 1.5 kg dried distillers grains (DDG), and 15 kg of pasture and forage sources, including fescue/prairie grass mixes

and seasonal species such as ryegrass (*Lolium perenne*), oats (*Avena sativa*), or Kikuyu (*Cenchrus clandestinus*), depending on availability. The concentrate composition varied slightly between batches but generally consisted of high-energy cereal grains, canola meal, and a mineral-vitamin premix formulated to support milk production and herd health.

Cows were maintained in a pasture-based system without built shelter, with continuous access to open pasture and natural tree shade. No active cooling strategies (e.g., fans, sprinklers, or misting systems) were implemented during the study period. The extent of shade was not quantitatively measured; however, cows had access to scattered trees within paddocks that provided intermittent shade throughout the day.

Milk sampling and HSP70 measurement

Milk samples were collected from all 20 cows at a consistent daily time point during afternoon milking (13:00-15:00 h) to standardise sampling; subsequent analyses focused on time-lagged relationships rather than real-time responses. Approximately 250 mL of milk were obtained in sterile snap-seal bottles using the in-line sampling port of the herringbone parlour (Waikato Milking Systems, Hamilton, New Zealand) to ensure equal representation from all four quarters. Milk samples were immediately placed in an ice box after collection and transported to the laboratory. Sample processing commenced within 30 min of collection. Milk was then centrifuged at $1,000 \times g$ for 10 minutes at 4 °C to isolate the skim fraction (Rakib et al., 2026b). HSP70 analysis was performed on freshly processed skim milk, and samples were not subjected to repeated freeze-thaw cycles before analysis. Two replicate aliquots from each sample were assessed for HSP70 concentration utilizing an in-house competitive ELISA as previously described by Pathirana and Garcia (2022). The assay has been previously validated for use in bovine milk, demonstrating acceptable performance and cross-reactivity with bovine HSP70 in this matrix (Pathirana and Garcia, 2022; Rakib et al., 2026b). In brief, 96-well polystyrene microtiter plates (Nunc Immuno Polysorp, Rochester, NY, USA) were coated using recombinant human HSP70 (ADI-NSP-555, Enzo Life Sciences, Farmingdale, NY, USA) and incubated for an overnight period at 4°C. Before starting the experiment, samples and standards were pre-incubated overnight at 4°C using a monoclonal anti-human HSP70 antibody (ADI-SPA-810, Enzo Life Sciences). The antibody-sample combinations were then transferred to the blocked wells. After incubation and rinsing, an HRP-conjugated goat anti-mouse IgG antibody (BML-SA204, Enzo Life Sciences) was added. The colorimetric reaction commenced with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, St. Louis, MO, USA) and was immediately

stopped with 1 N hydrochloric acid. Absorbance was measured at 450 nm using an ELISA plate reader (MR9600, Accuris, NJ, USA). The intra-assay and inter-assay coefficients of variation (CV) were 7.75% and 16.04%, respectively ($n = 3$ for each measurement). The assay demonstrated a detection limit of 31.25 ng/mL, with consistent quantification from 31.25 to 2000 ng/mL. Replicate data were averaged before statistical analysis to reduce technical variation.

Environmental data collection and THI calculation

Ambient temperature (T , °C) and relative humidity (RH, %) were measured at 1-min intervals throughout the study period using data from the Camden Airport Automatic Weather Station, located 3.17 km from the farm (BOM, 2024). The collected meteorological data were used for environmental evaluation and the subsequent validation of HW conditions. The temperature-humidity index (THI) was calculated using the following formula, which is widely used in dairy studies (NRC, 1971; Mader et al., 2006; Islam et al., 2020):

$$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26.8)]$$

For each sampling day, THI was calculated as 1-h averages from 1-min environmental data, resulting in fifty 1-h time points (-50 to 0 h) relative to sampling time that enabled time-lagged analyses.

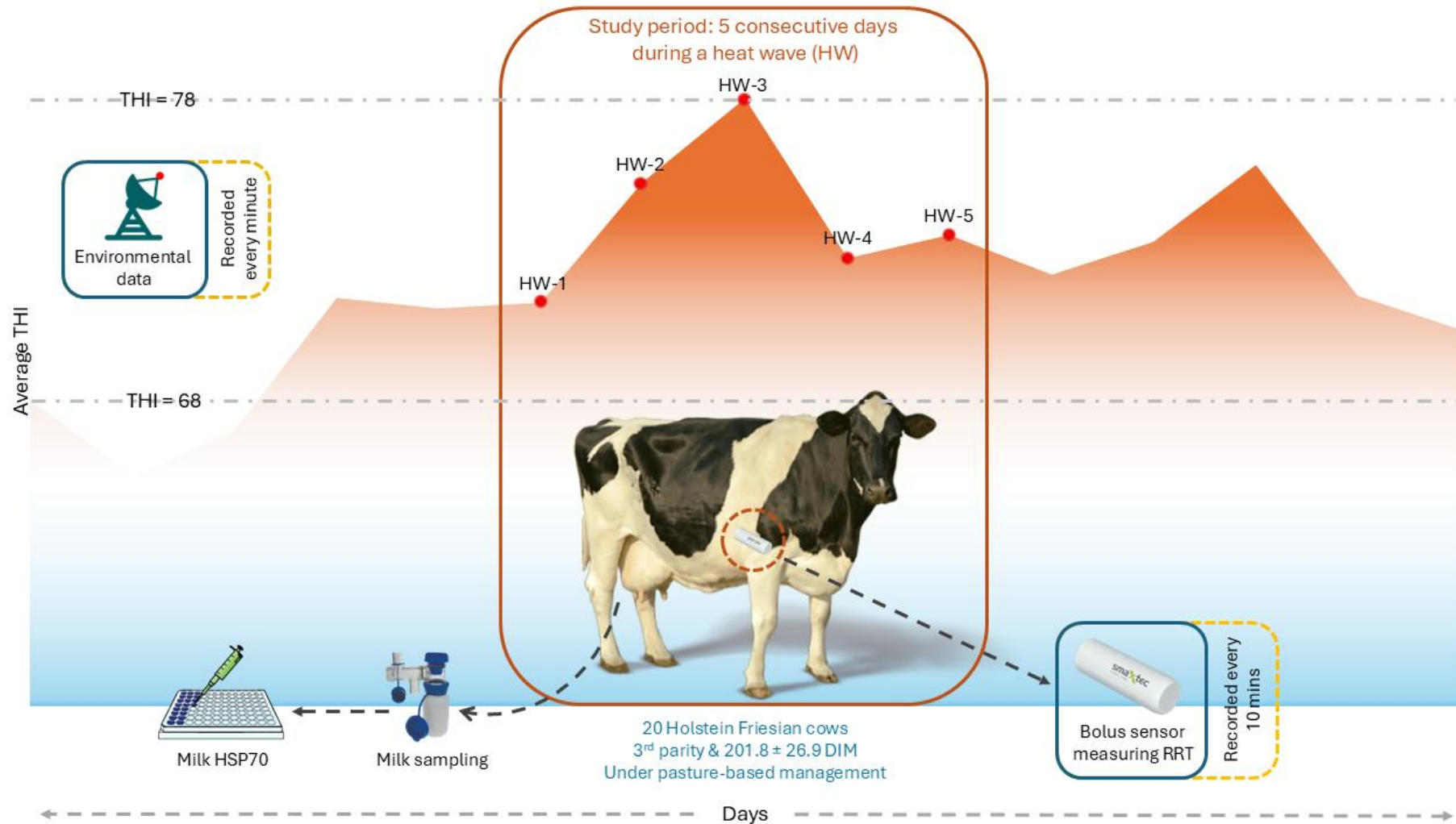


Figure 1: A schematic diagram of experimental design for evaluating the daily variation in milk HSP70 in dairy cows during HW.

Measurement of sensor-based RRT

Continuous RRT was recorded via smaXtec bolus sensors (model MX-2042; smaXtec Animal Care GmbH, Graz, Austria). The device records raw rumen temperature, which was subsequently processed using the manufacturer's proprietary algorithm to account for temporary temperature fluctuations associated with drinking events. These short-term fluctuations may not reflect changes in core thermal status and can introduce noise in time-lagged analyses. Therefore, corrected data were used for all analyses to provide a more physiologically relevant representation of internal thermal dynamics. Data were transferred via an antenna to a base station, subsequently stored in SmaXtec Messenger® software, and recorded at 10-minute intervals. For analysis, RRT data were averaged hourly, aligned to milk sampling time, and extended to a -50 h window for time-lagged based analyses.

Data processing and statistical analysis

All data processing and statistical analysis were conducted in R (version 4.5.0). Data wrangling was performed using packages from the *tidyverse* ecosystem, primarily *dplyr*, *tidyr*, *purrr*, *lubridate*, and *readr*. Milk samples were collected only during the HW period; therefore, the analysis focuses on day-to-day variation in milk HSP70 within the HW rather than direct comparisons with pre-heatwave baseline milk HSP70 concentrations. Milk sampling time (14:00 h) was defined as the temporal reference point for synchronizing all variables, representing the midpoint of the sampling window (13:00-15:00 h), as individual sampling times were not recorded due to variation in cow entry order into the milking parlor. A lag window of up to 50 h prior to sample collection was created to evaluate delayed correlations among environmental, sensor-based, and biomarker variables over the consecutive 5 days of HW (Figure 2). Considering the expected timing of physiological thermoregulatory responses and HSP70 synthesis, lag intervals indicated immediate (0-6 h), intermediate (~24 h), and delayed (~48 h) cellular responses were considered biologically relevant. Lagged associations were investigated throughout hourly intervals within this timeframe to identify the temporal range of response, and the comprehensive lag scan is presented as an exploratory analysis in the Supplementary Material.

Descriptive statistics, including mean, standard deviation (SD), coefficient of variation (CV), and range, were calculated using daily summaries. For environmental variables and RRT, summaries included a seven-day period comprising the two days prior to the HW (Pre-HW) and the subsequent five HW days, to capture pre-HW thermal conditions that may contribute to

accumulated heat load and influence delayed milk HSP70 responses during the early HW period. Milk HSP70 concentrations were calculated for each sampling day and limited to the five HW days, which represent the actual sampling period. Pearson correlations were calculated to visualise temporal patterns in pairwise associations across lag hours. These correlations were used for descriptive purposes only, as they do not account for repeated measurements within individual cows or the shared environmental exposure (THI) across animals. Time-lagged correlations were calculated between milk HSP70 concentration and both THI and RRT to illustrate potential temporal relationships. The association between THI and RRT was evaluated using RRT at the time of milk sampling (RRT_t0) to represent the simultaneous relationship between environmental heat load and physiological condition at sampling.

		Time of Sampling							
Indicators	Time (h)		09:00	10:00	11:00	12:00	13:00	14:00	
	Lag grid	-50		-5	-4	-3	-2	-1	0
THI		*	*	*	*	*	*	*	*
Milk HSP70									*
RRT		*	*	*	*	*	*	*	*
* = Data collected									

* = Data collected

Figure 2: A schematic diagram of a lag grid of 50h prior to sample collection for evaluating delayed correlations between THI, sensor-based RRT, and Milk HSP70 variables.

Inferential analyses were performed using univariable linear mixed models, incorporating cow ID as a random effect to address repeated measurements within cows. These models assessed the associations between milk HSP70 and either THI or RRT across the tested lag hours. Mixed models were fitted using *lme4* with *lmerTest* in the general form $outcome \sim predictor_lag + (1 | cow\ ID)$, using maximum likelihood estimation ($REML = FALSE$). Marginal and conditional R^2 values were calculated using *MuMIn* package. The model assessment also included the Akaike information criterion (AIC), the Bayesian information criterion (BIC), the root mean square error (RMSE), and the mean absolute error (MAE). Individual milk yield was not

available during the HW period; therefore, milk HSP70 concentrations were analysed without adjustment for potential dilution effects.

Receiver operating characteristic (ROC) curve analysis was conducted to explore the ability of milk HSP70 measured at sampling to classify prior HS status at the individual cow level. Here, HS status was defined using the lagged RRT measured 50 h before sampling, with HS classified as temperatures ≥ 39.5 °C, representing observed RRT patterns reported in cattle (Lees et al., 2019) and used to distinguish relatively elevated thermal states. This lag was selected because it corresponded to the strongest association between milk HSP70 and RRT in the lag analysis. Accordingly, the ROC analysis evaluates the ability of milk HSP70 to classify HS occurring approximately 50 h prior to sampling, rather than to diagnose HS in real time. The optimal milk HSP70 cut point was determined using the Youden index. Both ROC analyses and bootstrap confidence intervals were conducted using *pROC* package. The area under the curve (AUC), sensitivity, specificity, and their 95% confidence intervals were estimated using bootstrap resampling with 2,000 iterations performed at the observation level.

Results

Descriptive statistics of study variables

Daily descriptive statistics for environmental and physiological variables across a seven-day HW period, including two days before the HW (Pre-HW-1 and Pre-HW-2), and five HW days (HW-1, HW-2, HW-3, HW-4, and HW-5) are presented in Table 1, including the number of observations contributing to each summary. The ambient temperature (T, °C) varied during the study period, with daily mean values between 23.79 °C and 31.40 °C, and maximum temperatures peaking at 42.10 °C on HW-3. Heat load was sustained across the HW period, as THI values remained at or above the HS threshold throughout most days, including minimum values ≥ 68 during the later HW period. Relative humidity (RH) varied across the study period, with mean values ranging from 55.51% to 72.80%. Correspondingly, mean THI values ranged from 71.03 to 77.92, with the peak daily THI maximum of 84.89 observed on HW-3.

The RRT values ranged from 39.59 °C to 40.45 °C during the study period, but the daily maximum RRT exceeded 42 °C on several HW days, while CV remained low (1.24-2.78%), indicating minimal within-day variability compared to environmental parameters. Milk HSP70 concentrations were measured only on HW days and showed substantial variation among

sampling days. Mean concentrations ranged from 458.89 ng/mL to 858.74 ng/mL, with CV between 12.48% and 27.97%. The maximum concentrations of milk HSP70 exceeded 1,170 ng/mL on HW-1, HW-4, and HW-5, while the minimum values remained above 347 ng/mL across all sampling days.

Table 1. Descriptive statistics of environmental and physiological variables across two pre-HW days and five HW days.

Variables	Days	Mean	SD	CV%	Maximum	Minimum
T (°C)	Pre-HW-2	25.87	8.67	33.52	37.60	14.00
	Pre-HW-1	24.24	3.40	14.01	28.81	19.10
	HW-1	23.79	4.33	18.20	31.40	19.10
	HW-2	27.77	6.94	24.98	38.30	19.00
	HW-3	31.40	8.18	26.05	42.10	21.50
	HW-4	24.52	3.12	12.74	30.71	21.40
	HW-5	25.27	4.63	18.31	34.10	20.20
RH (%)	Pre-HW-2	63.51	28.72	45.23	99.00	23.00
	Pre-HW-1	62.19	19.20	30.87	91.00	38.00
	HW-1	70.88	13.92	19.63	86.00	48.05
	HW-2	66.01	26.74	40.52	100.00	27.00
	HW-3	55.51	27.59	49.71	89.00	14.00
	HW-4	71.46	8.83	12.35	83.00	50.05
	HW-5	72.80	16.06	22.06	94.00	46.00
THI	Pre-HW-2	71.38	9.07	12.70	81.44	57.20
	Pre-HW-1	71.03	3.17	4.46	75.53	65.64
	HW-1	71.23	4.84	6.80	79.75	65.60
	HW-2	75.16	6.28	8.36	83.58	66.11
	HW-3	77.92	5.82	7.47	84.89	69.58
	HW-4	72.70	3.39	4.66	78.95	68.94
	HW-5	73.44	4.80	6.53	82.55	68.02
RRT (°C)	Pre-HW-2	39.99	0.69	1.72	42.03	38.45
	Pre-HW-1	39.71	0.49	1.24	41.38	38.51
	HW-1	39.90	0.56	1.40	41.57	38.55
	HW-2	40.45	0.82	2.03	42.50	38.25

		HW-3	40.24	1.12	2.78	42.43	38.29
		HW-4	39.59	0.52	1.32	41.24	38.16
		HW-5	39.98	0.77	1.93	41.83	38.37
Milk (ng/mL)	HSP70	HW-1	858.74	240.18	27.97	1476.55	655.27
		HW-2	458.89	57.26	12.48	545.82	347.43
		HW-3	534.66	85.56	16.00	751.18	396.92
		HW-4	668.15	153.02	22.90	1171.53	495.11
		HW-5	810.11	142.63	17.61	1185.34	608.07

T = ambient temperature; *RH* = relative humidity; *THI* = temperature-humidity index; *RRT* = reticulorumen temperature; *HSP70* = heat shock protein 70; *HW* = heatwave; *Pre-HW* = days before the heatwave. *n* = number of observations contributing to each daily summary (1-min environmental data aggregated to hourly values; hourly *RRT* observations across cows; 20 cows per day for milk *HSP70*). *THI* values exceeded the HS threshold (≥ 68) for 16-24 h per day during the *HW* period, with continuous exposure (24 h/day) observed during *HW-3* to *HW-5*.

Time-lagged associations between THI, RRT, and milk HSP70

Figure 3 summarises descriptive pairwise correlations between *THI*, sensor-based *RRT*, and milk *HSP70* concentrations across lag hours to visualise temporal patterns. Formal statistical inference accounting for repeated measurements within cows was performed using time-lagged univariable mixed models. Associations between *THI* and milk *HSP70* were observed within the delayed (~48 h) window, with moderate to strong positive correlations and peak values occurring between 40 and 44 h ($r = 0.67$ - 0.69). Similarly, positive associations between *RRT* and milk *HSP70* were observed within a longer delayed (~48 h) window after heat exposure, with peak correlations occurring between 48 and 50 h ($r = 0.41$ - 0.51). In contrast, the relationship between *THI* and *RRT_t0* was strongest within the immediate (0-6 h) window ($r = 0.45$ - 0.53).

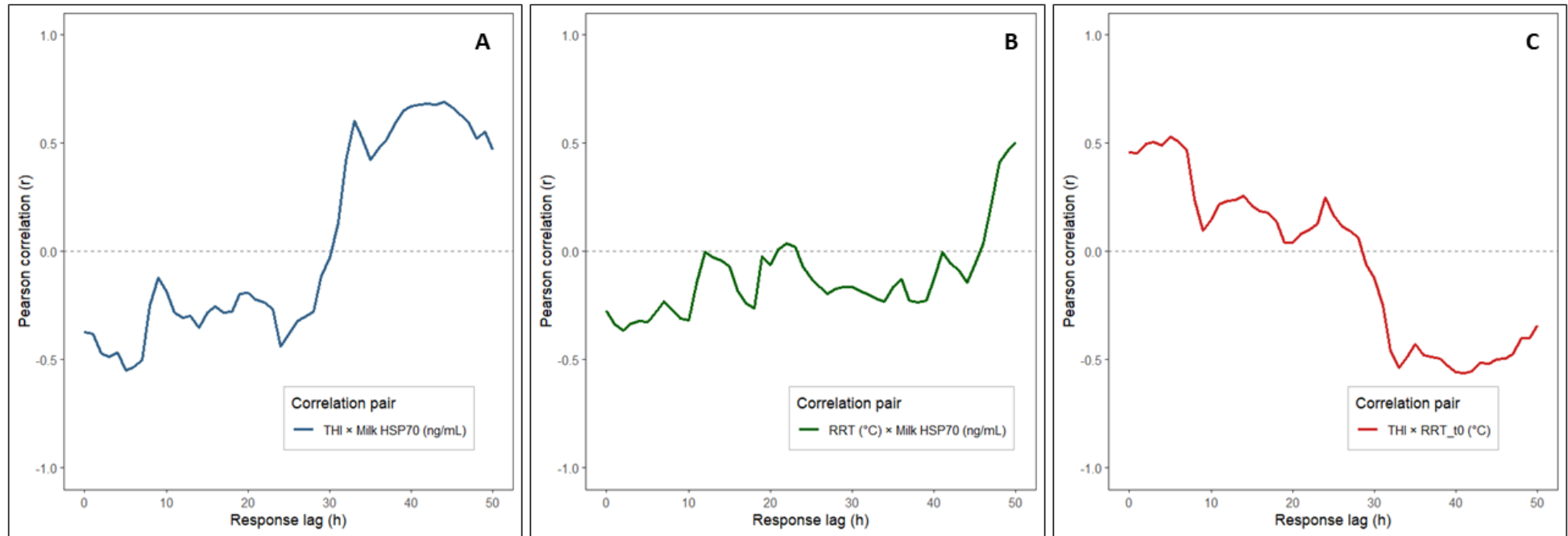


Figure 3. Time-lagged Pearson correlations illustrating descriptive temporal patterns between (A) temperature-humidity index (THI) and milk HSP70, (B) reticulorumen temperature (RRT) and milk HSP70, and (C) THI and baseline reticulorumen temperature at sampling (RRT_t0) during the heatwave period. Correlations are presented for visualisation of lag structure only; statistical inference was performed using time-lagged univariable mixed models accounting for repeated measurements within cows.

Time-lagged univariable mixed model results

Following the pairwise correlation analysis, the univariable linear mixed model associations between environmental THI, sensor-derived RRT, and milk HSP70 concentration across different lags are presented in Table 2.

Milk HSP70 concentration exhibited positive associations with THI within the delayed (~48 h) lag window. The estimated increase in milk HSP70 per unit rise in THI decreased with increasing lag within this interval, ranging from 41.56 ng/mL at 40 h to 33.92 ng/mL at 44 h. Model performance metrics showed gradual variation across this delayed window, with marginal and conditional R^2 values ranging from 45.09% to 47.72% and from 72.38% to 75.66%, respectively. Correspondingly, model fit statistics (AIC, BIC, MAE, and RMSE) varied modestly across this interval, indicating consistent model performance within the delayed response window.

Milk HSP70 concentration was also associated positively with RRT at extended lag intervals of approximately 48-50 h. The estimated effect sizes varied from 207.00 to 193.73 ng/mL per 1°C increase in RRT, with conditional R^2 values rising from 40.92% at 48 h to 51.71% at 50 h. Model fit showed a modest improvement with increasing lag, as indicated by decreasing AIC, BIC, MAE, and RMSE values, and all associations remained statistically significant ($P < 0.001$).

In contrast, RRT_t0 exhibited rapid responses to variations in environmental THI, with significant positive correlations with THI detected at short lag intervals of approximately 0-6 h. The estimated rise in RRT varied from 0.09 °C at 0 h to 0.08 °C at 6 hours for each unit increase in THI. Conditional R^2 values ranged from 48.15% to 57.21% across this interval, while prediction errors remained consistently low (MAE approximately 0.30-0.33 °C; RMSE approximately 0.39-0.41 °C). All associations were statistically significant ($P < 0.001$).

Table 2. Univariable linear mixed model results showing associations between environmental variables, sensor-based RRT, and milk HSP70 at predefined biologically relevant lag intervals corresponding to immediate (0-6 h) and delayed (~48 h) response windows during the HW period. Full results across all tested lag hours, including intermediate (~24 h) intervals, are provided in [Supplementary Table S1](#).

Outcome		Predictor (lag hours)	Estimate	SE	P-value	R^2_m (%)	R^2_c (%)	AIC	BIC	MAE	RMSE
Milk HSP70 (ng/mL)		THI (48)	34.69	4.72	<0.001	27.24	50.14	1317.55	1327.97	123.36	181.0
		RRT (48)	207.00	39.68	<0.001	21.00	40.92	1335.52	1345.95	141.48	193.48
RRT_t0 (°C)		THI (0)	0.09	0.01	<0.001	20.96	48.50	95.73	106.15	0.33	0.41
		THI (6)	0.08	0.01	<0.001	26.14	54.95	85.07	95.49	0.31	0.39

THI = temperature-humidity index; RRT = reticulorumen temperature; RRT_t0 = baseline reticulorumen temperature at sampling; HSP70 = heat shock protein 70; SE = standard error; R^2_m = marginal R-squared (variance explained by fixed effects); R^2_c = conditional R-squared (variance explained by both fixed and random effects); AIC = Akaike information criterion; BIC = Bayesian information criterion; MAE = mean absolute error; RMSE = root mean square error. MAE and RMSE are expressed in the same units as the corresponding outcome variable.

Diagnostic performance of milk HSP70 for HS detection

An exploratory ROC analysis was performed to assess the potential of milk HSP70 to classify HS. Heat stress (HS) was defined according to RRT assessed at a 50-hour lag, with HS classified as temperatures ≥ 39.5 °C, corresponding to the lag at which the strongest association between milk HSP70 concentration and RRT was observed. Therefore, this analysis reflects the ability of milk HSP70 measured at sampling to classify prior HS exposure, rather than to diagnose HS in real time. The ROC analysis produced an AUC of 0.888 (95% CI: 0.823-0.954). The optimum cut-off value determined by the Youden index was approximately 550 ng/mL. At this cut-point, milk HSP70 effectively identified 78.2% of HS cases (sensitivity = 0.782) and classified 90.9% of non-HS samples (specificity = 0.909).

Discussion

This study contributes to the understanding of milk HSP70 expression by integrating high-resolution environmental variables, sensor-derived continuous RRT, and daily milk HSP70 measurements over five consecutive days during an HW, beyond single-time-point observations. Results show that milk HSP70 exhibited a delayed but significant response to HS during a naturally occurring HW in a pasture-based dairy system, indicating cumulative cellular stress rather than immediate thermal response. Environmental indices such as THI are widely used as guidance tools to indicate potential heat load in dairy systems (Polsky and Von Keyserlingk, 2017). However, THI does not directly measure HS at the animal level and does not account for key modifying factors such as solar radiation, wind speed, or individual animal variability (Dunshea et al., 2013). These limitations restrict its ability to accurately reflect the thermal experience of individual cows, particularly under pasture-based conditions. However, composite indices, such as the Grazing Heat Load Index (GHLI), have been developed for improving these variables (Bryant et al., 2023) and have recently been updated for New Zealand dairy systems (Hitchman et al., 2025). In the present study, THI values during the HW frequently exceeded the commonly used HS threshold of $\text{THI} \geq 68$ (Collier et al., 2012), with a maximum daily mean of 77.92 and a range of 65.60 to 84.89, indicating sustained heat load over consecutive days.

The RRT provides continuous, less invasive monitoring of internal thermal conditions; however, changes in rumen temperature may be slower due to the large fluid volume of the rumen, which buffers rapid temperature fluctuations (Antanaitis et al., 2023). This study found

that the average RRT varied from 39.59 to 40.45 °C. These values are broadly comparable to previous reports in dairy cows under HS (Bewley et al., 2008; Liang et al., 2013), although differences in data processing may influence the magnitude of the observed variation. The RRT showed moderate real-time correlations with THI ($r = 0.46-0.53$), maintaining consistent model efficacy within the immediate (0-6 h) window ($P < 0.001$; $R^2_c = 48.15-57.21\%$). The observed minor delays probably reflect the thermodynamic resistance of the rumen and the impact of drinking events, which temporarily lower rumen temperature before returning to baseline, typically occurring within approximately 20 to 120 min after water consumption (Rogers Simmons et al., 1965; Brod et al., 1982; Yamada et al., 2001; Cantor et al., 2018).

In addition to environmental variables, biological factors such as productivity could influence RRT responses to HS. High-yielding dairy cows are generally reported to experience greater increases in internal temperature under elevated thermal conditions due to higher metabolic heat production (Purwanto et al., 1990). However, milk yield was not measured in this investigation; therefore, this effect cannot be validated within the existing dataset. Despite this limitation, RRT values exceeding 41-42 °C indicate considerable internal heat accumulation during the HW period. The persistence of high temperatures over consecutive days indicates a cumulative heat load, possibly enhanced by insufficient overnight cooling, potentially leading to increased physiological stress in the cows.

In contrast, the present study revealed that milk HSP70 concentrations ranged from 458.89 to 858.74 ng/mL during the HW period, with relatively low variability (CV: 12.48-27.97%), and peak concentrations exceeding 1,170 ng/mL on three of the five sampling days. In our previous study, conducted on HSP70 concentrations across various seasons, revealed significantly lower milk HSP70 concentrations (76.45-546.93 ng/mL) with greatest variability (CV: 53.76%), relative to blood (CV: 34.37%) and saliva (CV: 32.35%) samples (Rakib et al., 2026a). Because these datasets were obtained under different experimental designs, they cannot be directly compared statistically. However, the higher and more consistent milk HSP70 concentrations observed during the present HW suggest a more uniform cellular stress response associated with prolonged heat exposure. In contrast, seasonal conditions likely reflect more diverse environmental and physiological stressors.

Milk HSP70 showed a delayed response in relation to both external and internal HS indicators. Associations between THI and milk HSP70 were observed within a delayed (~48 h) window,

with peak correlations occurring between 40 and 44 h ($r = 0.67$ - 0.69 ; $P < 0.001$; $R^2_c = 72.38$ - 75.66%), whereas associations with RRT were most evident at a longer delayed (~ 48 h) window after heat exposure, with peak correlations occurring between 48 and 50 h ($r = 0.41$ - 0.51 ; $P < 0.001$; $R^2_c = 40.92$ - 51.71%). This delayed response aligns with the biological function of HSP70 as a stress-induced molecular chaperone. Prolonged HS disrupts protein homeostasis, increases oxidative stress, and initiates heat shock factor-mediated transcription, leading to increased intracellular synthesis of HSP70 prior to its detection in extracellular components like milk (Mosser and Morimoto, 2004; Mayer and Bukau, 2005; Bagath et al., 2019). The variability in milk HSP70 concentrations may be influenced by the local secretion dynamics within the mammary gland (Collier et al., 2008; Hu et al., 2016) collectively indicating that milk HSP70 reflects cumulative rather than immediate detection of HS. Moreover, these findings complement previous studies demonstrating increased milk HSP70 concentrations under HS conditions (Rakib et al., 2025; 2026a; Rakib et al., 2026b) and further expand them by characterizing daily variations over consecutive days of HW.

The validation of diagnostic thresholds for biological stress markers requires investigation across various herds, environmental conditions, and experimental settings, along with baseline data. In this study, HS was defined using RRT as a physiological reference, as sensor-derived RRT provides continuous, cow-specific measurements of thermal status and closely reflects CBT of the animal (Bewley et al., 2008; Lees et al., 2019). Based on this reference, a milk HSP70 cut-point of approximately 550 ng/mL was identified, indicating high sensitivity (approx. 78%) and specificity (approx. 91%) and efficiently distinguishing between HS and non-HS cellular conditions. However, this cut-point should be interpreted as exploratory and conditional on the RRT-based HS definition and selected lag structure applied in this study. This represents a preliminary step toward determining an operational milk-HSP70 cut-point for HS detection across consecutive HW days, which requires additional refinement through extensive, multi-seasonal studies.

Some limitations should be acknowledged. Because milk HSP70 measurements were collected only during the HW period, the present study evaluates variation within the HW rather than changes relative to a pre-HW milk HSP70 baseline. Furthermore, while lagged associations were explored across multiple hourly intervals, the lag analysis should be interpreted as exploratory. The identified lag windows represent approximate biological response times rather than precise causal delays, and results should be interpreted within predefined lag windows

rather than at the level of individual lag-specific estimates. In addition, environmental data were obtained from a nearby weather station, and although representative of local conditions, they may not fully capture microclimatic variation at the farm level. Individual milk yield was not recorded because the study farm lacked in-line milk metering during the HW event. While this lowered the ability to adjust HSP70 concentrations for potential dilution effects, the study focused on temporal patterns and lagged relationships within cows across multiple days, which are less sensitive to short-term fluctuation in milk volume. Future research that includes milk yield measurements is necessary to enhance the interpretation of milk HSP70 concentrations.

In addition, the study was limited to a single HW in a single herd, with milk sampling conducted at a single daily time point (afternoon milking); therefore, within-day variation was not assessed. Also, the identified cut-off values should be interpreted with caution and require further validation under different environmental conditions, herds, and management systems. Furthermore, the HS classification was based on a relative temperature threshold within the observed physiological range, and the ROC analysis should be considered exploratory, particularly as bootstrap resampling was performed at the observation level despite repeated measurements within cows.

Conclusion

This study provides one of the first detailed evaluations of daily variation in milk HSP70 concentrations in dairy cows during a HW. The results indicate that milk HSP70 responds to HW exposure with a significant temporal lag, indicating accumulated cellular stress rather than an immediate response to HS. Milk HSP70, combined with traditional environmental and sensor-derived indications, provides additional insight into prolonged HS in pasture-based dairy systems. Overall, the results suggest that milk HSP70 has potential value as a non-invasive indicator of HS during heat events.

Notes

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Ethical Statement

The study was conducted in accordance with ethical guidelines and received approval from the Animal Ethics Authority at The University of Sydney, Australia (Project Identifier No: 2022/AE002230).

Conflict of Interest

The authors have not stated any conflicts of interest.

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CHAPTER 8

General Discussion and Conclusion

General Discussion

Heat stress (HS) detection in dairy cows has traditionally relied on environmental indicators that quantify external heat load and physiological indicators that capture animal-level responses. While these methods measure environmental exposure and physiological adaptation, they provide limited insights into the underlying cellular stress processes. In contrast, heat shock protein 70 (HSP70) is a stress-related protein that is activated inside cells when cows are exposed to heat, reflecting the internal cellular stress response (Hyder et al., 2017; Hassan et al., 2019).

This research evaluated the potential of HSP70 as a non-invasive indicator of HS in dairy cows by investigating its analytical reliability, biological variability, and temporal behaviour in relation to established environmental and physiological indicators in pasture-based dairy production systems. Collectively, the findings indicate that milk HSP70 is analytically reliable, exhibits a relatively limited biological responsiveness to heat load, and is temporally distinctive from traditional HS indicators, positioning this protein as a potential complementary component within a multi-level HS detection framework.

The literature review in [Chapter 2](#) demonstrated the potential biological significance of HSP70 as a HS indicator in dairy cows, highlighting its critical role in cellular protein homeostasis and stress adaptation (Mosser and Morimoto, 2004; Mayer and Bukau, 2005). The study also identified that HSP70 can be detected in extracellular matrices, including milk and saliva, providing a foundation for non-invasive applications in dairy systems (Lamy et al., 2017; Rakib et al., 2026b).

However, the review highlighted critical limitations that restricted its practical application in commercial dairy systems. These included variability in analytical techniques, the lack of standardised reference ranges, inter-individual biological variation, uncertainty regarding sensitivity to HS, and the use of invasive sampling methods (i.e., blood, saliva) in most published studies. Therefore, the subsequent experimental chapters were accordingly designed to address these limitations by investigating the analytical reliability, biological variability, and temporal behaviour of milk HSP70 relative to conventional HS indicators.

Reliable and reproducible quantification is essential for the effective application of any biomarker in HS detection. Before investigating biological variation, it was necessary to ensure that HSP70 could be quantified reliably and consistently across different sample types. Commercial ELISA kits are widely available and demonstrate high sensitivity to HSP70; however, their limited working ranges require dilution of high-concentration samples, which can introduce additional variability. A recently developed in-house competitive ELISA (Pathirana and Garcia, 2022) provides a cost-effective alternative; however, its performance across various biological fluids has not been systematically compared to a commercial ELISA system. Therefore, [Chapter 3](#) compared the in-house competitive ELISA with a commercial ELISA kit in milk, blood, and saliva of dairy cows.

Variations between ELISA systems are common, particularly when assays use different formats and calibration systems (Findlay et al., 2000). The strong agreement observed between the two assays across all sample types represents an important validation finding. Although the in-house test systematically detected higher absolute concentrations, both systems showed strong correlations and consistently classified animals based on HSP70 levels (Rakib et al., 2026b), suggesting that biological interpretation was maintained. Similar biological differences have been reported in other ELISA comparisons, including HSP70 and infectious disease biomarkers, where absolute values varied but overall agreement remained strong (Njemini et al., 2005; Rastawicki, 2024).

Moreover, the consistently lower coefficients of variation in the in-house assay suggest enhanced precision and reduced sample sizes required to achieve the same statistical power, which is particularly relevant for extensive or long-term studies (Al-Saeedi et al., 2023; Gobena et al., 2025). Previous studies in other livestock species, including cattle, sheep, and poultry, indicate that well-validated in-house ELISAs can perform similarly to, or in some cases better than, commercial kits when optimised for specific sample types (Ferreira et al., 2002; Hurisa et al., 2025; Toaleb et al., 2025).

The broader working range of the in-house ELISA without routine dilution further strengthens its suitability for milk analysis (Rakib et al., 2026b), as dilution steps can introduce additional variability (Verch et al., 2016). In addition, the approximately 7-8-fold lower per-sample cost substantially improves the feasibility of large-scale or repeated sampling (Njemini et al., 2005; Hurisa et al., 2025). These data collectively indicate that the in-house ELISA offers both

analytical reliability and practical advantages, establishing the methodological foundation for the subsequent studies conducted in this research program.

While analytical reliability is important, biomarker validity also depends on appropriate sample handling. Until now, this was unknown for HSP70 in milk samples. Chapter 4 investigated the practical effects of milk processing and storage conditions on HSP70 quantification. Findings indicated that skimming milk before storage reduced HSP70 degradation, and refrigerated or frozen skim milk maintained stable concentrations during short-term storage. In contrast, prolonged storage led to substantial degradation, underscoring the importance of proper sample handling for reliable results (Rakib et al., 2025). These results are consistent with previous studies in dairy diagnostic procedures. For example, Charlier et al. (2005) reported that preserving whole milk samples at 4 °C for 4 days did not significantly affect ELISA detection of *Ostertagia ostertagi* antibodies compared with skim milk samples.

In addition to temperature and storage duration, the use of preservatives is another common approach to maintaining milk sample integrity. Bronopol is widely used to preserve milk samples and is frequently considered appropriate for certain ELISA experiments (Sweeney et al., 1994; Sanchez et al., 2002; Da Silva et al., 2017). However, in the present investigation, bronopol-preserved samples consistently exhibited reduced HSP70 concentrations, suggesting that the preservative may interact with HSP70 or affect the assay. Similar interference with bronopol has been documented in other ELISA-based assays, including C-reactive protein and anti-bovine viral diarrhoea antibodies (Wilson and Rood, 2012; Thomas et al., 2016). This indicates that preservative effects may vary across biomarkers, underscoring the need for biomarker-specific validation of preservative compatibility in ELISA systems. Overall, these results provide novel practical guidance on milk handling for research and future on-farm applications where HSP70 determination is required.

After the analytical and methodological foundations were established, Chapter 5 extended the investigation of the biological variability of HSP70 across milk, blood, and saliva in relation to environmental heat load. Significant seasonal variations were observed across all matrices, with maximum HSP70 concentrations found in the summer, consistent with previous studies on heat-induced cellular responses (Dangi et al., 2012; Manjari et al., 2015). Blood exhibited the highest HSP70 concentrations, followed by saliva and milk; however, milk HSP70 demonstrated the highest variability, indicating lower sensitivity or greater biological

interference than blood or saliva (Rakib et al., 2026a). This variation might indicate dilution effects associated with varying milk production (Boland et al., 2013) or mammary-specific secretion dynamics (Collier et al., 2008; Hu et al., 2016), which may limit its reliability as an individual HS indicator.

An association between days in milk (DIM) and milk HSP70 concentrations was reported in [Chapter 5](#) (Rakib et al., 2026a). Although part of the variation in concentration might reflect dilution effects associated with changes in milk yield, the models showed that DIM and average THI remained significant predictors, whereas milk yield was not significant after accounting for lactation stage (Rakib et al., 2026a). This suggests that the lactation stage may independently influence milk HSP70 dynamics, although the limited number of sampling time points in [Chapter 5](#) restricts the ability to draw firm conclusions about the underlying mechanisms. This complexity, however, is consistent with broader evidence that HSP70 responds not only to HS but also to other modifying factors, including oxidative, metabolic, and physiological stressors (Kregel, 2002; Rakib et al., 2024). Moderate correlations between milk and blood, and milk and saliva, indicate that milk HSP70 partially reflect systemic stress responses, while highlighting unexplored questions regarding the origin, transport, and timing of HSP70 appearance in milk (Rakib et al., 2026a).

These knowledge gaps, together with the biological complexity and limited suitability of milk HSP70 as a standalone indicator, led to the lag-based analyses described in [Chapter 6](#) and [Chapter 7](#). In [Chapter 6](#), we compared milk HSP70 with environmental and physiological indicators, including rectal temperature (RT), infrared thermography (IRT), and reticulorumen temperature (RRT) to evaluate their sensitivity, temporal lag, and usefulness. This analysis revealed that HS detection tools operate across distinct biological time scales. Real-time RT and IRT showed the maximum associations with environmental conditions (THI), indicating their sensitivity to short-term changes in heat load. Sensor-derived RRT indicated a relatively slower rate of internal heat accumulation, reflecting the influence of substantial fluid volume and thermal capacity of the reticulorumen environment (Rogers Simmons et al., 1965; Brod et al., 1982; Shirley et al., 2025). Together, these indicators primarily reflect environmental load and systemic thermoregulatory responses, while also highlighting that environmental indices alone cannot fully explain individual variation among cows under HS.

In contrast, milk HSP70 exhibited a consistent and substantial temporal delay, with the maximum correlation occurring at approximately 35 h after heat exposure. This behaviour indicates that milk HSP70 reflects delayed cellular stress responses of prior heat exposure, rather than immediate heat load. Unlike RT, IRT, and RRT, all of which demonstrate their sensitivity to short-term changes in environmental heat load, HSP70 requires transcriptional activation, protein synthesis, and subsequent extracellular release before detection (Mosser and Morimoto, 2004; Mayer and Bukau, 2005; Bagath et al., 2019). Experimental studies on bovine mammary epithelial cells indicate that HS rapidly stimulates HSP70 transcription, but the recovery of mammary gene expression might extend for up to 24 hours after exposure (Hu et al., 2016). Moreover, HS modifies mammary blood circulation, nutrient absorption, and oxidative balance (Guo et al., 2021; Yue et al., 2023; Zeng et al., 2024), factors that affect milk production outcomes over hours to days rather than immediately. The hormonal control of the blood-milk barrier could additionally impact the delayed expression of stress-related responses in milk (Stelwagen, 2025). Therefore, milk HSP70 represents a biologically distinct layer of HS assessment, indicating the subsequent cellular effects of extended heat exposure rather than the immediate HS.

This interpretation was further supported by the heatwave study in [Chapter 7](#). HSP70 levels remained elevated during prolonged heat exposure, indicating that milk HSP70 responds to sustained heat load rather than immediate temperature changes. Previous studies similarly indicate that extended heat exposure activates systemic and cellular stress mechanisms, including heat shock proteins and stress hormones (Min et al., 2015; Jo and Lee, 2025). This distinction is important because conventional indicators guide immediate management measures, such as activating cooling systems, whereas milk HSP70 may indicate the overall intensity and duration of cellular stress.

Moreover, the production responses reported in [Chapter 6](#) aligned with the delayed characteristics of cellular stress indicators. Milk yield had the most significant decline at 48 h lags subsequent to elevated THI and RRT, consistent with previous research that indicates milk yield typically decreases one to three days after HS events, rather than immediately (Wildridge et al., 2018; Li et al., 2021). This delay is consistent with the time expected for decreases in feed intake and metabolic changes to be reflected in production losses (Hagiya et al., 2019). In contrast, milk composition exhibited minimal and delayed changes, suggesting that yield is more susceptible to cumulative HS than compositional traits (Rakib et al., 2025).

Preliminary threshold analyses were performed to investigate whether milk HSP70 could differentiate between HS and non-HS cows. Under the specific sampling schedule implemented in [Chapter 6](#), its capacity to distinguish between groups was limited. However, during the prolonged heatwave discussed in [Chapter 7](#), discrimination significantly improved. This indicates that milk HSP70 functions more effectively when cellular stress is more substantially induced, as during prolonged heat exposure. The differences in threshold values across studies further suggest that thresholds might be influenced by the timing of sample collection, the intensity and duration of the heat load, and other factors. Because HS classification was based on reticulorumen temperature at a defined lag interval, these thresholds should be regarded as exploratory rather than conclusive. These findings collectively represent an important first step toward the application of milk HSP70 as a diagnostic tool, underscoring the need for further validation across herds, seasons, and production systems.

This thesis integrates analytical validation, environmental monitoring, and biological evaluation to investigate milk HSP70 as a potential indicator of HS in dairy cows. However, several analytical and methodological limitations should be considered when interpreting the findings and defining their scope of application.

First, the environmental evaluation was indirect. Heat load was calculated using data from a nearby automated weather station rather than on-farm microclimatic sensors, which might not precisely represent the thermal environment experienced by individual cows. This may have reduced the strength of associations between environmental and biological responses. In addition, although THI was used as a proxy indicator of HS in some of the studies, it reflects environmental load rather than individual physiological variation (Dunshea et al., 2013) and, therefore, may not fully capture the biological HS experienced by individual cows. Furthermore, environmental variables and RRT were continuously recorded, whereas milk HSP70, RT, and IRT were assessed at specific time points. This variation in temporal precision limited the ability to assess exposure and response with precision, potentially influencing the interpretation of lag-based associations.

Second, nighttime cooling and recovery were not specifically considered in the studies because the analytical approach focused on discrete lag intervals rather than cumulative exposure metrics, making it difficult to distinguish between accumulated heat load and delayed biological activity. Diurnal temperature variation plays a critical role in mitigating HS by

allowing animals to dissipate heat during cooler nighttime periods (Veissier et al., 2017). Therefore, not considering nighttime recovery may have influenced lag interpretation, as elevated milk HSP70 could reflect cumulative heat exposure or incomplete recovery rather than a completely delayed response.

In addition, biological interpretation remains complex. The modest proportion of variation explained by THI or RRT suggests that milk HSP70 reflects an integrated stress response rather than HS alone. As described in [Chapter 2](#), HSP70 expression is regulated by several interacting physiological factors, including oxidative stress, metabolic load, immune activation, and potentially subclinical mastitis, which were not quantified in this study. Furthermore, the cellular origin of milk HSP70 remains unknown. These features complicate its interpretation as a specific HS biomarker.

Finally, the external verification is limited. All experiments were conducted on a single herd and production system, limiting the applicability of the findings across breeds, climates, and management settings. In addition, individual variation in HSP70 responses across different environments was not evaluated. Therefore, further multi-herd and multi-environment investigations, including consideration of individual variation, are required to validate the reliability and applicability of these findings.

Despite these limitations, the findings collectively suggest that milk HSP70 might be used as a complementary indicator of HS when evaluated alongside conventional environmental and physiological indicators, rather than as a standalone diagnostic tool. Further studies should extend beyond HS alone to evaluate milk HSP70 as an integrative marker of overall physiological stress and health status in dairy cows, including potential associations with disease, ageing, somatic cell count, and immunological activation. Significant variations in HSP70 responses were observed among cows under similar environmental conditions, suggesting that animals may differ in how their cells respond to heat load. Further research is needed to determine whether these individual variations are consistent across lactations and environmental conditions, and to better understand the biological processes underlying them. If these conditions are validated, milk HSP70 responses might be beneficial to the phenotypic characterisation of heat resilience and potentially support future breeding strategies focused on improving long-term thermal adaptation.

Conclusion

This thesis provides the first integrated evaluation of HSP70 as a non-invasive biomarker of HS in dairy cows by integrating analytical validation, environmental monitoring, and biological interpretation under practical dairy production conditions.

This research summarised the current state of knowledge and identified limitations of commonly used environmental and physiological indicators, particularly their limited capacity to capture cellular-level stress responses. Collectively, the novel research reported in this thesis has made substantial contributions to the advancement of science in this field. It was demonstrated that HSP70 can be reliably measured in bovine milk under different biological and environmental conditions. Milk represents a practical, non-invasive sample for assessing HSP70 and is uniquely advantageous because it is already collected routinely on commercial dairy farms.

In addition, an in-house competitive ELISA was optimised and validated, showing strong agreement with a commercial assay while offering improved precision and lower cost, and is now available for future investigations. The evaluation of milk processing and storage conditions further demonstrated that skimming milk before storage and short-term refrigeration or freezing maintains stable HSP70 concentrations without requiring immediate analysis. Beyond analytical validation, this research advanced the understanding of the biological dynamics of milk HSP70. Concentrations were influenced not only by environmental heat load but also by physiological state, and a consistent temporal delay relative to environmental and physiological indicators was identified.

Overall, this thesis demonstrates the biological significance and potential applications of milk HSP70 for HS detection in dairy cows. Although not suitable as a direct, real-time indicator, milk HSP70 can provide insights into the long-term cellular effects of HS, opening opportunities to explore its potential as a cellular marker for genetic selection for heat resilience. These findings advance the current knowledge of cellular stress responses in dairy cows as climate change increases the frequency and intensity of HS events. It also underscores the importance of integrated HS assessment frameworks that integrate available physiological indicators with molecular biomarkers to provide a more comprehensive understanding of stress mechanisms in dairy cows.

This thesis provides a strong foundation for future research aimed at developing and validating milk-based cellular biomarkers within integrated HS monitoring frameworks. Ultimately, this research contributes to the development of climate-resilient dairy production systems by advancing the understanding and monitoring of cellular stress in dairy cows.

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APPENDIX

Supplementary Material

Supplementary Material: Chapter 7

Supplementary Table S1: Univariable linear mixed model results showing time-lagged associations between environmental THI, sensor-based RRT, and milk HSP70 variables across all tested lag hours (0-50 h) during the HW period.

Outcome	Predictor (lag hours)	Estimate	SE	<i>P</i> -value	R^2_m (%)	R^2_c (%)	AIC	BIC	MAE	RMSE
Milk HSP70 (ng/mL)	THI (0)	-34.61	7.56	<0.001	14.05	33.7	1340.24	1350.66	147.37	196.59
	THI (1)	-31.02	6.55	<0.001	14.81	34.65	1339.09	1349.51	146.18	195.73
	THI (2)	-31.52	4.97	<0.001	22.6	44.36	1326.29	1336.71	138.65	186.64
	THI (3)	-27.06	4.08	<0.001	23.98	46.08	1323.79	1334.21	136.01	184.98
	THI (4)	-28.06	4.51	<0.001	22.06	43.68	1327.25	1337.67	138.49	187.28
	THI (5)	-31.23	3.82	<0.001	30.7	54.45	1310.34	1320.76	133.25	176.67
	THI (6)	-36.47	4.73	<0.001	28.8	52.09	1314.37	1324.79	137.53	179.05
	THI (7)	-40.07	5.76	<0.001	25.45	47.91	1321.03	1331.45	141.02	183.19
	THI (8)	-27.81	9.62	0.005	6.4	24.17	1350.92	1361.34	151.22	205.08
	THI (9)	-17.55	13.01	0.181	1.5	18.07	1357.06	1367.49	157.56	210.31
	THI (10)	-22.46	10.58	0.037	3.61	20.7	1354.48	1364.9	154.47	208.08
	THI (11)	-35.16	10.75	0.002	7.98	26.14	1348.83	1359.25	150.59	203.35
	THI (12)	-34.97	9.75	<0.001	9.37	27.87	1346.94	1357.36	149.08	201.82
	THI (13)	-28.41	8.11	<0.001	9.0	27.41	1347.44	1357.86	150.6	202.23
	THI (14)	-32.82	7.65	<0.001	12.64	31.95	1342.31	1352.73	150.59	198.18

THI (15)	-22.65	6.9	0.002	8.02	26.19	1348.77	1359.19	153.92	203.3
THI (16)	-18.3	6.18	0.004	6.68	24.52	1350.55	1360.97	153.59	204.77
THI (17)	-19.29	5.88	0.002	8.02	26.19	1348.78	1359.2	151.75	203.31
THI (18)	-15.81	4.9	0.002	7.79	25.91	1349.08	1359.5	151.37	203.56
THI (19)	-9.41	4.22	0.029	3.96	21.13	1354.04	1364.46	155.9	207.7
THI (20)	-10.06	4.65	0.033	3.74	20.86	1354.31	1364.73	156.55	207.94
THI (21)	-12.69	4.97	0.013	5.09	22.55	1352.6	1363.02	155.84	206.49
THI (22)	-13.03	4.82	0.008	5.66	23.25	1351.88	1362.3	154.21	205.88
THI (23)	-15.49	4.99	0.003	7.27	25.26	1349.77	1360.19	152.55	204.12
THI (24)	-28.15	4.91	<0.001	19.69	40.73	1331.32	1341.74	145.12	190.09
THI (25)	-24.02	5.13	<0.001	14.55	34.33	1339.48	1349.9	147.38	196.02
THI (26)	-18.12	4.74	<0.001	10.46	29.23	1345.42	1355.84	149.94	200.62
THI (27)	-15.24	4.28	<0.001	9.25	27.73	1347.1	1357.52	149.38	201.95
THI (28)	-15.53	4.82	0.002	7.77	25.89	1349.1	1359.52	151.05	203.57
THI (29)	-6.35	5.07	0.214	1.3	17.82	1357.31	1367.73	158.68	210.53
THI (30)	-2.19	6.18	0.724	0.11	16.33	1358.74	1369.16	160.6	211.79
THI (31)	10.42	7.21	0.152	1.72	18.35	1356.8	1367.22	159.86	210.08
THI (32)	45.44	8.6	<0.001	17.48	37.98	1334.92	1345.34	138.43	192.66
THI (33)	83.0	8.54	<0.001	36.52	61.7	1296.52	1306.95	116.04	169.14
THI (34)	61.51	8.31	<0.001	27.44	50.39	1317.14	1327.56	124.03	180.74
THI (35)	52.9	9.82	<0.001	18.0	38.62	1334.09	1344.51	136.37	192.06

THI (36)	54.56	8.5	<0.001	22.97	44.82	1325.62	1336.04	129.94	186.19
THI (37)	48.51	6.66	<0.001	26.91	49.72	1318.21	1328.63	124.13	181.41
THI (38)	53.15	5.72	<0.001	35.02	59.83	1300.31	1310.73	118.23	171.11
THI (39)	48.93	4.22	<0.001	42.31	68.91	1279.87	1290.29	113.15	161.29
THI (40)	41.56	3.27	<0.001	45.09	72.38	1270.43	1280.85	112.85	157.37
THI (41)	39.49	2.97	<0.001	46.39	74.0	1265.62	1276.04	111.51	155.51
THI (42)	35.44	2.65	<0.001	46.54	74.18	1265.06	1275.48	108.06	155.3
THI (43)	31.17	2.35	<0.001	46.33	73.92	1265.86	1276.28	101.76	155.6
THI (44)	33.92	2.44	<0.001	47.72	75.66	1260.35	1270.77	100.95	153.58
THI (45)	36.24	2.92	<0.001	44.36	71.47	1273.03	1283.45	103.61	158.41
THI (46)	34.17	3.19	<0.001	39.71	65.67	1287.79	1298.21	108.37	164.87
THI (47)	34.56	3.62	<0.001	35.92	60.95	1298.06	1308.48	111.76	169.93
THI (48)	34.69	4.72	<0.001	27.24	50.14	1317.55	1327.97	123.36	181.0
THI (49)	37.75	4.63	<0.001	30.6	54.33	1310.55	1320.97	116.0	176.79
THI (50)	28.53	4.57	<0.001	22.15	43.79	1327.09	1337.51	127.38	187.17
RRT (0)	-228.86	41.58	<0.001	20.31	53.24	1337.05	1347.47	153.38	208.94
RRT (1)	-217.51	34.61	<0.001	22.91	55.45	1330.53	1340.95	147.45	203.19
RRT (2)	-201.07	30.25	<0.001	24.42	56.59	1327.35	1337.77	150.23	200.38
RRT (3)	-169.51	27.79	<0.001	21.65	53.49	1331.67	1342.09	151.31	202.65
RRT (4)	-154.96	26.07	<0.001	20.83	52.83	1332.98	1343.40	150.94	203.74
RRT (5)	-155.89	25.61	<0.001	21.09	53.58	1331.95	1342.37	150.44	203.52

RRT (6)	-141.35	27.39	<0.001	17.04	47.77	1338.60	1349.02	152.03	206.07
RRT (7)	-120.96	29.73	<0.001	12.30	39.75	1345.42	1355.84	154.73	207.92
RRT (8)	-122.65	27.84	<0.001	13.72	40.98	1343.06	1353.49	154.27	205.73
RRT (9)	-115.81	25.80	<0.001	13.84	38.47	1341.82	1352.24	149.61	202.06
RRT (10)	-97.72	24.38	<0.001	11.65	32.12	1344.41	1354.83	148.32	200.93
RRT (11)	-64.10	28.07	0.025	4.35	25.11	1354.07	1364.49	158.97	209.92
RRT (12)	-41.50	35.49	0.245	1.34	21.92	1357.71	1368.13	162.79	213.26
RRT (13)	-53.02	34.75	0.130	2.23	24.13	1356.92	1367.34	161.50	213.39
RRT (14)	-53.77	32.95	0.106	2.51	24.42	1356.60	1367.02	160.09	213.10
RRT (15)	-63.23	33.21	0.060	3.45	26.00	1355.77	1366.19	160.62	212.80
RRT (16)	-124.90	36.52	<0.001	9.95	36.79	1349.36	1359.78	159.42	210.80
RRT (17)	-180.13	38.78	<0.001	16.62	47.45	1342.65	1353.08	156.18	210.24
RRT (18)	-254.47	48.17	<0.001	21.07	53.77	1338.94	1349.36	158.02	211.02
RRT (19)	-72.62	69.49	0.299	1.28	20.61	1357.89	1368.31	159.66	212.69
RRT (20)	-84.06	62.90	0.185	2.03	21.60	1357.23	1367.65	160.04	212.22
RRT (21)	-35.50	56.53	0.532	0.46	18.48	1358.51	1368.93	161.06	212.51
RRT (22)	-14.65	54.58	0.789	0.08	16.99	1358.80	1369.22	160.79	212.20
RRT (23)	-25.40	54.06	0.640	0.25	17.75	1358.66	1369.08	160.45	212.38
RRT (24)	-59.63	44.80	0.186	1.72	20.59	1357.20	1367.62	158.75	211.74
RRT (25)	-69.49	36.98	0.063	3.15	22.65	1355.54	1365.96	156.77	210.53
RRT (26)	-78.15	32.20	0.017	5.00	25.79	1353.44	1363.87	155.37	209.39

RRT (27)	-86.53	28.72	0.003	7.33	30.21	1350.83	1361.25	154.94	208.50
RRT (28)	-77.06	27.23	0.006	6.60	29.73	1351.82	1362.24	155.93	209.59
RRT (29)	-74.85	27.05	0.007	6.30	29.54	1352.15	1362.57	156.29	209.96
RRT (30)	-79.08	27.78	0.005	6.65	30.30	1351.82	1362.24	156.05	209.99
RRT (31)	-84.61	28.47	0.004	7.32	30.63	1351.12	1361.54	154.85	209.12
RRT (32)	-79.96	26.97	0.004	7.14	29.36	1350.99	1361.41	152.54	208.14
RRT (33)	-72.16	24.87	0.005	6.63	27.08	1351.09	1361.51	148.45	206.95
RRT (34)	-69.96	23.66	0.004	6.80	26.70	1350.73	1361.15	147.97	206.24
RRT (35)	-72.67	27.06	0.009	5.87	28.45	1352.46	1362.88	153.27	209.72
RRT (36)	-112.29	33.85	0.001	9.31	38.52	1350.51	1360.93	156.25	214.08
RRT (37)	-155.60	30.88	<0.001	17.24	50.74	1340.62	1351.04	156.79	211.76
RRT (38)	-158.30	29.74	<0.001	18.65	53.22	1338.96	1349.38	156.53	212.27
RRT (39)	-158.10	32.11	<0.001	17.39	50.46	1341.41	1351.83	156.88	212.10
RRT (40)	-104.40	38.83	0.008	6.61	31.64	1352.87	1363.29	159.13	212.17
RRT (41)	-34.02	45.05	0.452	0.61	18.93	1358.34	1368.76	160.95	212.52
RRT (42)	-61.27	55.83	0.275	1.32	19.68	1357.72	1368.14	161.65	211.96
RRT (43)	-124.62	69.18	0.075	3.76	25.31	1356.03	1366.45	159.48	212.44
RRT (44)	-141.85	60.64	0.022	6.00	27.75	1353.98	1364.40	160.71	210.76
RRT (45)	-93.90	55.12	0.092	3.40	25.40	1356.41	1366.83	164.14	213.09
RRT (46)	-12.33	52.01	0.813	0.07	16.88	1358.81	1369.23	161.43	212.16
RRT (47)	101.57	50.44	0.047	4.52	19.03	1354.90	1365.32	157.38	207.26

	RRT (48)	207.00	39.68	<0.001	21.00	40.92	1335.52	1345.95	141.48	193.48
	RRT (49)	195.57	31.24	<0.001	25.26	46.61	1327.10	1337.52	132.90	187.72
	RRT (50)	193.73	27.24	<0.001	29.51	51.71	1319.86	1330.28	127.53	183.12
RRT_t0 (°C)	THI (0)	0.09	0.01	<0.001	20.96	48.50	95.73	106.15	0.33	0.41
	THI (1)	0.08	0.01	<0.001	20.68	48.15	96.27	106.69	0.33	0.41
	THI (2)	0.07	0.01	<0.001	24.66	53.10	88.27	98.69	0.32	0.40
	THI (3)	0.06	0.01	<0.001	25.78	54.50	85.86	96.28	0.31	0.40
	THI (4)	0.06	0.01	<0.001	24.02	52.31	89.61	100.03	0.32	0.40
	THI (5)	0.06	0.01	<0.001	27.96	57.21	80.96	91.38	0.30	0.39
	THI (6)	0.08	0.01	<0.001	26.14	54.95	85.07	95.49	0.31	0.39
	THI (7)	0.08	0.01	<0.001	22.03	49.83	93.65	104.07	0.32	0.41
	THI (8)	0.06	0.02	0.005	5.83	29.66	120.55	130.97	0.36	0.45
	THI (9)	0.03	0.03	0.291	0.87	23.48	127.24	137.67	0.37	0.46
	THI (10)	0.04	0.02	0.092	2.20	25.13	125.51	135.93	0.37	0.45
	THI (11)	0.06	0.02	0.012	4.75	28.31	122.06	132.48	0.37	0.45
	THI (12)	0.06	0.02	0.007	5.51	29.26	121.00	131.42	0.36	0.45
	THI (13)	0.05	0.02	0.006	5.72	29.52	120.71	131.13	0.36	0.45
	THI (14)	0.05	0.02	0.003	6.61	30.63	119.44	129.86	0.36	0.44
	THI (15)	0.04	0.01	0.017	4.36	27.83	122.60	133.02	0.37	0.45
	THI (16)	0.03	0.01	0.034	3.43	26.67	123.86	134.28	0.37	0.45
	THI (17)	0.03	0.01	0.040	3.24	26.43	124.12	134.54	0.37	0.45

THI (18)	0.02	0.01	0.121	1.87	24.73	125.94	136.36	0.37	0.45
THI (19)	0.00	0.01	0.662	0.15	22.58	128.17	138.59	0.38	0.46
THI (20)	0.00	0.01	0.654	0.16	22.59	128.16	138.58	0.38	0.46
THI (21)	0.01	0.01	0.379	0.61	23.15	127.59	138.01	0.38	0.46
THI (22)	0.01	0.01	0.263	0.98	23.62	127.10	137.53	0.38	0.46
THI (23)	0.02	0.01	0.150	1.61	24.40	126.28	136.70	0.37	0.45
THI (24)	0.03	0.01	0.004	6.14	30.04	120.12	130.54	0.37	0.44
THI (25)	0.02	0.01	0.058	2.76	25.84	124.76	135.18	0.37	0.45
THI (26)	0.01	0.01	0.192	1.33	24.05	126.66	137.08	0.38	0.46
THI (27)	0.01	0.01	0.272	0.95	23.57	127.15	137.57	0.38	0.46
THI (28)	0.01	0.01	0.482	0.39	22.88	127.87	138.29	0.38	0.46
THI (29)	-0.01	0.01	0.507	0.35	22.83	127.92	138.34	0.37	0.46
THI (30)	-0.02	0.01	0.152	1.60	24.39	126.30	136.72	0.37	0.45
THI (31)	-0.04	0.01	0.004	6.31	30.26	119.87	130.29	0.36	0.44
THI (32)	-0.11	0.02	<0.001	21.26	48.87	95.15	105.57	0.32	0.41
THI (33)	-0.16	0.02	<0.001	29.30	58.88	77.78	88.20	0.30	0.39
THI (34)	-0.13	0.02	<0.001	24.41	52.79	88.80	99.22	0.31	0.40
THI (35)	-0.12	0.02	<0.001	18.46	45.38	100.41	110.83	0.33	0.41
THI (36)	-0.12	0.02	<0.001	23.13	51.19	91.45	101.87	0.32	0.40
THI (37)	-0.10	0.01	<0.001	24.06	52.36	89.53	99.95	0.31	0.40
THI (38)	-0.10	0.01	<0.001	24.79	53.27	87.98	98.40	0.31	0.40

THI (39)	-0.09	0.01	<0.001	28.23	57.55	80.32	90.74	0.29	0.39
THI (40)	-0.08	0.01	<0.001	31.43	61.54	72.46	82.88	0.28	0.38
THI (41)	-0.07	0.01	<0.001	32.01	62.25	70.97	81.39	0.28	0.38
THI (42)	-0.06	0.01	<0.001	31.04	61.05	73.48	83.90	0.29	0.38
THI (43)	-0.05	0.01	<0.001	26.68	55.61	83.88	94.30	0.30	0.39
THI (44)	-0.06	0.01	<0.001	27.07	56.10	83.01	93.43	0.30	0.39
THI (45)	-0.06	0.01	<0.001	25.26	53.85	86.99	97.41	0.30	0.40
THI (46)	-0.06	0.01	<0.001	24.76	53.22	88.06	98.48	0.30	0.40
THI (47)	-0.06	0.01	<0.001	23.01	51.04	91.69	102.11	0.31	0.40
THI (48)	-0.06	0.01	<0.001	16.25	42.63	104.32	114.74	0.32	0.42
THI (49)	-0.06	0.01	<0.001	16.08	42.42	104.61	115.03	0.33	0.42
THI (50)	-0.04	0.01	<0.001	11.71	36.98	111.81	122.23	0.34	0.43

THI = temperature-humidity index; RRT = reticulorumen temperature; RRT_t0 = baseline reticulorumen temperature at sampling; HSP70 = heat shock protein 70; SE = standard error; R²m = marginal R-squared (variance explained by fixed effects); R²c = conditional R-squared (variance explained by both fixed and random effects); AIC = Akaike information criterion; BIC = Bayesian information criterion; MAE = mean absolute error; RMSE = root mean square error. MAE and RMSE are expressed in the same units as the corresponding outcome variable.